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"Novel approaches for prevention/eradication of *Pseudomonas aeruginosa* lung infections in murine models"

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A. ABSTRACT

Pseudomonas aeruginosa is one of the top three causes of opportunistic human infections in different categories of patients. Antibiotics are used as first line drugs for the treatment of these infections. However, frequently observed inefficacy of these treatments is linked to the high levels of intrinsic and acquired resistance of *P. aeruginosa* to these agents. Unfortunately during the last decades few novel anti-pseudomonas drugs have arrived to clinical phases. Moreover, in the past, several vaccine candidates against *P. aeruginosa* have been tested in pre-clinical trials, few have reached clinical phases but none of these has obtained market authorization. Thus, this scenario highlights the need of new therapeutic options for the prevention/eradication of *P. aeruginosa*. In the first part of this work the efficacy of a novel Protein Epitope Mimetic antibiotic named New Chemical Entity (NCE) was assessed in murine models of *P. aeruginosa* acute and chronic airways infection, including CFTR-deficient mice. A comparison between different administration routes (systemic versus pulmonary) was also considered. NCE demonstrated a remarkably efficacy in reducing the bacterial load and the inflammation in the lung when administered locally in all murine models tested. The efficacy of this novel antibiotic peptide was superior to ciprofloxacin, one of the current available treatments. Moreover pharmacokinetic studies confirmed that NCE reached favorable concentrations in the lung after pulmonary administration. These results supported the development of NCE as a potential novel inhaled therapy to treat *P. aeruginosa* airways infections.

In the second part of this work, novel vaccine candidates against *P. aeruginosa*, identified by the combination of advanced whole genomic approaches including the "reverse vaccinology", were tested in murine models of acute pneumonia. 32 vaccine candidates were tested for their ability to protect against a lethal dose of *P. aeruginosa*. 10 proteins showed an increase in the survival curves when compared with a negative control group. Further characterization suggested that the vaccine candidates were immunogenic, expressed in bacterial culture and surface exposed. When combinations of two proteins were tested in murine models, five of them showed a statistically significant increase in both the survival curves and mean survival time compared with a negative control group. The highest protection rate (50%) was achieved by the combination of two unknown proteins. Overall these results suggested that the combination of comparative genome analysis and innovative methods in vaccine design are valid tools for the identification of novel vaccine candidates against *P. aeruginosa*.

B. STATE OF THE ART

B.1 Epidemiology and pathogenesis of *Pseudomonas aeruginosa* respiratory diseases

Pseudomonas aeruginosa is an ubiquitous environmental Gram-negative bacterium. It is one of the top three causes of opportunistic human infections targeting a wide range of patients. The European Centre for Disease Prevention and Control (ECDPC) reports show that *P. aeruginosa* is responsible of 16% of healthcare associated infections causing millions of cases each year (Lyczak, Cannon et al. 2000; Boucher, Talbot et al. 2009). It rarely causes infections in immunocompetent individuals, when this occurs *P. aeruginosa* is normally eliminated by the host immune system. Alterations or disruption of defensive barriers like skin or mucosa (after surgery, serious burns, indwelling devices or mucosal flora alteration by broad spectrum antibiotic) or a compromised immune system may favor *P. aeruginosa* infections. *P. aeruginosa* can cause infections at different body sites including skin and soft tissues, urinary tract, brain, heart, bloodstream, cornea and respiratory tract. In humans, *P. aeruginosa* is able to cause acute and chronic airways infections. Acute airways infections are characterized by tissue injury, acute pneumonia, multiorgan failure and sepsis due to the dissemination of the environmental acquired strain throughout the body. This process can occur in hours or days (**Fig.1A**). This kind of infections mainly occur in patients with a compromised immune system, due to immunosuppressive therapies or underlying diseases, such as cancer or AIDS, or hospitalized patients. In hospitals *P. aeruginosa* is responsible of infections in intensive care units (ICUs), causing Hospital-acquired pneumonia (HAP) and, in ventilated patients, ventilator-associated pneumonia (VAP) and sepsis with a rate of mortality of about 38% (Stover, Pham et al. 2000; Chastre and Fagon 2002; Williams, Dehnbostel et al. 2010; Gellatly and Hancock 2013).

Chronic airways infections due to *P. aeruginosa* occur without injury and in the presence of biofilm structures that develop over days or weeks (**Fig.1B**). This kind of infection typically occur in patients suffering from chronic obstructive pulmonary diseases (COPD) or the hereditary diseases cystic fibrosis (CF). COPD is a destructive lung disease of which the predominant cause is excessive tobacco smoke but also less frequent causes associated with indirect cigarette smoke, air pollutants, biomass fuels, and genetic mutations. It afflicts about 14.2 million cases in the United States alone with an estimated 63 million people world-wide. COPD is characterized by increased mucus production, airways inflammation and obstructive bronchitis (Hassett, Borchers et al. 2014; Postma, Bush et al. 2015). CF is the most common lethal autosomal genetic disorder in Caucasian populations (Ratjen and Doring 2003). The disease is characterized by pathological changes in

secretory cells due to mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene that encodes for a chloride channel in epithelial membranes. Mutations in the gene encoding the CFTR protein result in a hyper absorption of sodium and water across epithelia, that leads to depletion of the periciliary liquid layer, dehydration of the mucus and inhibition of the mucociliary transport in lungs, causing progressive disability and early death. In this situation *P. aeruginosa* has been recognized as having the greatest role in morbidity and mortality leading to premature death in 90% of patients.

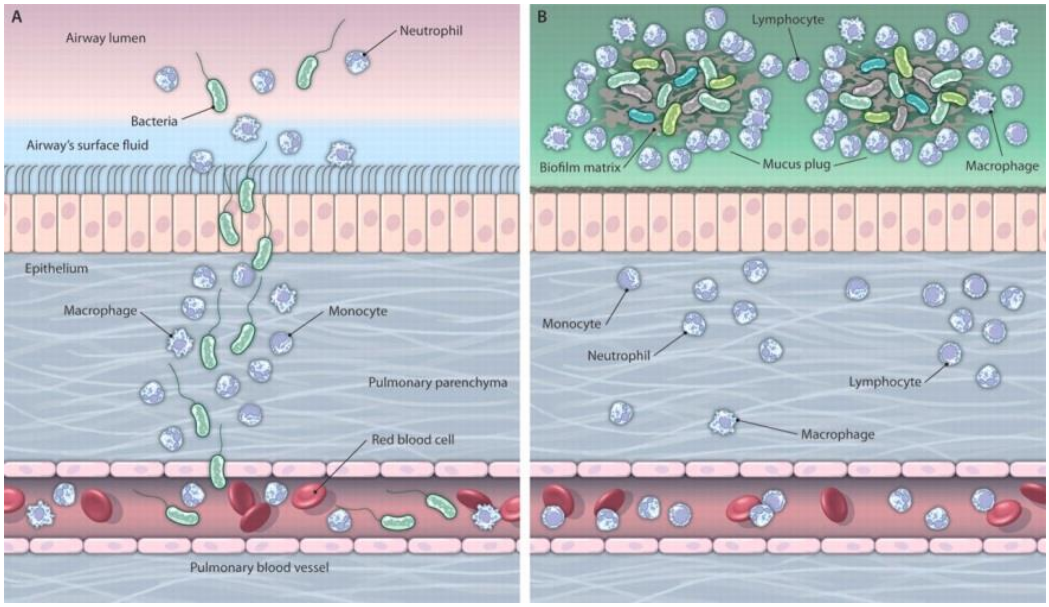

Figure 1: Acute and chronic infections caused by P. aeruginosa*. (A) Depicted here in the airways and pulmonary blood vessels, an acute infection caused by invasive and cytotoxic bacterial cells that can quickly progress to a systemic infection (septicemia) in immune-compromised patients. (B) Under chronic conditions, bacterial genetic variants may grow in biofilm structures in the airways of CF and COPD patients (Bragonzi 2010).*

B.2 *P. aeruginosa* virulence factors and adaptation during airways infection

P. aeruginosa is able to adapt to many different ecological niches and infect many different organism, including plants, amoebas, nematodes and vertebrate animals. Probably, this colonization capacity reside in its large genome that consist in a circular chromosome of around 6.3-Mbp encoding 5,570 predicted protein sequences (Stover, Pham et al. 2000). It has been observed that the phenotype of *P. aeruginosa* from acute infections differ substantially from those isolated from CF chronic infections (**Fig.2**) (Smith, Buckley et al. 2006).

In early stages of acute infection virulence factors of *P. aeruginosa* play an important role in bacteria-host interaction, invasion, colonization and dissemination. Many virulence factors has been linked to disease severity and worst clinical outcomes in infected patients (Sadikot, Blackwell et al. 2005).

Flagella and pili present in *P. aeruginosa* surface are involved in bacterial motility allowing bacteria to spread along hydrated surfaces facilitating the rapid airways colonization (Gellatly and Hancock 2013). They are also important adhesins involved in the initiation of an inflammatory response.

The lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria and is critical for *P. aeruginosa* virulence, playing an important role in bacteria-host interaction. LPS is composed by three domains: the lipid A, the oligosaccharide core and the O-specific polysaccharide or O-antigen. The variability of the O-antigen chains is the basis of the antigenic identification of *P. aeruginosa* serotypes. Lipid A component activates multiple pro-inflammatory pathways by TLR4 recognition (Gellatly and Hancock 2013).

Another major determinant of *P. aeruginosa* virulence is the type III secretion system (T3SS), that allows the bacterium to inject toxins directly into the host cell through a pore formed in the host cell membrane. Four effectors have been identified in *P. aeruginosa*: ExoY, ExoS, ExoT and ExoU. These effector proteins can modulate innate immune recognition of bacteria or target effector mechanisms of the innate immune system (Sadikot, Blackwell et al. 2005).

P. aeruginosa pathogenicity is also characterize by many others virulence factors. Among these proteases secreted into the extracellular space, such as elastase, alkaline protease or hemolysins, that contribute for invasion and dissemination of the bacteria by disrupting the respiratory epithelium, participate in cytotoxicity and are involved in specific strategies of immune escaping by degrading host complements proteins, antibodies and cytokines. Moreover *P. aeruginosa* secretes small molecules that have an inhibitory or toxic effect on immune system, like pyocianin, pyoverdine, pyochelin or rhamnolipids. Pyocyanin is a blu-green pigment that cause oxidative stress to the host and induces neutrophil apoptosis (Allen, Dockrell et al. 2005; Sadikot, Blackwell et al. 2005). Pyoverdin and pyochelin, the two major siderophores produced by *P. aeruginosa*, that regulate the secretion of other virulence factors including exotoxin A, endoproteases and itself while rhamnolipids are associated with neutrophil necrosis (Sadikot, Blackwell et al. 2005; Gellatly and Hancock 2013).

The production of several *P. aeruginosa* virulence factors is coordinated by Quorum Sensing (QS), therefore it is a key contributor to *P. aeruginosa* pathogenesis. The QS is a cell density monitoring mechanism that allows cell-to-cell signaling through both homoserine lactones and

quinolones that are auto-inducers that diffuse freely across bacterial membranes (Jimenez, Koch et al. 2012). *P. aeruginosa* mainly employ two systems: Las and Rhl. Through these systems the QS is involved in the control and regulation of multiple virulence factors like LasA and LasB elastases, exotoxin A, alkaline proteases, rhamnolipids production or T3SS assembly and production (Jimenez, Koch et al. 2012).

The transition from acute to chronic lung infection is characterized by the reduction of several bacterial factors, mainly involved in immunostimulation or host damage, and by the selection against invasive functions of *P. aeruginosa*. All these changes leads to more persistent phenotypes. The pathogen adaptation arises from several mechanisms of differential gene expression, gene mutation and extensive genomic rearrangements (Smith, Buckley et al. 2006; Bragonzi, Paroni et al. 2009; Bianconi, Milani et al. 2011; Marvig, Sommer et al. 2015).

The adaptive process is frequently associated with *P. aeruginosa* alteration in colony morphology, often characterized by conversion to morphotypes that overproduce alginate (mucoid morphotypes), lack of expression of some virulence factors, changes in surface antigens, increased antibiotic resistance and modulation of metabolic pathways (**Fig.2**).

The predominance of mucoid colony morphology mainly results from the exopolysaccharide alginate overproduction and absence of flagellin and pilin. The genetic mechanisms underlying *P. aeruginosa* transition to the mucoid form have been identified mainly in the mutational inactivation of the *mucA* gene. Mutations in *mucA*, carried by *P. aeruginosa* during chronic infection, activate the transcriptions of genes involved in alginate production, while negatively regulate several virulence factors, including pili, flagella, T3SS and Rhl QS. The conversion to mucoid phenotype promotes the biofilm mode of growth, a key factor for the persistence in the host pulmonary environment. In the mucoid form, *P. aeruginosa* is more difficult to be eradicated because it is highly resistant to antibiotics, as well as to the action of host immune defenses, for instance to macrophages and neutrophils-mediated phagocytosis and antibodies opsonization (Folkesson, Jelsbak et al. 2012)

Moreover, a down-regulation of the expression of several cytotoxic factors like pyocyanin, pyoverdin and elastase, is exerted by the pathogen with the final aim to chronically persist in the pulmonary milieu (Mahenthiralingam, Campbell et al. 1994). Likewise, modification of pathogen associated molecular patterns (PAMPs), mainly lipid A moiety of LPS, but also peptidoglycan muropeptides (Cigana, Curcuru et al. 2009), can hijack genes involved in innate response in order to evade immune surveillance and to favor *P. aeruginosa* persistence. The adaptive process is also supported by mutations in the *lasR* gene and the emergence of hypermutable strains characterized

by an increased spontaneous mutation rate, due to defect in genes involved in DNA repair systems (Oliver, Canton et al. 2000). Moreover, conversely to the early phase of colonization, when *P. aeruginosa* exhibited sensibility to antibiotics, adaptation processes leads to increased antibiotics resistance not only due to mucus protection but also to increased expression of efflux pumps (Sousa and Pereira 2014). Thus, the response of airway epithelia to the stimuli presented by mucoid adapted *P. aeruginosa* is less pro-inflammatory and, hence, may not be conducive to the effective pathogen elimination (Cobb, Mychaleckyj et al. 2004).

All these findings suggest that immune escape strategies may confer a selective advantage for the establishment of *P. aeruginosa* chronic colonization and persistence.

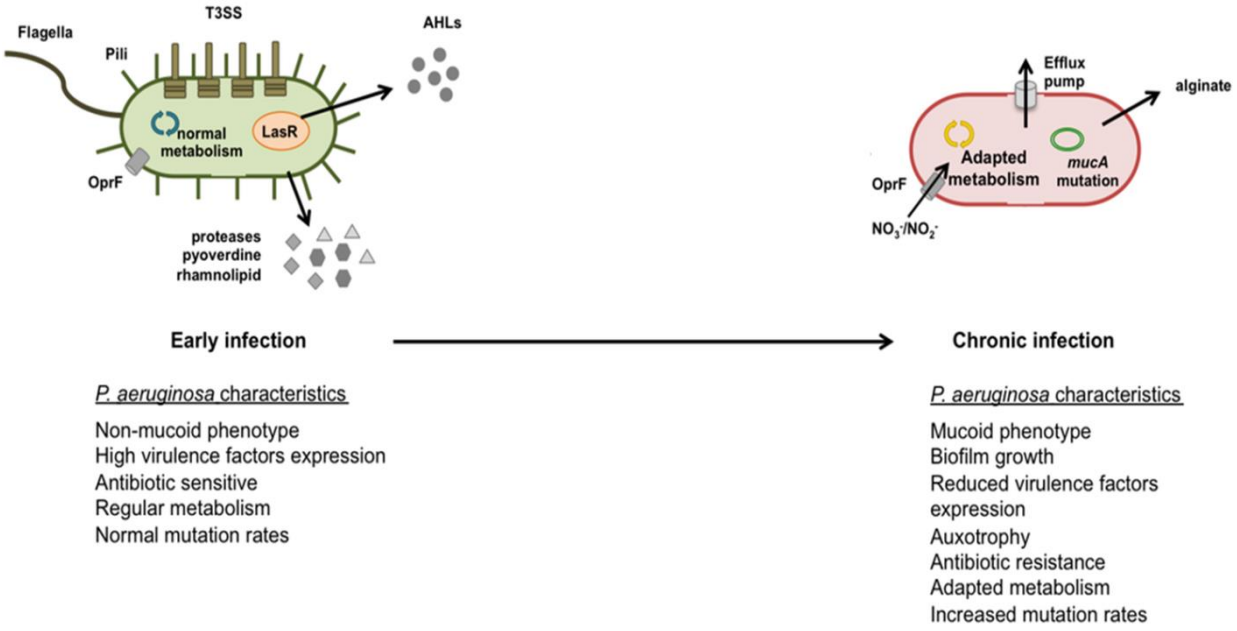

Figure 2: Representation of P. aeruginosa microevolution during infection in CF airways. *At early stage of infection, P. aeruginosa is fully equipped with cell-associated virulence factors, including flagella, pili, T3SS and secreted virulence factors (e.g. proteases, pyoverdine, and rhamnolipids) and exhibit antibiotic sensitivity. At the chronic stage of infection, P. aeruginosa is fully adapted to CF environment and exhibits a variety of adaptations, including overproduction of alginate, loss of the implicated virulence factors for initial infection establishment, resistance to antibiotics (expression of efflux pumps), and adapted metabolis (Sousa and Pereira 2014).*

B.3 Therapies against *P. aeruginosa* infections

B.3.1 Antibiotic therapy: state of the art

As mentioned before, *P. aeruginosa* is a pathogen difficult to eradicate. Antibiotics are used as first line drugs for the treatment of *P. aeruginosa* infections. The therapy is directed towards a

decrease in the bacterial load, passive release of proinflammatory substances and a reduction in the consequent inflammatory response. Initial phases of *P. aeruginosa* airways infections are often easy to resolve with antibiotic therapy but progressive airways colonization by more resistant and adaptive *P. aeruginosa* strains could lead to the establishment of untreatable chronic infection and the decline of the lung function. It is estimated that about 25-45% of adult CF patients are chronically infected with multiresistant bacteria within their airways (Lechtzin, John et al. 2006).

For the management of *P. aeruginosa* infections current treatment guidelines recommend single or combined antibiotic therapies (Doring, Conway et al. 2000). For this purpose a wide range of molecules are available, from broad-spectrum antibiotics such fluoroquinolones, aminoglycosides, carbapenems (e.g. imipenem, meropenem, doripenem) and third generation cephalosporins (e.g.ceftazidime) to more selective drugs against Gram-negative like monobactam aztreonam and piperacillin (a derivative of penicillin) alone or in combination with tazobactam (Doring, Conway et al. 2000; Giamarellou and Kanellakopoulou 2008; Rahal 2008). For the tratment of more resistant strains recently polymyxin B and polymyxin E (colistin) have been "re-introduced" (Page and Heim 2009).

Antibiotics can be classified based on their target or their mechanism of action. The aminoglycosides (like gentamicin, tobramycin or amikacin) inhibit the protein synthesis by binding the ribosomal subunit of the bacteria. Quinolones (ciprofloxacin) bind to the alpha subunit of DNA gyrase (topoisomerase II), which maintains the ordered structure of the chromosome inside the cells, thus prevent cell replication. Beta-lactams (e.g. piperacillin, ceftazidime, imipenem, meropenem and aztreonam) inhibit the peptidoglycan-assembling interacting with the trans-peptidases located on the outer face of the cytoplasmic membrane. Polymyxins (colomycin, colistin) bind to phospholipids in the cytoplasmic membrane, altering the membrane permeabilization and destroying its barrier function that leads to cellular death (**Table 1**).

The lack of a comparative randomized double-blinded studies showing significant differences in efficacy between antimicrobial agents make difficult the choice of the better therapy. The guidelines from the Infectious Diseases Society of America (IDSA) and the American Thoracic Society (ATS) on the management of community and hospital-acquired pneumonia advocate a therapeutic selection based on the severity of the infection, awareness of underlying risk factors and co-morbid diseases, recognition of the epidemiology and resistance phenotypes in individual settings, and knowledge of pharmacokinetic–pharmacodynamic parameters (El Solh and Alhajhusain 2009).

The recommended choice consits in the combination of beta-lactams antibiotics toghether with an aminoglycoside or fluoroquinolone. However, especially during chronic infections, polymixins are given as last option. Colistin is present in the market from several decades ago, but it was rarely

used due to polymyxins known toxicity. Interaction between cationic chains of colistin and bacterial LPS favor an increase of membrane permeabilization leading to the cellular death (Canton, Cobos et al. 2005; Giamarellou and Kanellakopoulou 2008). Also the macrolid azithromycin is used againts chronic *P. aeruginosa* infections due to their capacity to decrease the number of exacerbations and improve the pulmonary function. It has been reported that macrolides limit the quorum-sensing interbacterial signals and capacity of bacteria to form biofilms (Doring, Conway et al. 2000; Canton, Cobos et al. 2005).

Class	Agent	Advantages	Limitations	Mechanism of action
Penicillin	Ticarcillin Carbenicillin Piperacilin Tazobactam	Synergistic with aminoglycosides against *P. aeruginosa*	May induce beta-lactamase in *P. aeruginosa*	Inhibit the peptidoglycan-assembling interacting with the trans-peptidases located on the outer face of the cytoplasmic membrane
Cephalosporin	Ceftazidime Cefoperazone	Can be used as single agent against *P. aeruginosa*	May induce beta-lactamase in *P. aeruginosa*	
Carbapenem	Imipenem Meropenem	Very broad spectrum of activity against Gram-negative bacteria including *P. aeruginosa*	May induce beta-lactamases; rapid development of resistance	
Aminoglycoside	Gentamicin Tobramycin Amikacin	Synergistic with beta-lactams antibiotics against *P. aeruginosa*	Narrow therapeutic/toxic ratio; penetrate poorly into cerebrospinal fluid	Inhibit the protein synthesis by binding the ribosomal subunit of the bacteria
Quinolone	Ciprofloxacin	Can be given orally	Contraindicated in children under 16 years of age	Bind to the alpha subunit of DNA gyrase, thus prevent cell replication
Polymyxin	Colistin	Very active and little resistance development	Possible toxicity concerns; used largely in cystic fibrosis patients	Bind to phospholipids in the cytoplasmic membrane, altering the membrane permeabilization and destroying its barrier function.

Table 1: Antibiotics commonly used in the treatment of P. aeruginosa infections. *Modiefied from Hancock and Speert 2000.*

B 3.2 Mechanisms of antibiotic resistance

P. aeruginosa has been described as one of most dangerous multidrug resistant (MDR) ESKAPE pathogens (together with *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baummannii,* and *Enterobacter s*pecies (Boucher, Talbot et al. 2009). *P. aeruginosa* resistance to antibiotics often lies in intrinsic bacteria factors like low permeability of the outer membrane, the constitutive expression of efflux pumps or antibiotic-inactivating enzymes (Hancock 1998; Mesaros, Nordmann et al. 2007).

If we focus our attention in the mode of action of the major classes of antibiotics used to treat *P. aeruginosa* infections mentioned before, glaringly obvious that all of these molecules have to cross the cell wall to reach their targets. In fact, the innate resistance of *P. aeruginosa* to these classes of antibiotics has been generally attributed to the low permeability of its outer membrane together with

the efficient removal of antibiotic molecules that do penetrate by the action of efflux pumps. The permeability of the *P. aeruginosa* outer membrane is 10 to 100 folds lower from other gram-negative bacteria like *Eschericchia coli* (Hancock and Speert 2000). In particular outer membrane porins play an important role in limiting the access of hydrophilic antibiotics to the periplasmic space (Page and Heim 2009). Porins downregulation further decrease the possibility to enter in the cell for antibiotic molecules, an example of that is the lost of OprD, that is associated with resistance to imipenem (Lambert 2002).

The efficient removal of antibiotic molecules that do penetrate by the action of efflux pumps is an intrinsic resistance factor closely correlated with the outer membrane (Hancock and Speert 2000). In fact mutations in one of the three component of this system have been correlated with an increase of susceptibility to some drugs (like quinolones) while antibiotic pressure can induce expression of other efflux pumps (Kohler, Michea-Hamzehpour et al. 1997; Poole and Srikumar 2001; Aeschlimann 2003).

Often different mechanisms of resistance are express contemporary in the cell showing a cross-resistance phenomenon. One classical example is the co-regulation of OprD porin and MexEF-OprN pumps that are under the control of common regulators acting in opposite ways, (Kohler, Michea-Hamzehpour et al. 1997). *P. aeruginosa* harbors enzymes able to inactivate or modify antibiotics, as described for aminoglycosides or beta lactams (Mesaros, Nordmann et al. 2007). The capacity of produce beta lactamase or enzymes capable to modify aminoglycosides is intrinsic but probably also acquired, in fact the ESBLs (extended spectrum beta lactamases), are widespread in the bacterial population in recent years (Livermore 2002). Moreover aminoglycosides inactivating enzymes are codify at plasmid levels (Lambert 2002).

Another well-known mechanism of resistance in *P. aeruginosa* consist in mutate the antibiotic target, as happens for the DNA gyrase (Mesaros, Nordmann et al. 2007).

Convergence of multiple resistance mechanism in one strains, for exaple the antibiotic-inactivating enzymes and target mutation, results in multidrug resistance (resistant to three or more anti-pseudomonal drug classes) or pan-resistance (resistance against all common antibiotic classes); both phenomena observed with increasing frequency in *P. aeruginosa* (Souli, Galani et al. 2008).

All these resistance mechanisms compromise the mangement of infections by *P. aeruginosa*, that is able to develop resistance also during the course of the therapy. Hypermutable strains were detected in up to 37% of the CF patients with *P. aeruginosa* chronic infection, in which a strong link between hypermutation and antibiotic resistance was also observed (Oliver, Canton et al. 2000). Hypermutable strains have an increase mutation rate due a defects in the methyl-directed mismatch repair (MMR), a post-replicative repair system that corrects errors on newly synthesized

DNA strands to ensure the fidelity of chromosome replication. Antibiotic pressure can select hypermutable strains increasing the likelihood that resistance emerges and stabilizes contributing to lung damage during long-term persistence (Livermore 2002; Alcala-Franco, Montanari et al. 2012).

Unfortunantly chronic *P. aeruginosa* infections are not erradicable by antibiotic therapy. As mentioned before, *P. aeruginosa* adaptation to the lung implies, by others, mucoid phenotype, non motility and biofilm formation. Also if a heterogeneous population is present inside di biofilm, regarding antibiotic susceptibility and resistance, exopolymeric matrix materials form a barrier that limits antimicrobial penetration to cells residing within a biofilm.

B.3.3 New antibiotic therapies

As described above, intrinsic and acquired resistance of *P. aeruginosa* made the wide arsenal of antibacterial drugs available in the market inefficacious to treat *P. aeruginosa* lung infections resuwting in a rapid increase in resistant rates and appearance of MDR or pan resistant phenotypes. Although this alarming scenario only a very small number of new anti-*Pseudomonas* drugs are currently in late stage of pre-clinical or clinical development (**Table 2**) (Page and Heim 2009).

Arrived to the phase II in the last years are described only two cephalosporins, CXA-101 from Calixa (http://www.prnewswire.com/news-releases/calixa-therapeutics-announces-positive-phase-1-results-for-cxa-101-a-novel-intravenous-cephalosporin-antibiotic-with-excellent-anti-pseudomonal-activity-61916077.html) and NXL104/ceftazidime by Novexel (http://www.novexel.com/includes/cms/_contenus/mod_press_releases/09_NXL_01_Phase_II_cIAI_Final_EN.pdf). CXA-101 has demonstrate an excellent potency against MDR *Pseudomonas* while the NXL104/ceftazidime combination showed potent protection against many serine beta-lactamases.

Other drugs have reach the clinical phase I. Among these BLI-489/piperacillin is active against some classes of beta-lactamase and the lipopeptide CB-182,804, that showed a pre-clinical potent bactericidal activity against MDR bacteria.

Many are the advantages of the use of cationic peptides: often kill microorganisms rapidly, do not easily trigger the emergence of resistant mutants, show synergistic effects in combination with conventional antibiotics and can often activate the host innate immunity without displaying immunogenicity (Bragonzi 2010). POL7080 and the New Chemical Entity (NCE) studied in this thesis, from Polyphor, are the first of a new class of antibiotics derived from the protein epitope mimetic (PEM) Technology. NCE was chosen as coded name due to current patent rights of the original name of the peptide. This family peptides mimics protegrin-I (PG-I), a naturally occurring antimicrobial peptide shown to have a potent activity against *P. aeruginosa*. They consist in cyclic

14-mer peptides that contain both natural and unnatural amino acids that targets the ß-barrel protein LptD, involved in the outer-membrane biogenesis being essential for the LPS transport to the cell surface, thus blocking bacterial growth. In preclinical studies both compounds were highly active on a broad panel of *Pseudomonas* bacteria (Srinivas, Jetter et al. 2010). POL7080 has successfully completed clinical Phase I demonstrating the clinical safety and tolerability the peptide (http://www.polyphor.com/products/pol7080).

Other antimicrobial compounds like novel lactams and beta-lactams inhibitors, peptides and peptide-mimetics, efflux inhibitors and modulators of virulence are studied in pre-clinical phases (**Table 2**) (Page and Heim 2009).

Class	Agents	Advantages	Stage of development	References
Cephalosporin	CXA-101	Excellent potency against MDR *Pseudomonas*	II-Phase	Takeda S et al. 2007 Zamorano L et al. 2010
Cephalosporin-inhibitor	NXL104/ceftazidime	Potent protection against many serine beta-lactamases	II-Phase	Miossec et al. 2007 Livermore DM et al. 2008
Lipopeptide	CB-182,804	pre-clinical potent bactericidal activity against MDR bacteria	I-Phase	
Lactams and beta-lactamase inhibitor	BAL30072	Activity against broad range of Gram-negative. Potent activity against *P. aeruginosa*	Preclinical	Page MG et al. 2010
	NXL105	Activity against MDR *P. aeruginosa*	Preclinical	
	BAL30376	Activity against broad range of Gram-negative. Potent activity against MDR *P. aeruginosa*	Preclinical	Page MG et al. 2007 Bowker KE et al. 2007 Schmitt-Hoffmann A et al. 2007 Page MG et al. 2011
	BLI-489/piperacillin	Active against some classes of beta-lactamase	I-Phase	Venkatesan AM et al. 2004 Petersen PG et al. 2009
	Arenicin-3	Potent activity and strong bactericidal activity against broad range of Gram-negative, *including P. aeruginosa*	Preclinical	Sandvang D et al. 2008 Cooper M et al. 2014
Peptides and peptide mimetics	POL7080	Highly active on a broad panel of P*seudomonas* bacteria. Clinical safety and tolerability tested.	I-Phase	Srinivas, Jetter et al. 2010
	NCE	highly specific against *P. aeruginosa* including MDR, mucoid and hypermutable clinical isolated	Preclinical	This study
	RTA3	moderate activity against a range of Gram-negative bacteria, including *P. aeruginosa*. Synergistic with rifampicin, erythromycin and polymixin	Preclinical	Hawrani A et al. 2010

Table 2: Selection of most promising anti-Pseudomonal drugs in development.

To bypass the problem of intravenous administration of antibiotics (and their potential toxicity when given over prolonged periods of time) obtaining at the same time a high drug concentration in the lung, aerosolization of drugs has been suggested for the treatment of pulmonary *P. aeruginosa* infections. In the last years many efforts have been made in this field and novel formulations of existing antibiotics including aminoglycosides, beta-lactams and fluoroquinolones alone or in combinations has been test in pre-clinical and clinical studies. Currently chronic airways infection

caused by *P. aeruginosa* can be treated with inhaled antibiotics such as inhaled tobramycin, aztreonam or colistin. Considering that only a small portion of the antibiotic is deposited in the lungs and that the size of nebulized droplets and particles carrying the antibiotics is a critical point for the lung deposition many efforts have been made also in the development of novel aerosol delivery devices. The particle size (1 to 5 μm), the lung dose delivered (a range between 3% and 8% up to >50%) and the administrations time (10 to 20 minutes to <2 or 3 minutes) have all improved during recent years as treatment of lung infections with antibiotics administered by inhalation therapy has become standard care in CF centers (Doring, Conway et al. 2000; Geller 2009; Kesser and Geller 2009; Hoiby 2011).

B.4 Immunotherapy against *P. aeruginosa* infections

B.4.1 Current state

Although chemotherapies showed initial efficacy in the management of *P. aeruginosa* infections they are not able to definitively eradicate the bacterium. Alternative therapeutic options to the prevention and control of these infections may include immunotherapy. In the past many efforts have been made in this direction and several vaccine candidates against *P. aeruginosa* have been tested in pre-clinical trials, only few have reached clinical phases but none of these has obtained market authorization (**Table 3**) (Doring and Pier 2008; Sharma, Krause et al. 2011).

Several virulence factors of *P. aeruginosa* have been used as targets against immunotherapy. Among these flagella has been widely used. Flagellin is the main component of flagella, it is divided into two types a (heterogeneous type) and b (serologically uniform), so a broadly successful vaccine has to be bivalent. An attenuation of pneumonia was observed in rats receiving human antiflagellar monoclonal antibodies (Landsperger, Kelly-Wintenberg et al. 1994). Mono-or bivalent flagella vaccines have been test in clinical phases demonstrating to be safety and to elicit protective long-lasting antibodies but the response remained modest raising also the question of protections against no-flagellated *P. aeruginosa* strains (Doring and Pier 2008; Sharma, Krause et al. 2011).

Also pili has been considered a reasonable target to develop *P. aeruginosa* immunotherapy (Sheth, Glasier et al. 1995; Hahn, Lane-Bell et al. 1997; Cachia, Kao et al. 2004). However, pilus antigens are serologically heterogeneous and there is no cross reactivity of antigens targeting pili across different *P. aeruginosa* strains (Hahn, Lane-Bell et al. 1997; Cachia, Kao et al. 2004). A chimeric vaccine incorporating both pilin and non-toxic modified toxin A successfully reduced bacterial adherence in pre-clinical studies (Hertle, Mrsny et al. 2001), even so there was no human studies with pilin vaccines.

The LPS has remained the most widely characterized and investigated vaccine antigen in the last 50 years because of its surface accessibility and high immunogenicity. A LPS-based vaccine, (Pseudogen®) was tested in phase III with a high adverse reaction rate probably due to the pyrogenic and toxic effects of lipid A when LPS is administered in a purified state. To avoid the LPS toxicity approaches like inclusion of LPS in liposomes or the use of non-toxic polysaccharide part in vaccine preparations, like the O- polysaccharide portion, were used (Doring and Pier 2008; Sharma, Krause et al. 2011). However several problems have been also described with vaccines based on the variable O-specific polysaccharide chains. Various O-antigen based vaccines have been tested over decades also in phase II, like the Aerugen® (an octavalent O-polysaccharide conjugate vaccine), with limited success, in fact immunization elicited by O-antigen based vaccines is lacking in protection even when multiple O-antigens from different serotypes are conjugated (Hatano and Pier 1998; Pier 2003; Lang, Horn et al. 2004). To circumvent this difficulty one strategy was the use of multiple serotype conjugates that can be further conjugated with another target such as exotoxin A (Lang, Horn et al. 2004). Recently an increase in the IgG2 to the O-antigen that inhibit the antibody-mediate-killing have been described in patients (Wells, Whitters et al. 2014).

Due to the mucoid alginate–producing phenotypes of *P. aeruginosa* commonly found in chronic airways infections and to the relative conservation between strains the mucoid exopolysaccharide (MEP or alginate) has been selected as an attractive vaccine antigen. Human and animal studies demonstrated the role of MEP-specific opsonizing antibodies in facilitating bacterial clearance (Sharma, Krause et al. 2011). Human monoclonal antibodies directed against alginate have been also took into account to an efficacy vaccine. They were effective in a murine model of lethal pneumonia and even against non-mucoid strains producing undetectable levels of alginate (Pier, Boyer et al. 2004). However, being the alginate alone poorly immunogenic, also in this case strategies of conjugation to proteins, like exotoxin A, to enhance immune responses were used (Doring and Pier 2008). Despite the preclinical encouraging results a successful clinical product has not been yet developed.

Another interesting target to vaccine development has been the T3SS, in particular the PcrV protein. PcrV is a component of the T3SS, located in the bacterial surface, required for the translocation of the effectors proteins. In murine models of lung infection and burn mouse vaccination against the PcrV induced protective immunity, decrease lung inflammation and injury (Sharma, Krause et al. 2011). The KB001 by KaloBios Pharmaceuticals consist in a high-affinity antibody fragment that binds the PcrV protein inhibiting the activity of the T3SS, and so reduce

pathogenicity of *P. aeruginosa* and its toxicity to host cells (Page and Heim 2009). Since 2013 KB001 has being tested in clinical phase II in CF patients (http://kalobios.com/pr_01102013).

Whole-cell killed and live-attenuated vaccines present multiple bacterial antigenic components and can thus potentially induce diverse immunologic effectors against *P. aeruginosa*. Oral human immunization with killed *pseudomonas* vaccine showed to be safety and to increase pseudomonas-specific serum antibodies, mainly IgA, that promoted opsonophagocytotic killing of the bacteria in healthy volunteers and to reduce significantly the bacterial load in the sputum of patients with bronchiectasis (Sharma, Krause et al. 2011).

Vaccination with highly conserved outer membranes proteins (OMP) has been a good choice to avoid the heterogeneity related to different strains. *P. aeruginosa* OprF and OprI have been widely use to vaccination demonstrating that elicit cross-reactive, opsonizing and protective antibodies in animal model or humans (Sharma, Krause et al. 2011). OprF seems to play a key role in *P. aeruginosa* adaptation to host immune response (Wu, Estrada et al. 2005) while OprI adhere to mucosal surfaces and probably facilitate the antigen delivery to antigen presenting cells acting as a mucosal carrier (Loots, Revets et al. 2008). In humans OprF and OprI have been usually administered as recombinant vaccine (OprF/I). The CFC-101 vaccine composed of Opr extract from four *P. aeruginosa* strains, induced Opr-specific antibody titer with opsonophagocytic activity and increased *P. aeruginosa* clearance in blood in healthy volunteers and burn patients proving the promising capabilities of this antigens (Doring and Pier 2008; Sharma, Krause et al. 2011).

Other interesting vaccines used in pre-clinical studies with encouraging results include DNA vaccines and viral vector vaccines.

Antigens	Advantages	Limitations	Stage of development	References *
LPS and O-polysaccharide	Generation of high levels of opsonic antibodies	High heterogeneity, Low immunogenicity, Pyrogenicand toxic	I-III Phase	21, 31–34, 36, 40
MEP	Low heterogeneity	For CF use only	I Phase	45, 51, 52
Outer Membrane proteins	Highly conserved and immunogenic Anti-OprF inhibits quorum-sensing through IFNγ binding to *P. aeruginosa*	No significant drawback	I/II Phase	137, 139, 142, 143,146
Flagella	Moderate heterogeneity, Adjuvant effect through TLR5	Loss of flagella in CF variants	I-III Phase	80–82
Pilin	High immunogenicity	High heterogeneity, Hidden receptor binding site	Preclinical	94, 95, 98, 103
PcrV, Exotoxin A and proteases	Neutralizes cytotoxic effects and pathology	Less effective in bacterial clearance	Preclinical	105, 106, 108, 110,111
Killed	Presentation of multiple antigens to immune system	Toxicity	I Phase	56, 57
Live attenuated (*P. aeruginosa* ΔaroA)	Presentation of multiple antigens toimmune system	Residual virulence	Preclinical	58, 59, 61, 62
Attenuated *Salmonella enterica* delivered O-antigen or OprF-OprI	Efficient activation of mucosal immunity	Residual virulence	I/II Phase	68–70
Ad vector delivered OprF	High immunogenicity and adjuvant properties	Pre-existing anti-Ad immunity	Preclinical	157–159
Novel OMP	Highly conserved in clinical isolates	No significant drawback	Preclinical	This study

Table 3: Potential antigens for P. aeruginosa*. Modificated from Sharma, Krause et al 20011.*References: **21**. Erridge C. et al 2002; 31. Langford DT, et al 1984; **32**. Jones RJ et al 1978; **33**. Jones RJ et al 1979; **34**. Jones RJ et al 1980; **36**. Cryz SJ Jr, Lang A et al. 1997; **40**. Lang AB et al. 2004; **45**. Pier GB et al. 1994; **51**. Kashef N et al. 2006; **52**. Theilacker C et al. 2003; **137**. Mansouri E et al. 2003; **139**. Baumann U et al. 2004; **142**. Baumann U, et al. 2007; **143**. Sorichter S et al. 2009; **146**. Lee NG et al. 2000; **80**. Doring G et al. 1995; **81**. Doring G et al. 2007; **82**. Saha S et al. 2007; **94**. Ohama M et al. 2006; **95**. Hertle R et al.2001; **98**. Horzempa J et al. 2008; **103**. Kao DJ et al. 2007; **105**. Sawa T et al. 1999; **106**. Holder IA et al. 2001; **108**. Chen TY et al. 1999; **110**. Shiau JW et al. 2000; **111**. Manafi A et al. 2009; **56**. Cripps AW et al 1997; **57**. Cripps AW et al. 2006; **58**. Kamei A et al. 2011; **59**. Priebe GP et al. 2002; **61**. Priebe GP et al. 2003; **62**. Zaidi TS et al. 2006; **68**. DiGiandomenico A et al. 2004; **69**. DiGiandomenico A et al. 2007; **70**. Bumann D et al. 2010; **157**. Krause A et al. 2011; **158**. Worgall S et al. 2007; **159**. Worgall S et al. 2005.*

B.4.2. Novel approaches for vaccine development

In spite of the diversity of the strategies adopted and the efforts made, the traditional approaches to vaccine discovery have been shown to be ineffective. Previous clinical trials have shown that CF patients are immunocompetent and actually do mount an immune response to multiple *P. aeruginosa* antigens (Doring and Pier 2008). However, the antigens inducing the immune response, and/or alterations in the bacterium itself made this response ineffective at clearing *P. aeruginosa* from the lung of these individuals. The fact that right now no vaccine is available for clinical use against *P. aeruginosa* infections is a controversial topic for the scientific community. The question raised was if *P. aeruginosa* is an antigenically variable microorganism that can escape to the immune recognition and/or induces immunological unresponsiveness as is seen with other bacteria such as Borrelia, Bordetella or Neisseria.

The ideal protective antigens are those particularly expressed during the specific infectious process, present in a wide range of strains and exposed on cell surface, which makes the target easily accessible by the immunotherapy. As described above, *P. aeruginosa* adaptation to environmental changes comprise phenotypic variations that include variability of the cellular components (like proteins) among different strains and different phases of infection (**Fig.2**). This changes could represent a serious obstacle to the production of a globally effective anti-*P. aeruginosa* vaccine (Doring and Pier 2008).

Although successful in several cases, one of the biggest limitations of the conventional vaccine development approach is that it requires cultivation of the pathogens and its dissection using biochemical, immunological and microbiological methods, that is time-consuming and failed to provide a solution for many human pathogens (like those difficult to growth in laboratory conditions). For these reasons the new Era for the vaccines design take advantage of emerging

genomic technologies that allow to predict new antigens in silico, independently of their abundance and to explore novel functions of *P. aeruginosa* for infections control without the need to grow the microorganism in vitro (Grandi 2006). The reverse vaccinology, which has been successfully applied in the last few years, has revolutioned the approach to vaccine research (Scarselli, Giuliani et al. 2005). The first example of the potential reverse vaccinology was the identification of novel antigens of Meningococcus B as possible candidates for a novel and effective vaccines. The same approach has been successfully applied to other important human pathogens demonstrating the feasibility to develop vaccines against any infectious disease (Mora, Veggi et al. 2003). The success of genomic-based strategies for vaccine development is highly dependent on the criteria used for the in silico selection of the potential antigens. Several approaches can be used to mine genomic sequences, and the appropriate combination of various algorithms and the critical evaluation of the information generated are essential for the proper selection of the antigens (Mora, Veggi et al. 2003), protein's subcellular localization can provide valuable clues as to its function; subcellular localization prediction also allows researchers to identify potential diagnostic, drug and vaccine targets (Grandi 2006). This approach consented to identified novel *P. aeruginosa* antigens evaluated *in vivo* in this thesis.

B.5 Murine models of *P. aeruginosa* lung infections

Animal models are an essential step between *in vitro* testing and clinical studies. They are a valuable tool for the study of pathophysiology, pharmacology and efficacy therapy. Acute and chronic *P. aeruginosa* lung infection models have been established in several animal models, including mice (Cash, Woods et al. 1979; Johansen 1996). Acute pneumonia in mice are mainly obtain by intratracheal or intranasal administration of planktonic *P. aeruginosa* cells. The bacterial pulmonary colonization obtained by this method is transitory. The dose and the frequency of planktonic cells administration will drive into an acute lung infection, with a rapid clearance of the bacteria, or death by acute sepsis (**Fig.3**) (Jackson, Southern et al. 1967; Southern, Mays et al. 1970). The lung pathology of this kind of infection is characterized by inflammatory cells infiltrated in the lung, in particular neutrophils that leads to tissue damage. Bacteria are localized adhered to the bronchial epithelial cells and in the alveoli (**Fig.4**). Animal models of acute pneumonia are a precious instrument for studies of virulence and immunity.

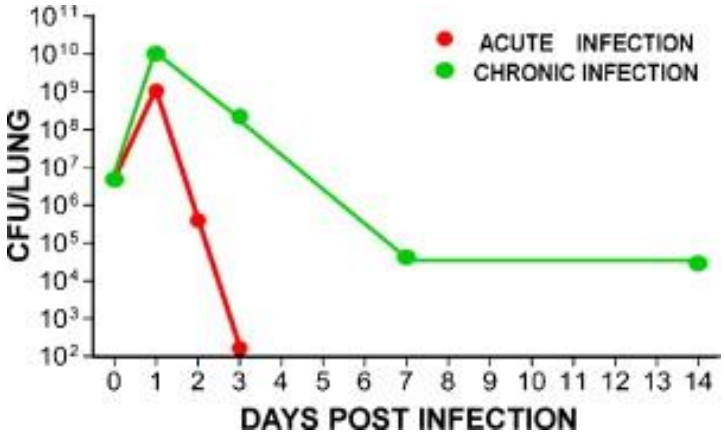

Figure 3: Pulmonary persistence of P. aeruginosa in C57BL/6NCrlBR mice. *Mice were infected with 5 × 10^6 CFU/lung of the strain PAO1 either in planktonic suspension (acute infection) or embedded in agar beads (chronic infection) and sacrificed at different time points to determine the lung bacterial load (Bragonzi 2010).*

For the achievement of persistent chronic infections, bacteria must be inoculated in an immobilizing agents, like agar, agarose or seaweed alginate (Bragonzi 2010), or alternatively a stable mucoid *P. aeruginosa* strain could be used for the infection (Hoffmann, Rasmussen et al. 2005). In this way, the model mimics the micro-aerobic conditions presents in lungs patients with CF and COPD due to the mucus accumulation. Lung pathology associated with *P. aeruginosa* chronic infection in mice is characterized by bronchopneumonia, mucus plugging, epithelial metaplasia, fibrosis and alveolar exudate with inflammatory cells, all signs that reflects an advanced chronic pulmonary disease similar to those observed in patients (**Fig.4D**) (Bragonzi 2010). It has been demonstrated that during chronic infection in C57Bl/6 mice exist a correlation between inflammation and body weight loss suggesting that pro-inflammatory cytokines in the lung may have systemic effect that affect CF patients (van Heeckeren, Tscheikuna et al. 2000). The agar beads mouse model of chronic infection with *P. aeruginosa* has been deeply characterize in our laboratory: during the first days post infection, there is a proliferation of the bacteria inside the agar beads that results in an increase in the bacterial density followed by a decrease of load to finally reach a stable pulmonary infection (≈10^4 CFU) at day7 post infection. With this model the bacterial load is able to persist unchanged up to one month (**Fig.3**) (Bragonzi, Paroni et al. 2009).

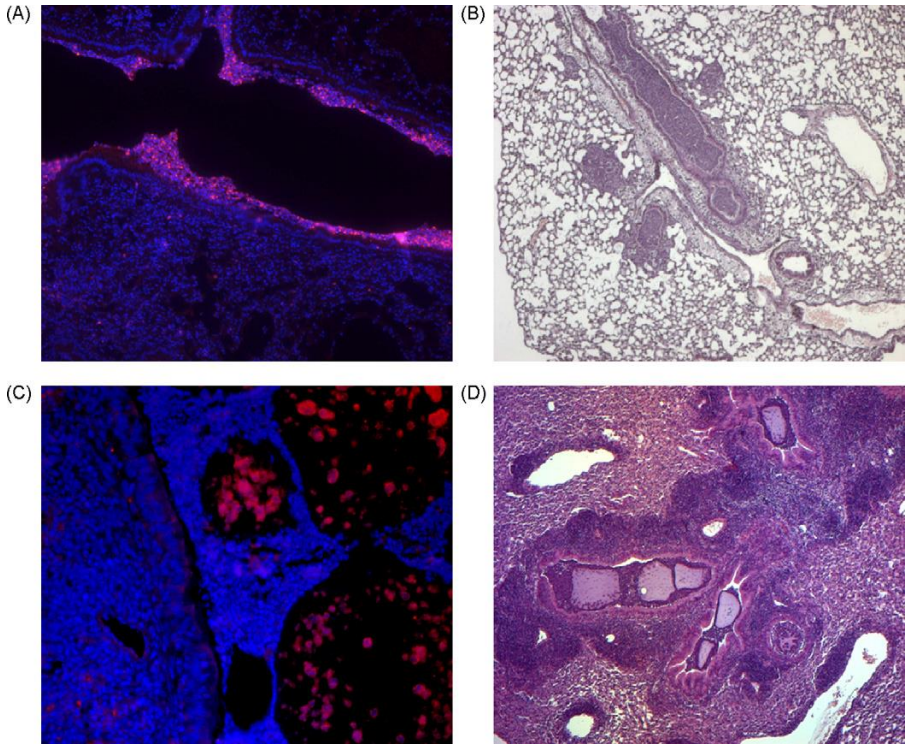

Figure 4: Murine lung histology and localization of P. aeruginosa bacterial cells in acute or chronic infection. *Mice were infected with 5x106 CFU/lung of P. aeruginosa strain PAO1 (A and B) and clinical strain isolated from a CF patient embedded in agar beads (C and D). After 1 day (acute infection; A and B) and after 14 days (chronic infection, C and D), the lungs were removed and stained with H&E (B and D) or with specific antibody against P .aeruginosa strains (red) (A and C) (Bragonzi 2010).*

Important to take into account for the achievement of the desired pulmonary infection, together with the dose of bacteria challenged, is the *P. aeruginosa* strain used for the infection. For example presence or absent of different virulence factors as well as alginate production may influence in the read outs. *P. aeruginosa* longitudinal strains isolated from CF patients at different time points of the infection were tested for their pathogenicity in mouse models of acute and long-term chronic bronchopulmonary infections. Several *P. aeruginosa* strains showed different read out in terms of mortality, severity of the lesions, clearance and percentage of chronicity in a mouse model of chronic infection (Bragonzi, Paroni et al. 2009). *P. aeruginosa* RP73 is a clinical strain isolated from a CF patient 16.9 years after the onset of infection. Long-term lung infection with RP73 in a murine model of chronic pneumonia resulted in a low mortality, high chronicity, compared with PAO1 reference strain (**Fig.5**), and severe lesions that resembles those of patients with advanced chronic pulmonary disease. This murine model most closely mimics the course of the human disease and can be used both for studies of the pathogenesis and for the evaluation of novel therapies (Jeukens, Boyle et al. 2013; Facchini, De Fino et al. 2014).

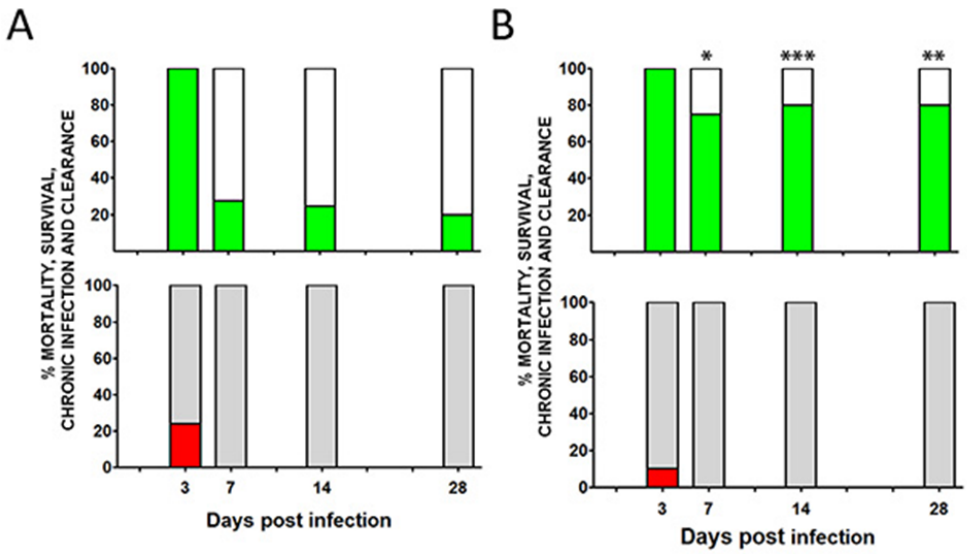

Figure 5: Time course of P. aeruginosa chronic infection with PAO1 reference strain and RP73 clinical strain. *C57BL/6NCrl (20-22 g) male mice were infected by intratracheal injection with 1 to 5 x 10^6 CFU of P. aeruginosa strain PAO1 (**A**) or RP73 (**B**) embedded in agar-beads. For each time-point, histograms represent the percentage mortality induced by bacteremia (red) and survival (grey) or the percentage of animals that cleared the infection (white) and those able to establish a chronic infection (green). Surviving mice were euthanized at the indicated time-points, and the lungs were harvested, homogenized, and cultured on TSA plates to determine the bacterial load. Statistical significance by Fisher's test is indicated: * p<0.05, ** p<0.01, *** p<0.001(Facchini, De Fino et al. 2014).*

Cystic fibrosis is the first human genetic disease to benefit from the directed engineering of three different species of animal models (mice, pigs, and ferrets). Several CF murine models have been generated and characterized during the years, from those completely Knock out for the CFTR to those bring the main human CF mutations (ΔF508 or G551D). All these murine models present an altered chlorine conductance although manifesting different pathological phenotypes. The CF mice don't represent the human lung pathology of the CF patients and they not become infected spontaneously. One big limitation of the CF mice reside in the severe gut pathology that leads to gut obstruction. The CFTR-deficient mice used in my thesis are the *$Cftr^{tm1UNC}$TgN(FABPCFTR) (Bragonzi 2010).*These knock-out mice bring an insertion in the exon 10 that prevents the formation of the CFTR protein already at mRNA level. This mouse model have been gut corrected by the insertion of the human transgene hCFTR that allow a standard survival rate and the facilitated maintenance of the colony (Zhou, Dey et al. 1994; Bragonzi 2010).

C. AIM OF THE PROJECT

Pseudomonas aeruginosa is one of the top three causes of opportunistic human infections in different categories of patients. Antibiotics are used as first line drugs for the treatment of these infections. However, frequently observed inefficacy of these treatments is linked to the high levels of intrinsic and acquired resistance of *P. aeruginosa* to these agents that leads to the rapid emergence of MDR strains. In spite of the urgent need of new drugs to treat MDR bacterial infections, few novel anti-pseudomonal drugs or modifications of existing molecules have arrived to clinical phases recently. Moreover, in the last years, several vaccine candidates against *P. aeruginosa* have been tested in pre-clinical trials, few have reached clinical phases but none of these has obtained market authorization. Thus, this scenario highlights the need of new therapeutic options for the prevention/eradication of *P. aeruginosa*.

In this context, starting from a novel antibiotic and vaccine candidates against *P. aeruginosa*, my PhD work aims to test these therapies in pre-clinical models of infection. Specific aims of this work were to test:

- **the efficacy of the a novel peptidomimetic antibiotic against *P. aeruginosa* in mice.** To address this goal the pre-clinical efficacy of a New Chemical Entity (NCE) was tested in acute and chronic mouse respiratory infection models by systemic or local drug administration. Mice were challenged with the reference PAO1 strain or MDR-RP73 strain and treated with the NCE or ciprofloxacin, an approved antibiotic currently used in clinics, by subcutaneous (s.c.) or pulmonary administrations (i.t.). Body weight, bacterial count and lung inflammation were evaluated at different time points.

- **the protection of novel vaccine candidates against *P. aeruginosa* in mice.** Novel vaccine candidates against *P. aeruginosa* were selected previously by "reverse vaccinology" and by a combination of advanced whole genomic approaches. In this work, recombinant proteins, were tested alone or in combination in murine models for their ability to protect against lethal doses of *P. aeruginosa* reference strain PAO1. Characterization of the most promising proteins in terms of immunogenicity, localization and conservation in a collection of *P. aeruginosa* clinical isolates was carried out.

D. MAIN RESULTS

D.1 *In vitro* activity of a New Chemical Entity (NCE) against *P. aeruginosa* from CF patients

Previous studies demonstrated a potent and selective *in vitro* antibacterial activity of a New Chemical Entity (from now NCE) against *Pseudomonas* spp (Srinivas, Jetter et al. 2010). In the laboratory where this thesis was carried out, the *in vitro* activity of NCE was tested against a panel of *P. aeruginosa* strains from different sources, including sequential CF clinical isolates, reference laboratory strain (PAO1), one of the most abundant genotype (PA14) and the highly transmissible Liverpool epidemic strain (LESB58). The results were obtained in collaboration with POLYPHOR Ltd. The minimal inhibitory concentration (MIC) of NCE against *P. aeruginosa* strains was compared to approved antibiotics (**Table 4**). MIC values of NCE ranged between < 0.0005 - 0.125 μg/mL, with a median of 0.04 μg/mL, for all isolates confirming the potent *in vitro* activity of the compound against a large panel of CF isolates, and showing no difference in activity against mucoid, non-mucoid or hypermutable isolates. In particular, the MDR RP73 *P. aeruginosa* isolate, showed to be resistant to MER, IMP, COL, CAZ, and GEN while was sensitive to NCE. Based on these results, RP73 *P. aeruginosa* isolate was chosen to test the therapeutic efficacy of NCE in models of respiratory infections during my PhD thesis.

Origin	**Strain**	**Genotype/ Phenotype**	**Antibiotic resistance**							
			NCE	**COL**	**GEN**	**CIP**	**CAZ**	**MER**	**TOB**	**IPM**
Reference	ATCC 27853		0.03	0.5	1	0.5	2	0.5	0.25	4
Reference	PA01		0.125	0.125	1	0.125	2	2	0.25	4
Reference	PA14		0.015	0.125	0.5	0.06	2	0.25	2	ND
Epidemic	TB		0.03	1	4	0.125	4	0.25	0.5	4
Epidemic	LESB58		0.06	1	**16**	**2**	**>32**	**4**	1	**8**
CF patient 1	RP1 [e]	A	0.03	2	2	0.25	4	0.5	2	4
	RP2 [e]	A	0.03	0.5	4	0.125	4	1	0.5	4
	RP73 [l]	B	0.125	0.125	**8**	0.5	**16**	**8**	2	ND
	RP74 [l]	B / hypermutable	0.03	0.5	**8**	**8**	**>32**	**8**	0.5	**32**
CF patient 2	AA2 [e]	Θ	0.06	1	**8**	0.25	**8**	2	2	2
	AA11 [i]	Θ / mucoid	0.06	0.125	0.5	0.125	0.5	ND	0.25	ND
	AA12 [i]	Θ / mucoid	0.06	0.06	0.5	0.125	0.5	ND	0.25	ND
	AA43 [l]	Θ / mucoid	0.125	0.5	4	0.25	**8**	1	1	1
	AA44 [l]	Θ	0.03	0.25	**8**	0.5	2	1	1	1
CF patient 3	NN1 [e]	Γ	0.03	0.5	**>32**	1	8	2	**32**	4
	NN2 [e]	Γ	0.03	0.5	**>32**	1	**16**	2	**>32**	4
	NN83 [l]	Δ1 / hypermutable	0.06	1	**>32**	**2**	**32**	**8**	**>32**	**32**

	NN84 [l]	Δ1	0.06	1	**16**	**8**	8	**16**	2	**32**
CF patient 4	BST1 [e]	N	0.06	2	2	0.125	4	0.125	0.5	4
	BST2 [e]	M2	0.03	0.25	0.5	0.06	2	0.06	0.06	0.5
	BST44 [l]	M2 / hypermutable	0.06	0.5	**8**	0.25	**16**	**16**	1	**8**
	BST45 [l]	N	0.06	2	4	0.125	4	0.5	0.25	2
CF patient 5	SG1 [e]	A	0.06	0.5	1	0.125	4	2	0.5	2
	SG2 [e]	Γ / hypermutable	0.06	0.06	4	0.125	**32**	ND	0.5	ND
	SG57 [l]	A	0.06	0.5	4	0.25	4	1	2	2
	SG58 [l]	A	0.03	0.5	4	0.125	4	0.5	0.5	2
CF patient 6	BT1 [e]	Δ2 /hypermutable	0.06	0.06	**32**	0.125	8	ND	**32**	ND
	BT2 [e]	Δ2 / mucoid	0.06	1	1	1	1	0.5	0.125	2
	BT72 [l]	Δ2 / mucoid	0.06	1	**8**	0.125	2	0.25	1	1
	BT73 [l]	Δ2 / mucoid	0.03	0.06	0.125	1	1	ND	0.06	ND
CF patient 7	TR1 [e]	I	0.03	0.5	2	0.125	2	0.5	0.25	0.5
	TR2 [e]	I	0.03	0.5	2	0.25	2	0.5	0.25	1
	TR66 [l]	I	0.06	1	**16**	0.5	**32**	**8**	4	**32**
CF patient 8	KK1 [e]	M1	0.06	1	1	0.125	4	0.5	0.25	2
	KK2 [e]	M1	0.06	1	2	≤0.03	1	0.06	0.5	2
	KK27 [i]	M1	≤0.0078	0.5	1	≤0.03	2	0.25	0.5	1
	KK28 [i]	M1 / mucoid	0.06	1	**8**	0.25	2	0.06	1	1
	KK71 [l]	M1	0.06	1	**8**	**2**	**32**	**16**	1	**32**
	KK72 [l]	M1	0.06	2	**8**	**2**	**32**	**32**	2	**32**
CF patient 9	MF1 [e]	K	0.03	0.5	0.25	0.06	4	0.5	0.125	2
	MF2 [e]	K / hypermutable	0.06	1	2	0.5	8	2	1	4
	MF24 [i]	K	0.06	0.25	**16**	0.5	**>32**	2	4	2
	MF25 [i]	K / mucoid	0.125	0.25	**16**	0.5	**16**	1	4	2
	MF51 [l]	K	0.06	0.5	**16**	**2**	**32**	**16**	4	**32**
	MF52 [l]	Λ	0.03	0.25	**32**	1	8	0.25	**8**	2

Table 4: In vitro activity of NCE and comparators against a panel of P. aeruginosa CF isolates. *MIC values (μg/mL) were determined by the microdilution method in cation-adjusted Müller-Hinton (MH-II) broth, according to the CLSI guidelines (COL= colistin; GEN= gentamicin; CIP= ciprofloxacin; CAZ= ceftazidime; MER = meropenem; TOB= tobramycin; IMP= imipenem). Resistance is indicated in bold (http://www.eucast.org/clinical_breakpoints/). MIC for NCE are indicated in grey. Time of isolation is indicated as "e": early; "i": intermediate; "l": late. Relevant P. aeruginosa strains genotype and phenotype are indicated as previously described (Bragonzi 2006; Bragonzi 2009), and the MDR-RP73 strain used for in vivo efficacy studies is indicated in grey. ND: not determined.*

D.1.1 Therapeutic efficacy of NCE against acute *P. aeruginosa* respiratory infection in murine models

To provide evidence of therapeutic efficacy in models of respiratory infections relevant for VAP or CF patients, murine models of *P. aeruginosa* acute respiratory infection and long-term chronic infection including CF mice were employed for my PhD thesis. The schedule of the experiments is reported in the **Figure 6**.

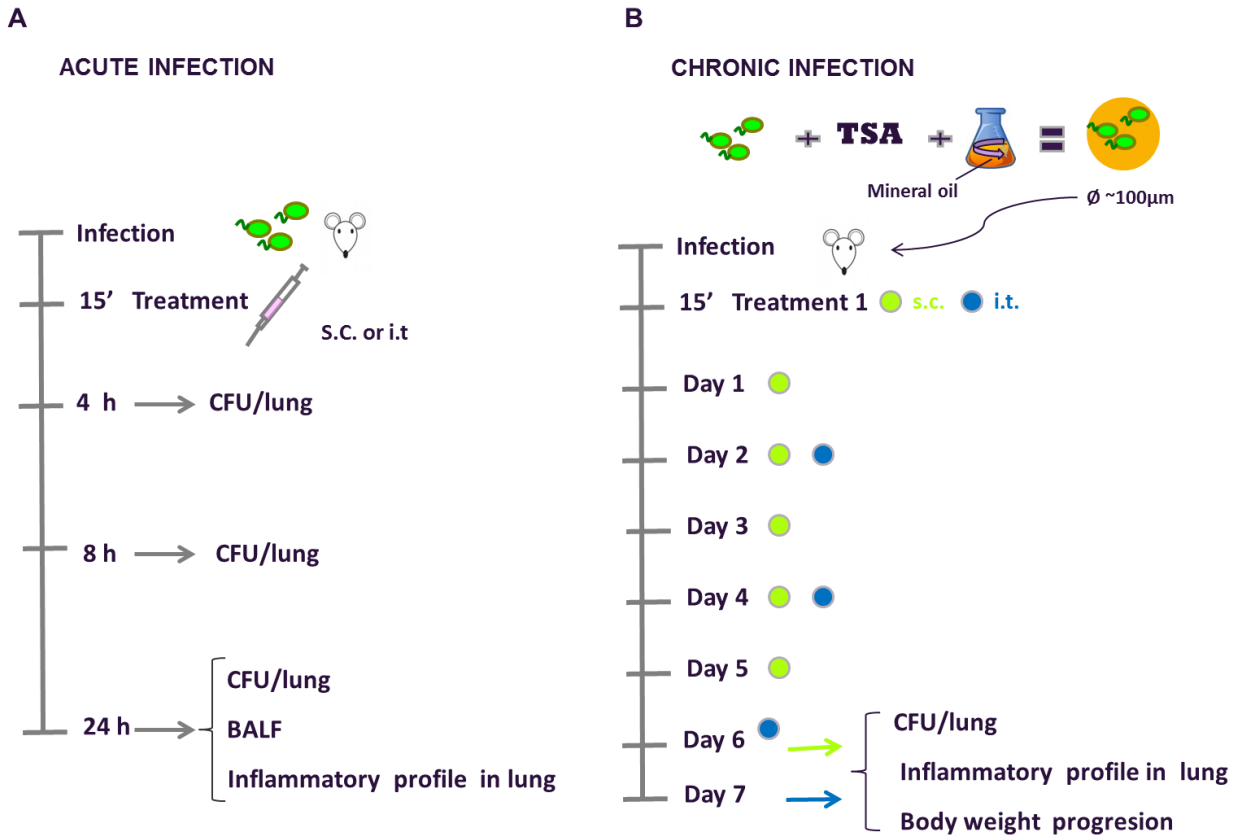

Figure 6: Schedule of treatment with antibiotics in murine models of acute and chronic P. aeruginosa infection and read-outs. *At day 0, C57Bl/6 (8-10 weeks) male mice were infected with P. aeruginosa planktonic cells to mimic acute infection (**A**) or embedded in agar beads to achieve long-term chronic infection (**B**). Schedule of treatment with antibiotics were: i) single dose by s.c. or i.t. route for acute infection or ii) repeated daily s.c. administration or every two days by aerosol for chronic infection. Read-outs of the disease progression were body weight recorded daily (for chronic infection), CFUs counts (4, 8 or 24 h for acute infection or 6/7 days after chronic infection), total and differential cell counts (24 h for acute infection), and cytokines and chemokines analysis assayed at the time of sacrifice (24 h for acute infection or 6/7 days after chronic infection).*

First, the antibacterial efficacy of NCE in a murine model of *P. aeruginosa* acute respiratory infection, previously described (Facchini, De Fino et al. 2014), was tested. C57Bl/6 mice were

challenged with $1x10^7$ colony forming units (CFUs) of *P. aeruginosa* RP73 by intratracheal (i.t.) inoculation. Mice were treated 15 minutes after infection as scheduled (**Fig.6A**) with a subcutaneous (s.c.) injection of 20 mg/kg of NCE or 80 mg/kg ciprofloxacin (CIP), as positive control. A group of mice was treated with saline as placebo control. S.c. treatment was chosen as standard antibiotic administration. Doses of each compound were chosen based on the respective MIC value (**Table 4**) (Saux 1994) and dose response of preliminary experiments (data not shown). The antibacterial effect of NCE and CIP in a time course (4, 8 and 24 hours post-treatment) is shown in **figure 7A**. A slight reduction of lung bacterial load was observed in NCE-treated mice in comparison to saline-treated controls after 8 hours post-treatment (**Fig.7A**). The same trend was observed after 24 hours in both CIP- and NCE-treated mice. However, no statistical significance was reached suggesting that the s.c. route of antibiotic administration may be not optimal.

Next, the pulmonary delivery of antibiotics was investigated as specific application for respiratory infection in CF patients. In this case after the acute infection, performed as described above, mice were treated with 8 mg/kg CIP, 2 mg/kg NCE or saline by i.t. administration (**Fig.7B)** (optimal dose was chosen based on dose response experiments (data not shown)). When NCE was administered i.t., a significant 1 $\log_{10}$ CFUs reduction at 8 hours post-treatment was observed with compared to saline-treated mice. At 24 hours, both NCE and CIP-treated mice showed a significant lower number of bacterial cells in the lungs respect to placebo. NCE displayed higher efficacy as compared to CIP, with 3 $\log_{10}$ and 1 $\log_{10}$ CFUs reduction compared to saline-treated mice, respectively.

Although NCE was effective against the *P. aeruginosa* MDR-RP73 isolate, the complete eradication of the bacterial cells was not achieved. Thus, the antibacterial activity of NCE was tested against the susceptible PAO1 reference strain. C57Bl/6 mice were challenged with $1x10^6$ CFUs of *P. aeruginosa* PAO1 by i.t. inoculation and treated either with 2 mg/kg CIP or 2 mg/kg NCE by i.t. administration, based on the respective MIC values (**Table 4**). A group of mice was treated with saline as placebo group. At 24 hours post-treatment, PAO1 bacterial cells were almost completely cleared from the lungs both in NCE and CIP-treated mice (**Fig.7C**).

In collaboration with POLYPHOR Ltd, the concentration of NCE was determined in lung tissue and plasma to study the absorption and bio-distribution of the compound after either 20 mg/kg s.c. or 2 mg/kg i.t. administration in C57Bl/6 mice. As shown in **Figure 7C**, the compound reached favorable concentrations in the lung after i.t. administration, with rather low systemic exposure.

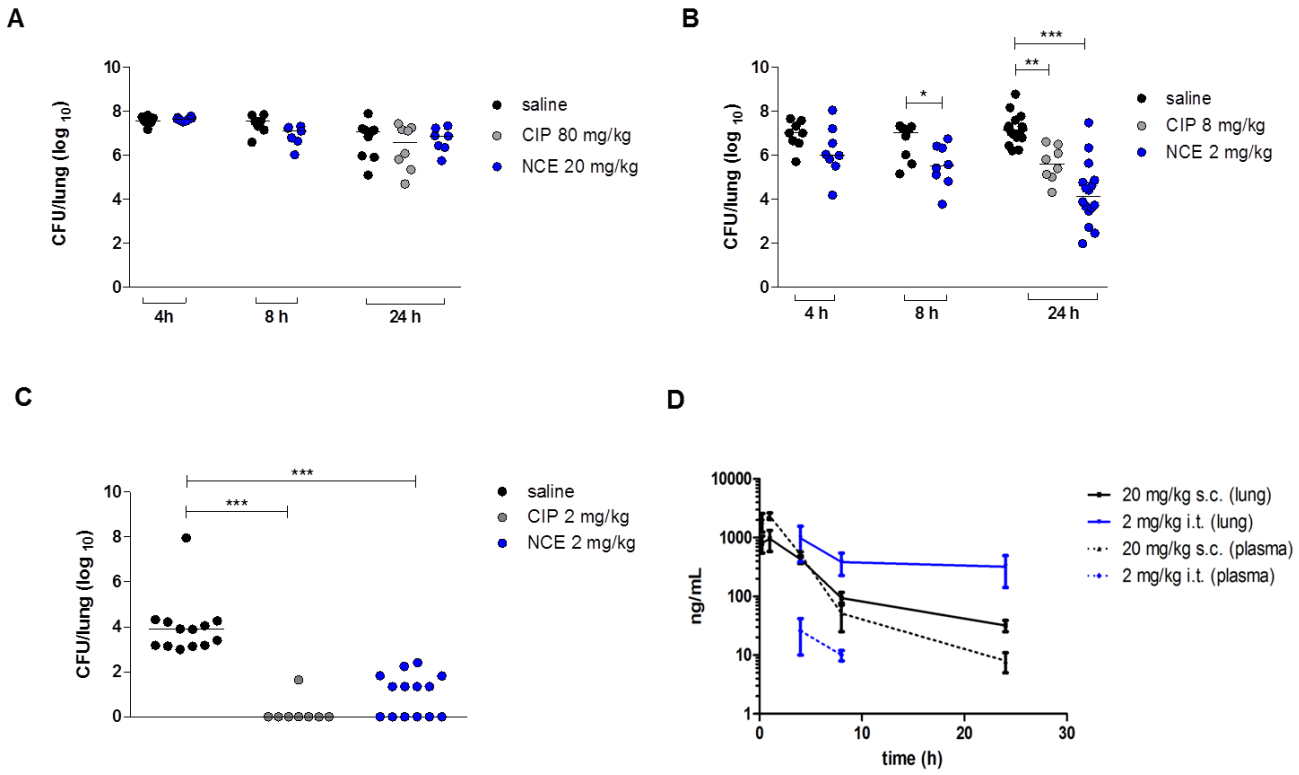

Figure 7: In vivo efficacy and pharmacokinetic of NCE against P. aeruginosa in a mouse model of acute airway infection. *C57Bl/6 (8-10 weeks) male mice were infected i.t. with 1 x 10^7 CFUs of planktonic MDR-RP73 (**A, B**). After MDR-RP73 infection, saline, 80 mg/kg CIP or 20 mg/kg NCE was administered by s.c. route (**A**) while saline, 8 mg/kg CIP or 2 mg/kg NCE was administered by. i.t. route (**B**). C57Bl/6 (8-10 weeks) male mice were infected i.t. with 1 x 10^6 CFUs of planktonic PAO1. After infection, saline, 2 mg/kg CIP or 2 mg/kg NCE was administered by. i.t. route (**C**). Mice lungs were recovered after 4, 8 (**A, B**) or 24 hours (**A-C**), homogenized and plated on Tryptic Soy Agar (TSA) plates to determine bacterial load (**A-C**). Concentration of NCE after 4, 8 or 24 hours from treatment was measured in lung tissue and plasma (**D**). Dots represent individual mice measurements and horizontal lines represent the median values (n=6-14). The data are pooled from two to three independent experiments. Statistical significance by Mann Whitney t-test or one-way ANOVA followed by Dunnett's analysis is indicated:* $p<0.05$, ** $p<0.01$, *** $p<0.001$.*

D.1.2 Modulation of the inflammatory response after treatment with NCE in acute *P. aeruginosa* respiratory infection

To define the effect of NCE i.t. administration on the airway inflammatory response, the broncholaveolar lavage fluid (BALF) was recovered after 24 hours of MDR-RP73 or PAO1 acute infection where total and differential leukocyte recruitment were counted. Results of the cells count showed that mice treated with NCE had significantly less total cells compared to placebo group, indicating a reduction of inflammation. In contrast, CIP-treated mice had no difference in the number of recruited cells when compared to negative controls. In particular, mice infected with

MDR-RP73 or PAO1 and treated with NCE showed a significant decrease in neutrophils, while the amount of monocytes/macrophages was unchanged (**Fig.8A** and **8B**). In the case of CIP-treatment a significant decrease of neutrophils was observed exclusively in PAO1 infected mice but not for MDR-RP73.

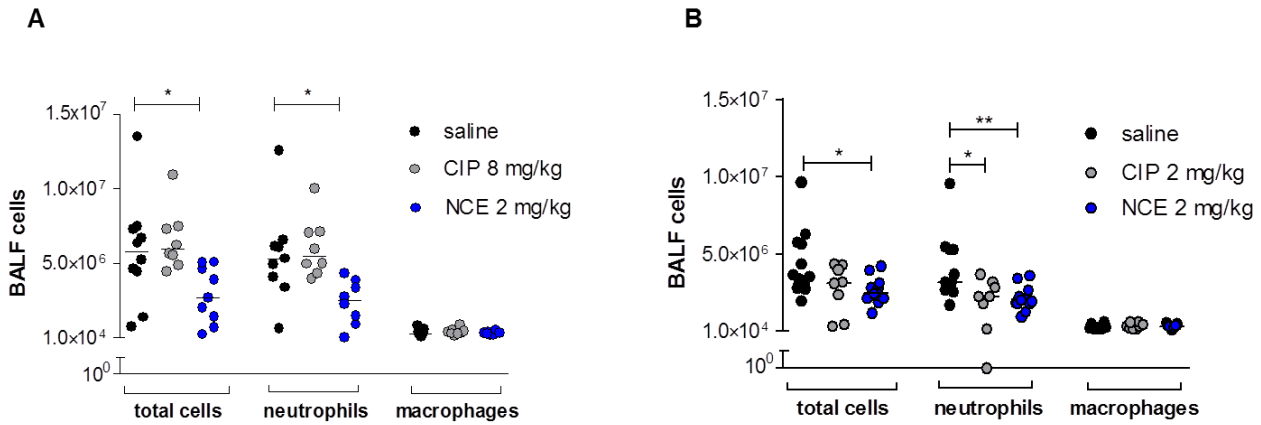

Figure 8: Lung inflammatory response after P. aeruginosa acute airway infection in mice treated i.t. *C57Bl/6 (8-10 weeks) male mice were infected and treated i.t. according to schedule in Fig. 6A with MDR-RP73 (**A**) or PAO1 strain (**B**). BALF was recovered after 24 hours of infection and total cells, neutrophils and macrophages count was performed (**A, B**). Dots represent individual mice measurements and horizontal lines represent the median values (n=8-11). The data are pooled from two independent experiments. Statistical significance by One-way ANOVA followed by Dunnett's analysis is indicated: * $p<0.05$, ** $p<0.01$.*

The concentration of cytokines and chemokines in murine lung homogenate were measured by ELISA. CXCL2/MIP-2, CCL2/JE, and CXCL1/KC production in the lungs was significantly reduced in mice infected with MDR-RP73 (**Fig.9A**) or PAO1 (**Fig.9B)** and treated with NCE compared to saline-treated controls. The only exception was IL-1β which was reduced significantly only in PAO1 infected mice. The same trend of reduction was observed in lung homogenates of CIP-treated mice (**Fig.9)**.

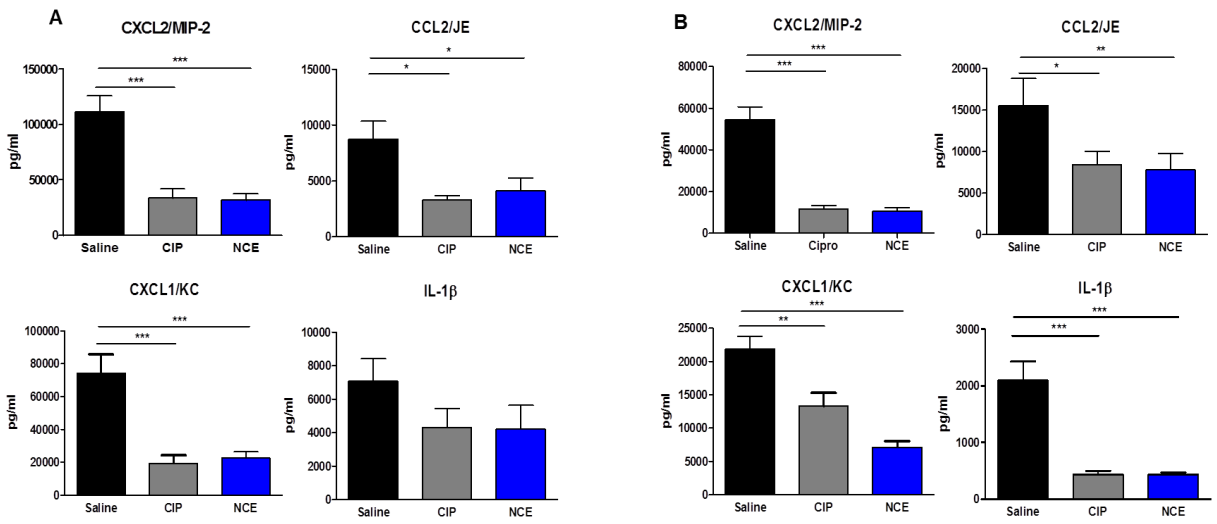

Figure 9: Cytokines and chemokines after P. aeruginosa acute airway infection and antibiotic treatment. *C57Bl/6 (8-10 weeks) male mice were infected and treated according to schedule in Fig.6A. MIP-2, JE, KC and IL-1β levels were measured by ELISA in lung homogenates after 24h of P. aeruginosa acute infection with MDR-RP73 (**A**) or PAO1 strain (**B**). Data represent mean values±SEM of mice (n=6-11) pooled from two to three independent experiments. Statistical significance by One-way ANOVA followed by Dunnett's analysis is indicated: * p<0.05, ** p<0.01,***p<0.001*

D.1.3 Therapeutic efficacy of NCE in *P. aeruginosa* chronic lung infection in murine models

The antibacterial activity of NCE was evaluated in a murine model of *P. aeruginosa* chronic lung infection. As described before this model mimic a chronic infection similar to the one typically established in the lungs of CF patients (Bragonzi 2010). $1x10^6$ CFUs of MDR-RP73 were embedded in agar beads and inoculated by i.t. injection in mice according to established procedures (Bragonzi A 2005; Facchini, De Fino et al. 2014). Starting from the day of the infection, a group of mice was treated daily with a single s.c. dose either of 80 mg/kg CIP or 20 mg/kg NCE and compared to saline-treated controls according to the treatment schedule shown in **Figure 6B**. Body weight loss was recorded in the first two days after infection in all the groups of mice (**Fig.10A**). Thereafter, a faster increase in body weight was observed in NCE-treated mice compared to saline-treated mice. NCE-treated mice recovered almost completely their initial body weight six days post infection. In contrast, the CIP- and saline-treated mice did not recover their body weight completely. At day 6 post infection the CFU counts in the lungs were significantly reduced in mice treated with NCE in comparison to the saline-treated group while no significant differences were observed between CIP-treated mice and controls (**Fig.10B**).

To evaluate the efficacy of NCE administered by aerosol in a model of chronic lung infection, the commercial MicroSprayer^{TM} aerolizer (Penn Century) was used. Treatment with either 8 mg/kg

CIP or 2 mg/kg NCE or saline was started 15 minutes after infection and repeated every second day for a total of four administrations according to the treatment schedule shown in **Figure 6B**. After an initial weight loss observed in the first two days, NCE and CIP-treated mice had a faster and significant increase compared to the saline-treated group of mice, recovering almost completely their initial body weight at day 7 post infection (**Fig.10C**). At day seven mice treated with both NCE and CIP showed a significant reduction of the bacterial load (1 log_{10} and half-log_{10} CFUs reduction respectively) in comparison to the saline-treated group (**Fig.10D**). Even if a significant reduction in the bacterial load was achieved in mice treated with NCE administered both s.c and by aerosol, the local treatment demonstrated to exert a higher efficacy suggesting a better potential therapeutic effect of NCE.

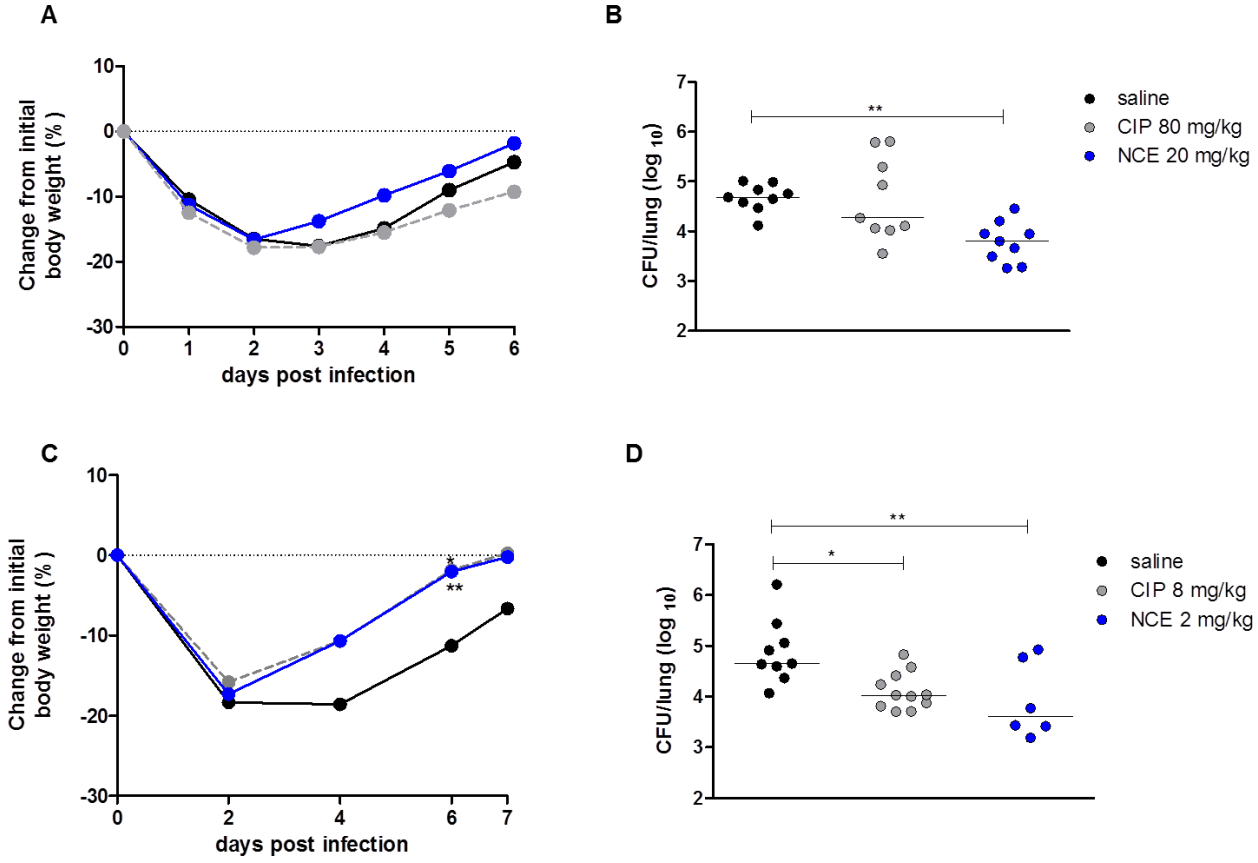

Figure 10: Efficacy of NCE against P. aeruginosa RP73 in a murine model of chronic lung infection after s.c. and aerosol administration. *C57Bl/6 (8-10 weeks) male mice were challenged with 1x10^6 P. aeruginosa RP73, embedded in agar beads, by i.t. inoculation. Mice were then treated s.c. daily with saline, CIP (80 mg/kg), or NCE (20 mg/kg) (**A, B**). Aerosol treatment was performed by Penn Century every two days with saline, CIP (8 mg/kg), or NCE (2 mg/kg) (**C, D**). Before each administration, mice were weighed and changes from initial body weight were averaged for each group (**A, C**). At day six for s.c. treatment and*

*at day seven for aerosol treatment, mice were euthanized, lungs excised, homogenized and plated onto TSA plates to determine bacterial load (**B, D**). Dots represent individual mice measurements and horizontal lines represent the median values (n=6-11). The data are pooled from two independent experiments. Statistical significance by two-way ANOVA with Bonferroni post-test is indicated in the body weight curves, one-way ANOVA followed by Dunnett's analysis is indicated in the CFUs/lung:*
** p<0.05, ** p<0.01.*

D.1.4 Modulation of the inflammatory response after treatment with NCE in *P. aeruginosa* chronic lung infection

Cytokines and chemokines profiles in the lung tissue of *P. aeruginosa* infected mice treated by aerosol, as the most promising treatment, were measured. Results showed that the levels of CXCL1/KC and CXCL2/MIP-2 were significantly reduced in lung homogenate of mice treated with NCE and CIP, compared to saline (**Fig.11**). Levels of IL-1β were reduced significantly only in NCE treated mice while levels of JE were reduced but differences did not reach statistical significance neither in NCE- nor in CIP-treated mice. These data indicates that pulmonary administration of NCE is effective in modulating host response in addition to bacterial burden.

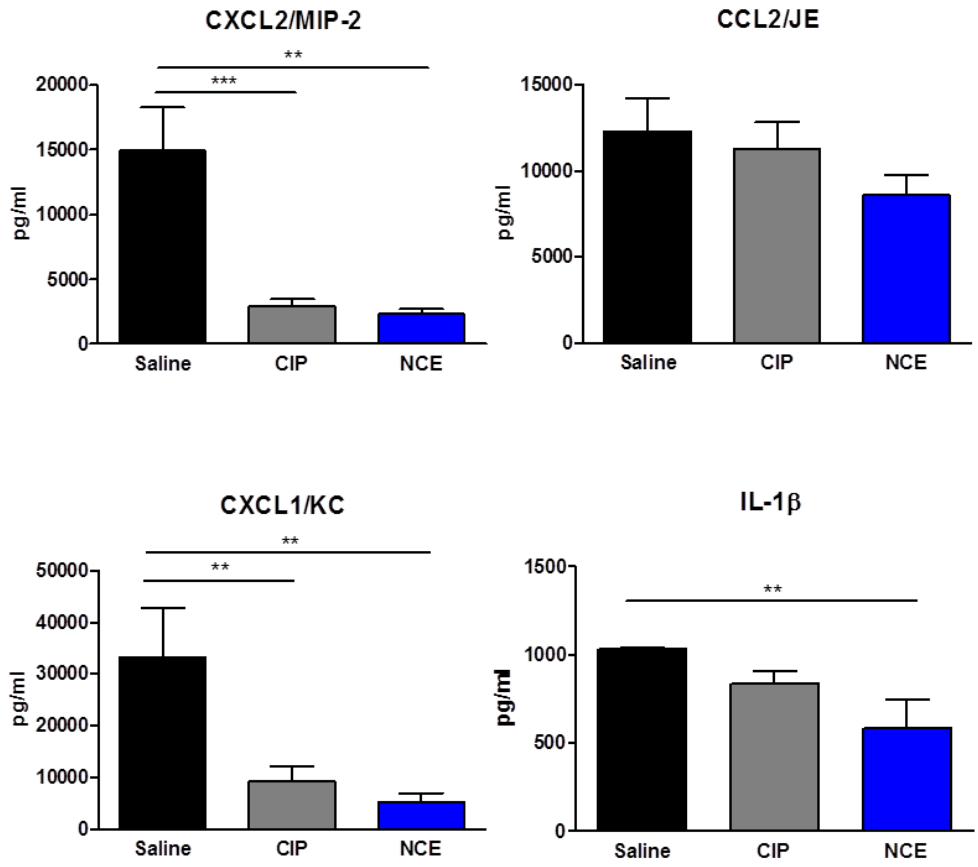

Figure 11:. Cytokines and chemokines after P. aeruginosa chronic airway infection and pulmonary antibiotic administration. *C57Bl/6 (8-10 weeks) male mice were infected and treated according to schedule in Fig.6B. MIP-2, JE, KC and IL-1β levels were measured by ELISA in lung homogenates after 7 days of P. aeruginosa chronic infection with MDR-RP73. Data represent mean values±SEM of mice (n= 6-11) pooled from two to three independent experiments. Statistical significance by One-way ANOVA followed by Dunnett's analysis is indicated: ** p<0.01,***p<0.001.*

D.1.5 Antibacterial and anti-inflammatory effect of treatment with NCE against *P. aeruginosa* chronic lung infection in CFTR-deficient mice.

Since the therapeutic efficacy of NCE may be relevant for CF patients, chronic infection with MDR-RP73 was established also in a CFTR-deficient mice. The mouse model used in these experiments is the *Cftr^{tm1UNC}TgN(FABPCFTR) (Bragonzi 2010).* These knock-out mice bring an insertion in the exon 10 that prevents the formation of the CFTR protein already at mRNA level. Like all CF mice does not represent the human lung pathology of CF patients and they not become infected spontaneously. In particular this mouse model have been gut corrected by the insertion of the human transgene hCFTR that allow a standard survival rate and the facilitated maintenance of the colony *(Zhou, Dey et al. 1994; Bragonzi 2010). Cftr^{tm1UNC}TgN(FABPCFTR)* (CF) and their wt congenic mice (non-CF) were challenged by i.t. inoculation of 1x10^6 CFUs of MDR-RP73,

embedded in agar beads, and treated by MicroSprayer™ aerolizer (Penn Century) 10-15 minutes after infection and then every second day, for a total of four administrations, either with saline or 2 mg/kg NCE.

Body weight loss was observed in the first two days after infection both in saline- and NCE-treated mice (**Fig.12A**). While saline-treated mice did not recover weight until day seven, mice treated with NCE gained weight from the second day onwards, with a significant difference compared to the saline-treated mice from day four. After seven days of chronic infection, mice treated with NCE showed a significant reduction in the bacterial load in comparison to the saline-treated group (**Fig.12B**). No significant differences in bacterial load between CF or non-CF mice has been observed indicating that the treatment is effective in different genetic background and is not influenced by CF environment.

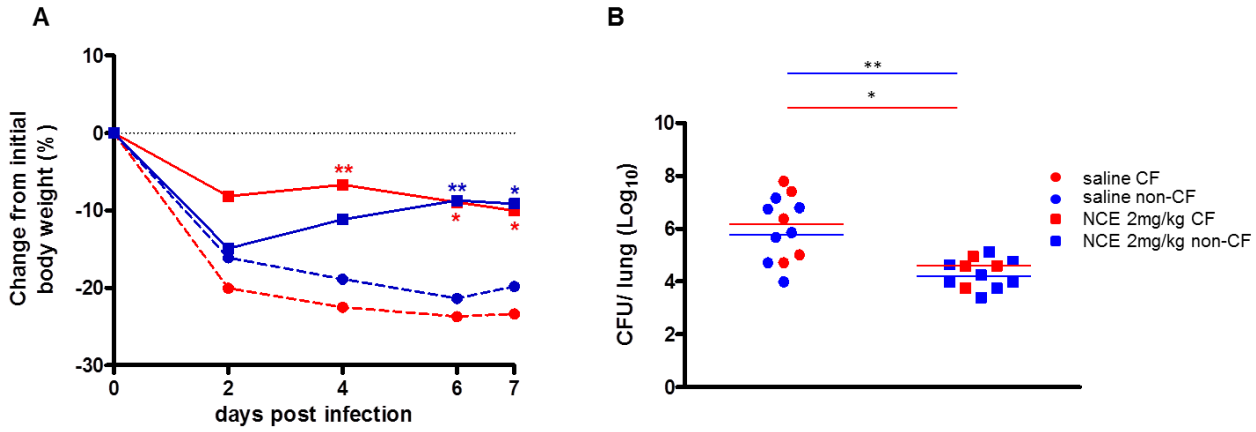

Figure 12: Efficacy of NCE against P. aeruginosa RP73 in CF murine model of chronic lung infection after endotracheal nebulization with MicroSprayer ™ aerolizer. *Cftr^{tm1UNC}TgN(FABPCFTR) and their wt congenic mice (11-17 weeks old) were challenged with 1x10^{6} P. aeruginosa RP73, embedded in agar beads, by i.t. inoculation. Next, mice were treated by MicroSprayer ™ aerolizer after infection and then every second day with a single dose of saline or NCE (2 mg/kg) for a total of four administrations. Before each administration, each mouse was weighed. Changes from initial body weight were calculated for each group of mice (**A**). At day seven, mice were euthanized, lungs excised, homogenized and plated on TSA plates to determine bacterial load (**B**). Dots represent individual mice measurements of CF mice (red) (n=9) or wt (non-CF) congenic mice (blue) (n=15) and horizontal lines represent the median values. The data are pooled from two independent experiments. Statistical significance by two-way ANOVA with Bonferroni post-test in the body weight curve graph, and one-way ANOVA followed by Dunnett's analysis in the CFUs/lung graph is indicated. * p<0.05, ** p<0.01.*

The levels of CXCL2/MIP-2 were significantly reduced in lung homogenates of both CF and non-CF mice treated with NCE when compared with saline-treated mice, IL-1β was significantly reduced only in CF mice but not in non-CF mice, while KC and JE were reduced but did not reach statistical significance (**Fig.13**).

Taken together, these results suggest a therapeutic effect of NCE also in CF mice.

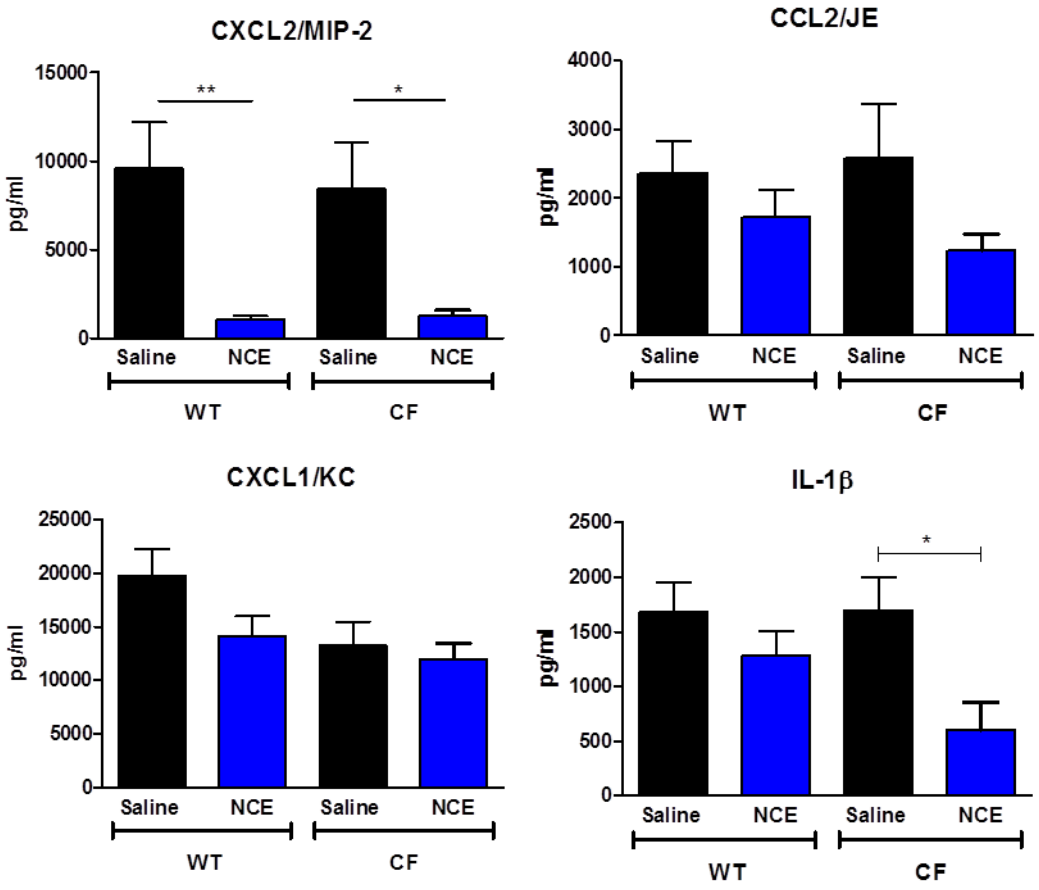

Table 13: Cytokines and chemokines after P. aeruginosa RP73 chronic airway infection in CF and non-CF mice treated with NCE by pulmonary administration. *$Cftr^{tm1UNC}$TgN(FABPCFTR) (CF) and their wt congenic mice (non-CF) (11-18 weeks) were infected with RP73 embedded in agar beads and treated with saline or NCE 2 mg/kg by Penn Century according to schedule of Fig. 6B. Lungs were collected after 7 days of infection and MIP-2, JE, KC and IL-1β levels were measured by ELISA on lung homogenates. Data represent mean values±SEM. The data are pooled from mice from two independent experiments (n=4-8). Statistical significance by Mann-Whitney U test analysis is indicated: * p<0.05, ** p<0.01*

D.2 Selection of novel vaccine candidates against *P. aeruginosa* in murine model of acute respiratory infection

In years before my PhD, the genomic sequence of reference strain PAO1 (Stover 2000) was analyzed *in silico* to identify open reading frames (ORFs) that potentially encoded novel surface-exposed or exported proteins. This work was carried out in the laboratory of Dr. Bragonzi with collaboration of Novartis-Vaccine. Among 5,570 ORFs, there were identify 196 secreted or outer membrane proteins, 124 periplasmic proteins, 2111 inner membrane proteins and 2327 citoplasmic proteins, while 812 ORFs were not assigned to a sub-cellular localization and were annotated as unknown. For the following analysis attention was pointed on 320 proteins belonging to the outer membrane or to the periplasmic space. To verify that the selected proteins were not unique to the PAO1 strain, conservation of the antigens were evaluated among seven sequenced *P.aeruginosa* strains deposited in Pseudomonas Genome Database for Comparative Analysis. Conserved proteins were then checked for similarity to those present in human and mice and similar epitopes were excluded. Next, selected proteins were grouped by ranking score based on their gene expression profile under aerobic and anaerobic growth carried out in this work, their immunogenicity (Montor 2009) and relevance for *in vivo* infection as reported by other databases (Potvin 2003). The final database included 53 antigens of which 29 proteins of known and 24 unknown functions. Successful expression in *E. coli* of 33 ORFs (62.3%) as His-tag fusions were obtained and finally 32 ORFs (60.4 %) were obtained in enough amount for mice immunization (**Fig.14A**).

During my PhD, novel identified vaccine candidates against *P. aeruginosa* were tested for efficacy in a murine model of *P. aeruginosa* acute lung infection reported in **Figure 14B**. Vaccination protocols were set up in these models.

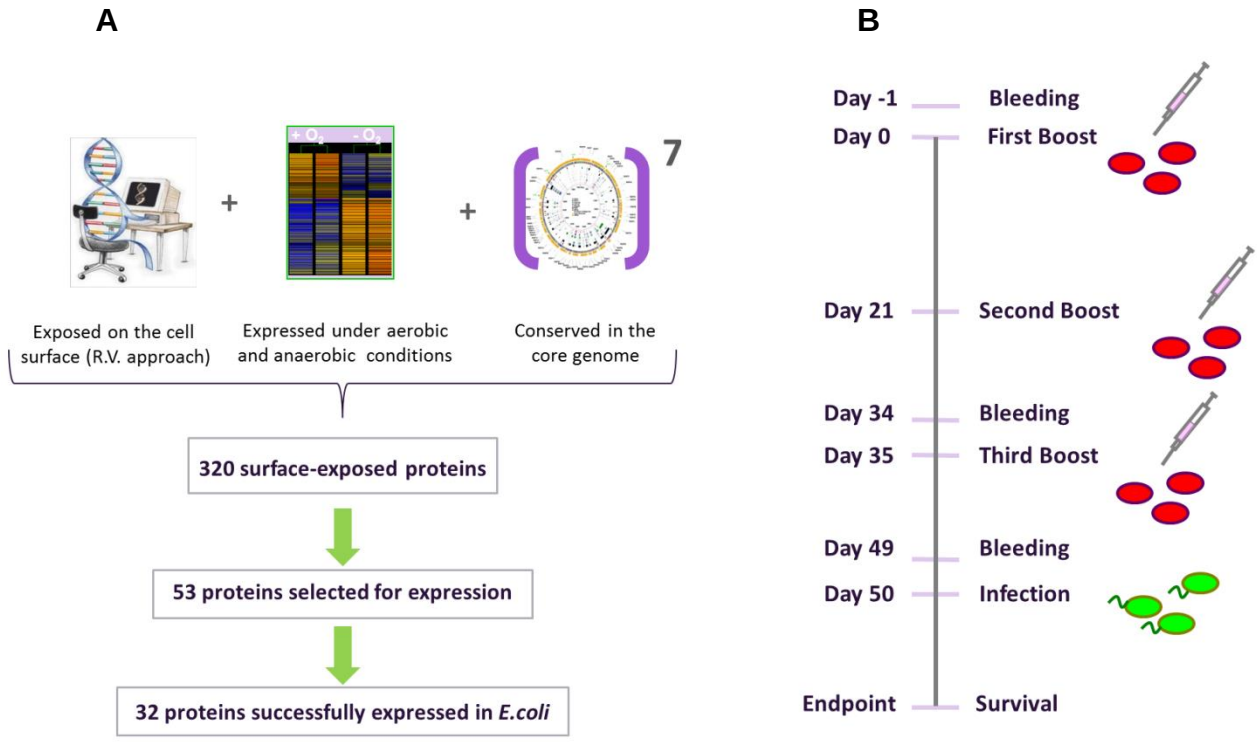

Figure 14: Strategy for the identification of novel vaccine candidates against P. aeruginosa and their validation in murine model of acute P. aeruginosa infection. *By the combination of "reverse vaccinology" and advanced whole genomic approaches, novel vaccine candidates were identified. 32 proteins were successfully expressed in E.coli and purified for mice immunization (**A**). At day 0, 21 and 35 groups of 10 C57Bl/6 male mice (5 weeks) were immunized intraperitoneally (i.p.) with 10 µg of recombinant protein/s adsorbed in alum (alone or in combinations of two of them). To obtain antisera, mice of all groups were bleed at day -1, day 34, and day 49. As negative control, 10 mice each immunization round were injected with alum alone, while as positive control 10 mice each immunization round were boosted with 10^7 cfu of heat inactivated PAO1 strain. On day 50, mice were challenged with $5x10^6$ CFU (first lethal dose) of planktonic P. aeruginosa PAO1 homologous strain to mimic acute infection. Mice were monitored for survival for 120 hrs at intervals of twelve hours and compared with un-vaccinated and PAO1 vaccinated control groups (**B**).*

D.2.1 Protection to *P. aeruginosa* acute pneumonia after immunization with single antigens or their combinations

Protection after immunization with selected antigens was tested in a mouse model of acute pneumonia. C57Bl/6 mice were immunized intraperitoneally (i.p.) with 10 µg of selected proteins, challenged intratracheally (i.t.) with a lethal dose of *P. aeruginosa* ($5x10^6$ CFU/lung) and monitored for survival as schedule in **Figure 14B**. Proteins PA number were coded due to current patent rights of the application number:13798638.6-1403. Vaccination with ten single antigens showed a shift in

the survival curve (survival up to 25% at day 5) when compared with a negative control group (alum alone) (mortality 100% within day 2), although this was not always significant. In particular PSE10, PSE11, PSE21, PSE27, PSE21, PSE44, PSE47, PSE52, PSE53 and PSE54 (**Fig.15A**) where selected for further investigation while the other 21 proteins tested did not differ substantially from the negative control.

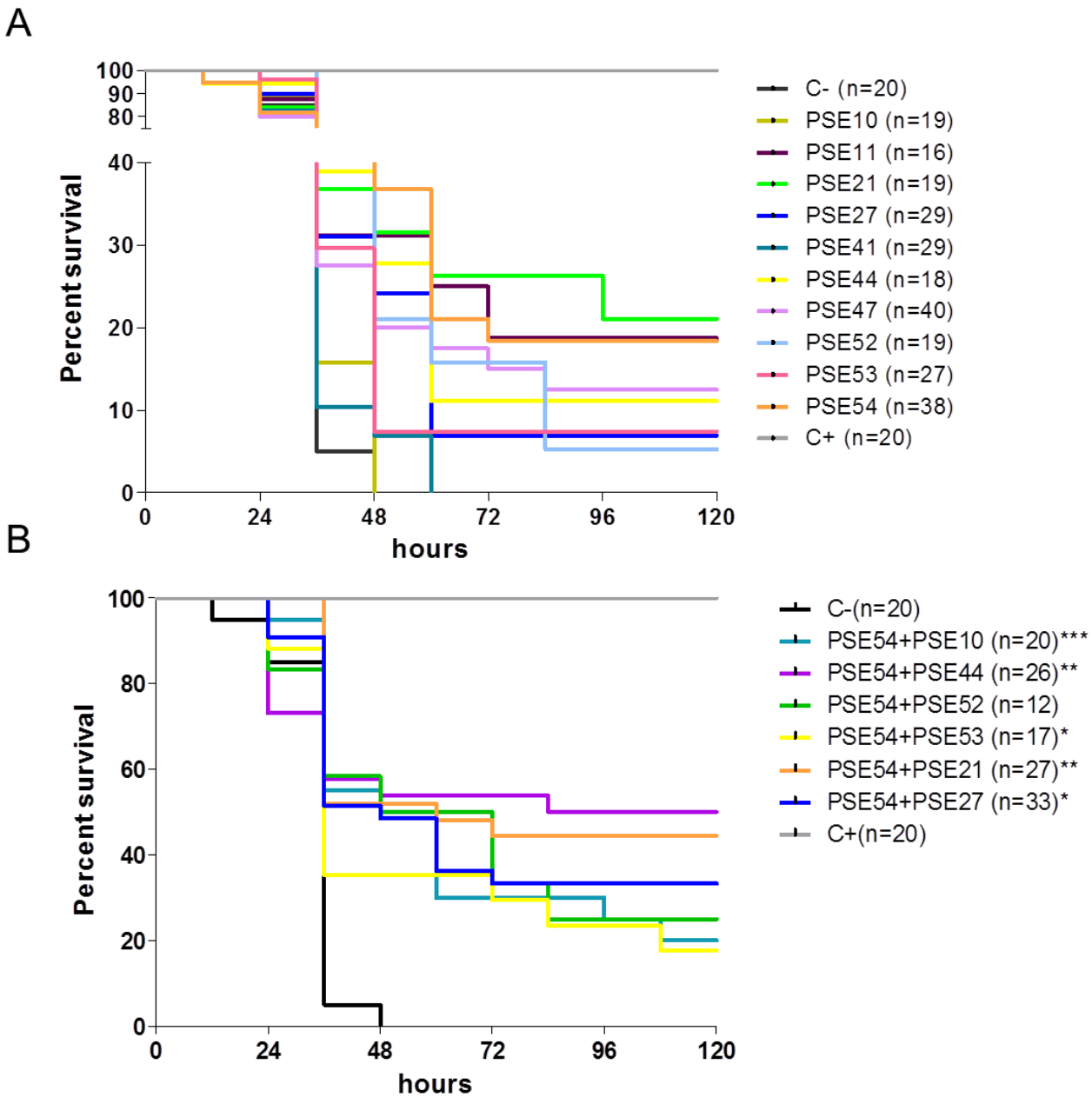

***Figure 15**: **Survival curves of the ten single antigens selected as vaccine candidates and best combinations of two proteins.** C57Bl/6 male mice were challenged with PAO1 ($5x10^6$ CFU) two weeks after vaccination with ten single antigens (A) or cocktails (B) (colored lines). Black line and grey line indicate survival curves of representative mice from negative control (immunization with alum alone) and PAO1 heat inactivated groups respectively. A moderate increase in the survival was observed for mice vaccinated with single P. aeruginosa antigens respect to the negative control (A) while a significant increase was observed with five*

*proteins combination (B). Results are represented in Kaplan–Meier survival curves and analyzed by the Mantel-Cox test against negative control group : *< 0.05, ** < 0.01, ***< 0.001. n refers to the number of animals.*

Given that survival of mice to *P. aeruginosa* infection in mice vaccinated with single antigen was modest, we combined the most promising proteins two by two. 22 combinations were used to vaccine mice followed by *P. aeruginosa* infection as described above. Five out 22 protein combinations (PSE54 + PSE10, PSE54 + PSE44, PSE54 + PSE21, PSE54 + PSE53, PSE54 + PSE27) showed a significant increase in both survival curves (Mantel-Cox test p value < 0.0002, 0.0019, 0.0027, 0.015 and 0.015 respectively) and mean survival time (one way ANOVA p value < 0.01) (**Fig. 15B and Fig.16**). The best antigens combination was PSE54 + PSE44 showing an increase in survival up to 50%.

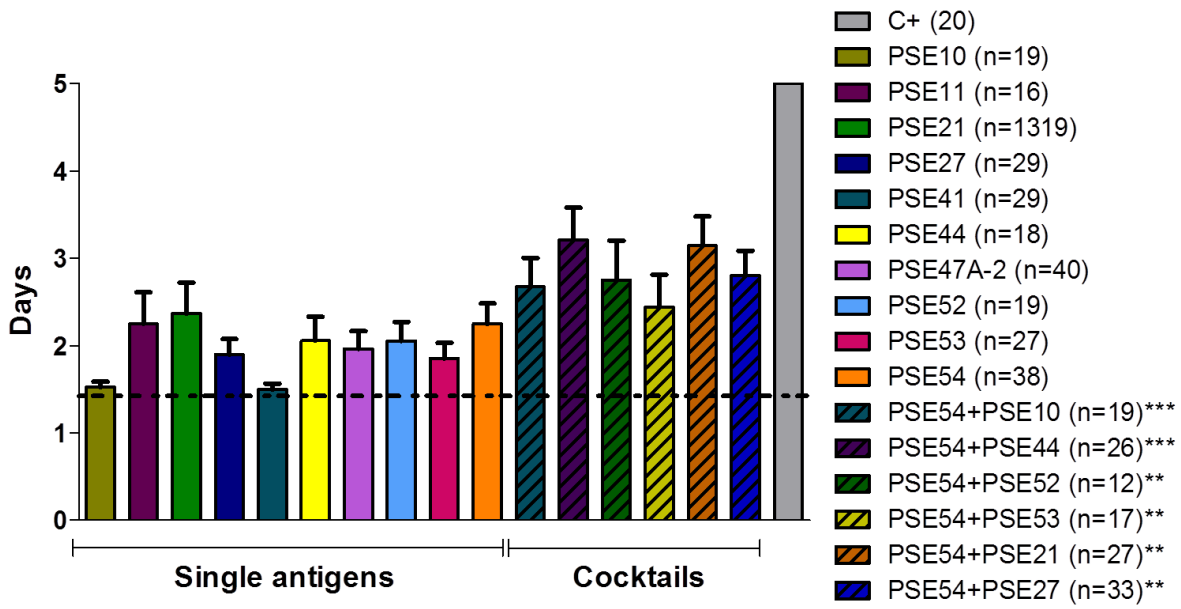

Figure 16: Mean survival time of the ten single antigens selected as vaccine candidates and best combinations of two proteins. *Mean survival time was calculated based on the survival curves. Bars represent mean values of single antigens (colour bars) and combinations (colour striped bars) and PAO1 heat inactivated (grey bar). Error bars represent the standard error of the mean (SEM). Dashed line indicates the mean survival time of representative mice immunized with alum alone. Results are analyzed by one way ANOVA against negative control group : ** < 0.01, ***< 0.001. n refers to the number of animals.*

D.2.2 In vitro characterization of selected antigens

To further characterize the antigenic potential and effective cellular localization of the selected antigens PSE10, PSE11, PSE21, PSE27, PSE21, PSE44, PSE47, PSE52, PSE53 and PSE54, the antisera obtained immunizing with the recombinant proteins were tested in western blotting (WB) and *P. aeruginosa* immunofluorescence. Results showed that all the antisera were able to recognize the recombinant proteins in WB, demonstrating the capacity of the vaccine candidates to induce specific antibody production. Next, to understand if antibodies were able to recognize also native proteins, WBs against whole cell extracts of the homologous *P. aeruginosa* strain PAO1 and the clinical isolate MDR-RP73, both grown in stationary and exponential growth phases, were carried out. Also in this case all the antisera of the selected proteins were able to recognized the native proteins of both PAO1 and MDR-RP73 *P. aeruginosa* strains. Results are summarized in **Table 5**.

protein	Western Blot			Immuno-fluorescence	Amino acid identity among CF collection (number of strains with the full length gene sequence/ total n° of strains)
	Recombinant protein	PAO1	RP73		
PSE10	+	+	+	+	100% ± 0% (19/19)
PSE11	+	+	+	+	99.6%± 0.3% (19/19)
PSE21	+	+	+	+	99.8% ± 0.2% (17/19)
PSE27	+	+	+	+	99.3% ± 0.2% (16/19)
PSE41	+	+	+	+	100% ± 0% (19/19)
PSE44	+	+	+	+	99.1% ± 0.8% (18/19)
PSE47	+	+	+	+	98.8% ± 0.5% (11/19)
PSE52	+	+	+	+	99.7%± 0.3% (19/19)
PSE53	+	+	+	+	99.9% ± 0.1% (18/19)
PSE54	+	+	+	+	98.8% ± 0.3% (17/19)

Table 5. In vitro characterization of the 10 vaccine candidates. *Solubilized recombinant proteins and whole cell lysates of the homologous strain PAO1 and the clinical isolate RP73, both in stationary and exponential growth phases, were separated by sodium dodecyl sulphate (SDS)-PAGE and electrotransferred onto nitrocellulose membranes (0.45µm) using a mini transblotter (Bio-Rad) following the manufacturer's instructions. Membranes were blocked 1 hour at RT in blocking buffer. Specific pooled sera from each group of mice collected from immunized mice at day 49 were used O/N at 4°C. Specific antibodies were detected with polyclonal rabbit anti- mouse. Bands at expected weight for the proteins were observed and indicated as +. Localization of proteins was performed on PAO1 growth spread on a coated slide. After fixation with 4% PFA and incubation in blocking solution, bacteria were incubated with mouse anti sera (1:50) and specific rabbit anti P. aeruginosa cell wall (1:800). Primary antibodies were labelled with anti-mouse Fab-*

Jackson and Texas Red-labeled goat anti-rabbit. Co-localization of proteins with the cell wall is indicated as +.[#] *Average ± standard deviation.*

To determine whether the selected proteins were effectively expressed and exposed on the cell surface of bacterial cells a double immunofluorescence was carried out with the murine antisera and a specific antibody for *P. aeruginosa* anti-cell wall. Co-localization of the two signals suggested that the presence of proteins in the bacterial cell surface. All the selected antigens were surface exposed supporting the robustness of the screening method used in this study (**Table 5 and Fig.17**).

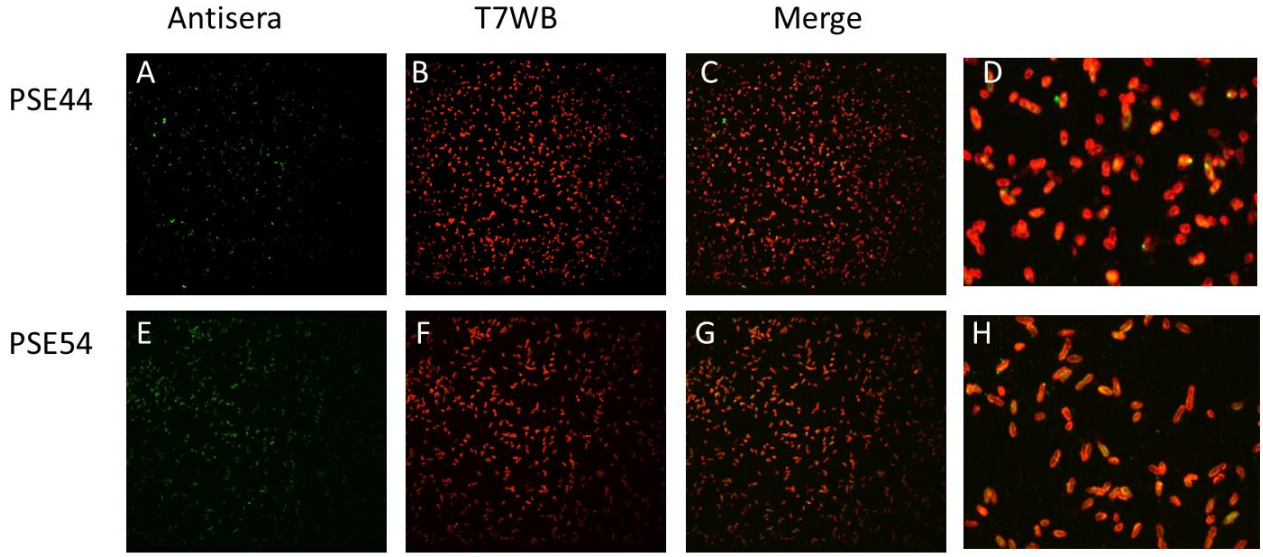

Figure 17: Cellular localization of vaccine candidates PSE44 and PSE54 by immunofluorescence*. Immunofluorescence staining observed with confocal microscopy shows localization of antigens (green) PSE44 (A), PSE54 (E), and bacteria cell wall (red) (B) (F) in PAO1. Co localization of both signals (yellow) indicates that proteins PSE44 and PSE54 are surface expose (C) (D) and (G) (H) respectively*.

D.2.3 Conservation of selected antigens among a collection of CF clinical isolates

To test suitability of the selected proteins as candidate antigens for conferring protections against different *P. aeruginosa* strains and not just against the homologous strain, we used a collection of 19 CF clinical strains isolated at the onset of infection and after years of chronic colonization. Total gene sequences of the 10 proteins were obtained in most of the 19 strains. To summarize, PSE10, PSE11, PSE41 and PSE52 showed 100% ± 0%, 99.6 % ± 0.3%, 100% ± 0%, and 99.7%± 0.3% average of amino acid identity to the PAO1 sequence, respectively, within the 19 *P. aeruginosa* CF clinical isolates. PSE44 and PSE53 showed 99.1% ± 0.8%, 99.9% ± 0.1%, identity to the PAO1 sequences, respectively, within 18 CF clinical isolates. PSE21 and PSE54 showed 99.8% ± 0.2%, 98.8% ± 0.3%, identity to PAO1 sequences, respectively, within 17 CF clinical isolates. PSE47 showed 98.8% ± 0.5%, identity to PAO1 sequence, respectively, within 11 CF clinical isolates

whereas PSE27 showed 99.3% ± 0.2% of amino acid identity in the 16 strains analyzed (**Table 5 and Table 6**). The results suggest that these proteins may induce immunity against most strains of *P. aeruginosa* including the late adapted strains isolated from CF patients.

Recombinant proteins	**aa Mutation**	***P. aeruginosa* clinical isolates**
PSE10	-	
PSE11	Q_{53}-M	PAO1 vs AA2, AA43, AA44
	P_{450}-Q	
	P_{238}-A	PAO1 vs MF1, MF51
	T_{82}-A	PAO1, SG58 vs SG1, SG57, AA2, AA43, AA44
	F_{88}-L	PAO1 vs TR1, TR66, TR67, KK1, KK71, KK72, BST2, BST44
	S_{478}-G	PAO1 vs AA2, AA43, AA44, MF1, MF51
PSE21	A_{641}-V	PAO1 vs MF1, MF51
	N_{437}-D	PAO1 vs TR1, TR66, TR67, KK1, KK71,BST2
	T_{534}-S	PAO1 vs AA2, AA43, AA44, TR1, TR66, TR67, MF1, MF51, KK1, KK71, BST2
PSE27	N_{195}-S	PAO1 vs AA2, AA43, AA44, BT2, BT73, AA2, AA43, AA44, BST2, BST44, KK1, MF1, MF51, TR1,TR66,TR67
	K_{55}-E	PAO1 vs SG1, SG57, SG58, BT2, BT73, AA2, AA43, AA44, BST2, BST44, KK1, MF1, MF51, TR1, TR66, TR67
	Q_{314}-L	
	H_{516}-R	PAO1 vs SG1, SG57, SG58, BT2, BT73
	D_{580}-E	
	L_{609}-I	

	T_{585}-I	PAO1 vs BST2, BST44, KK1
	R_{416}-S	PAO1 vs BST44
	A_{486}-T	
	D_{538}-E	
	V_{600}-E	
PSE41	-	
PSE44	D_{169}-E	PAO1 vs BT2, BT72, BT73
	S_{111}-N	PAO1 vs TR1, TR66, TR67
	S_{241}-N	PAO1 vs MF51
PSE47	S_{23}-C	PAO1 vs SG1, SG57, SG58
	N_{293}-S	
	V_{367}-L	
	N_{748}-Y	
	E_{964}-K	
	H_{992}-R	PAO1 vs BT2, BT72, BT73
	N_{748}-D	BT2 vs BT72
	N_{748}-Y	BT2 vs BT73
	N_{187}-Q	PAO1 vs KK1
	S_{280}-T	PAO1 vs MF1

	K_{292}-S	
	H_{187}-Q	PAO1 vs MF1, BST 44
	N_{318}-K	PAO1 vs BT2, BT72, BT73, TR1, TR67
	V_{39}-A	PAO1 vs SG1, SG57, SG58, BT2, BT72, BT73, TR1, TR67,
	S_{629}-N	PAO1 vs SG1, SG57, SG58, BT2, BT72, BT73, KK1, BST44
	A_{633}-T	
	G_{643}-A	
	G_{700}-A	
	M_{701}-T	
	T_{801}-M	PAO1 vs SG1, SG57, SG58, BT2,BT72,BT73, KK1
	S_{818}-G	PAO1 vs SG1, SG57, SG58, BT2, BT72, BT73
	V_{878}-F	
	N_{915}-S	
	A_{882}-T	PAO1 vs TR1, TR67, MF1
	G_{916}-S	
	S_{917}-G	PAO1 vs SG1, SG57, SG58, BT2, BT72, BT73, TR1, TR67, MF1
	N_{403}-S	PAO1 vs SG1, SG57,SG58, BT2, BT71, BT72, TR1, TR67, MF1, KK1, BST44
	L_{958}-V	
PSE52	A_{138}-E	PAO1 vs SG1,SG57, SG58, BT2, BT72, BT73, MF1, MF51

PSE53	A_{421}-T	PAO1 vs MF1, MF51
PSE54	S_{142}-C	PAO1 vs SG1, SG57,SG58
	T_{12}-A	PAO1 vs BT2, BT72, BT73
	E_{104}-C	PAO1 vs TR1, TR66, TR67
	S_{110}-N	PAO1 vs MF1, MF51
	E_{114}-D	PAO1 vs AA2, AA43, AA44, KK1, KK71, KK72
	S_{128}-N	PAO1 vs SG1, SG57,SG58, BT2, BT71, AA2, AA43, AA44, TR1, TR66, TR67, MF1,MF51, KK1, KK71, KK72, BST2

Table 6: Amino acid substitution between PAO1 and a collection of P. aeruginosa clinical isolates. *A collection of 19 CF clinical strains isolated at the onset of infection and after years of chronic colonization were sequenced for the 10 vaccine candidates. Nucleotide sequences were translated into the amino acid (aa) sequences and compare with PAO1 protein sequence. Forward (For) primers and reverse (Rev) primers used for the gene amplification/sequencing are listed: PA1178: For-TAGAAAGCCTAGACCCTACTTG, Rev- TCTTCCACTACCAGCAGTTT; PA1248: For- CTGCTCAATTACCTGTTCAAG, Rev-AAGACAAACTACCGAAGACACT; pa5112: For- CTGATCGAGCGCGACAATAC, Rev-GTGTCGTCCTCGTACTCACG; pa0328: For-ATGAAACGGTCCGCATCCTG Rev-CGAGCCGAACCTGTTCTACGT; PA2407: For- CATTGATCGCACATCGACTC, Rev- GTCTTGACCAGCGAACTCTTG; PA3526: For- GATACATCCTTCGTATTTGGAC, Rev- CATTCGGGAAATTACAGAGG; PA4082: For- CAGAGAGATACCCGTAGGAGTT, Rev-CTCATGGAACTCTCCAAGATT; PA4765: For- GAAGTTCGCTATTTTCAACCAT, Rev- GTATCTTCGGCAAGCTCCTG; PA5047: For-GTATCTGGCTGGAGATGGAC, rev- TTTATTGCTTGTTGGACAGAC; PA5340: For- CTCGAGTAAGCCGGATGTTC, Rev- GCGGACTGTACTTCCTCTGG.*

E. CONCLUSIONS AND FUTURE PROSPECTS

The continuous increasing rate of infections caused by antibiotic-resistant bacteria is an alarming issue nowadays all around the world. The so called ESKAPE pathogens, the most dangerous multidrug resistant ones, literally escape to the effect of available treatments (Boucher, Talbot et al. 2009). *Pseudomonas aeruginosa* is part of this category. It is a Gram-negative pathogen, considered one of the top three causes of opportunistic human infections being able to target a wide range of patients at different body sites. Of particular interest are *P. aeruginosa* airways infection that affect mainly immunosuppressed patients, patients in intensive care units which may develop ventilator-associated pneumonia (VAP) due to the ventilation or diseases characterised by airways inflammation and altered mucus production like cystic fibrosis (CF) or chronic obstructive pulmonary disease (COPD) (Williams, Dehnbostel et al. 2010). Despite this worrisome situation in the last decades the number of drug candidates in clinical development relevant for *P. aeruginosa* infections are really low and most of the them are modifications of existing molecules. Thus, the development of antibiotics with new mechanism of action and/or alternative therapies remain at present the best alternatives for the treatment of *P. aeruginosa* infections. During my PhD, I focused my attentions in the pre-clinical evaluation of a New Chemical Entity (from now NCE). This NCE is a synthetic peptide identified by the innovative technology of protein epitope mimetics (PEMs). PEM technology was applied to identify a new class of synthetic molecules that exert potent antibacterial activity with novel mode of action. Peptidomimetics are compounds whose essential elements mimic a natural peptide or protein in 3D space and which retain the ability to interact with the biological target and produce the same biological effect. They have been designed to circumvent some of the problems associated with a natural peptide like stability against proteolysis (duration of activity) and poor bioavailability (Vagner, Qu et al. 2008). NCE is a peptidomimetic specifically effective against *P. aeruginosa* from different origins. In particular it has demonstrated to be active in a nanomolar range against a large collection of *P. aeruginosa* strains isolated from CF patients, including mucoid and hypermutable isolates, whose phenotypes have been associated to antibiotic resistance and poor prognosis or respiratory function in CF patients (Bonfield, John et al. 2005; Ferroni, Guillemot et al. 2009).

One critical point in the passage form pre-clinical efficacy evaluation of an antibacterial agent to human clinical trials is the animal model used to assess efficacy and safety (Coller and Califf 2009). In this context the best choice is to select the infection model that most closely mimics the course of human disease. Acute infection conditions are often characterized by heavily injure or kill the host, with multiorgan failure that can occur within hours or days. On the other hand, chronic infections

occur without rapid injury of the host and in the presence of biofilm structures, a population of microorganisms that aggregates on a matrix which develops over days or weeks that contain bacterial genetic variants. To investigate the efficacy of NCE in treating clinical conditions such as VAP or life threatening infection in CF patients, animal models of acute and chronic *P. aeruginosa* respiratory infection have been used (Bragonzi 2010; Facchini, De Fino et al. 2014). Another important issues to take into consideration should be the route of drugs administration and the endpoints of the study. The route of drugs administration should be those that most closely reproduce the clinical settings and the endpoints must be clinically relevant. Two different administration routes were tested for NCE and based on human clinical response to infection, we defined two endpoints: infection and inflammation. To evaluate the potential therapeutic effect of NCE, taking into account all the critical points described above, acute and chronic pneumonia model of *P. aeruginosa* infections have been used. In particular MDR-RP73 clinical isolate, strain isolated after years of colonization from a CF patient, and the PAO1 reference laboratory strain were used. Ciprofloxacin (CIP) was chosen as positive control because is one of the most effective clinically-approved antibiotics. First, treatments were administered by subcutaneous (s.c.) injection in a murine model of acute lung infection with the MDR-RP73 clinical isolate. S.c. administration in acute infection showed no significant efficacy in reducing the bacterial load in lung both for NCE- and CIP- treated mice, suggesting that this route of administration may be not optimal to treat this kind of respiratory infections. Then, treatments were administered locally by intratracheal injection (i.t.) in the same murine model. I.t. administration of NCE showed a significant reduction of the bacterial load in the lung demonstrating a high efficacy of the drug in mice challenged with a MDR strain. Despite the bacterial load reduction was significant against the MDR-RP73 strain, it was not complete, emphasizing the difficulty of potent drugs to obtain the complete eradication of *P. aeruginosa* patho-adaptive strains in the context of chronic infections. When the reference strain PAO1 was used as infectious agent the treatment achieved the complete eradication of the bacteria confirming a good profile of the drug. In general, the treatment with NCE demonstrated to be more efficacious than treatment with CIP highlighting the greater effectiveness of this new drug candidate compared to an antibiotic commonly used in clinics. The inflammatory profile in the airways suggested a positive impact of the treatment on pulmonary lung physiology, in particular the neutrophils recruitment and concentration of chemokines/cytokines such as IL-1β, CXCL1/KC, CXCL2/MIP-2 and CCL2/JE was reduced in mice treated with NCE. PK studies showed that NCE reaches favorable concentrations in the lung after i.t. administration, with rather low systemic exposure.

Inhaled administration of antibiotics is an excellent alternative to circumvent problematic associated to intravenous or oral drug administration like side-effects of drugs, related to prolonged periods of treatments, and at the same time contributes in reaching higher efficacy due to increased local concentrations. The administration of antibiotics by the inhaled route is a widely recognized treatment in patients with CF and bronchiectasis, and has recently being tested for the treatment of pneumonia, including VAP (Ratjen 2010). In the last years many efforts have been made in this direction offering new formulations of existing drugs for aerosol therapy. Inhaled aminoglycosides (e.g. tobramycin, gentamycin), polymyxins (e.g. colistinmethate sodium), and aztreonam lysinate have been described. Tobramycin inhalation solution is currently the only aerosol antibiotic approved for the treatment of bacterial infections in patients with CF. Aerosol administration of tobramycin potentially reduce systemic toxicity and a clinical benefit has been shown over several cycles of treatment (Ramsey BW 1999). However, its long-term use has been described to be associated with the selection of multiple-antibiotic-resistant *P. aeruginosa* strains (Sermet-Gaudelus I 2002; Merlo CA 2007). The encouraging results of tissue distribution of NCE combined with the preliminary evidence of improvement of efficacy following i.t. delivery compared to s.c. administration in the acute infection model, supports the pulmonary administration as possible therapeutic approach for the treatment of chronic lung infections with this new drug candidate. For these reasons the pre-clinical testing of NCE was extend to a more complex models of chronic infection including CF mice. Long-term severe *P. aeruginosa* airway infection was achieved challenging mice with the MDR-RP73 clinical embedded in agar beads. The agar beads mouse model has been widely characterized in our laboratory demonstrating not only to provide micro-anaerobic conditions for bacterial growth and biofilm formation, as those present in the mucus of CF patients, but also reflecting an advanced chronic pulmonary disease similar to those observed in patients (Worlitzsch D 2002; Bragonzi A 2005; Bragonzi 2010). This model represents a precious tool and has been used for pre-clinical testing of candidate anti-bacterial and anti-inflammatory molecules (Paroni, Moalli et al. 2013; Doring, Bragonzi et al. 2014). It has been reported that the use of aerosol, generated by a conventional human adopted nebulizer, resulted in a low deposition efficiency of drugs in rodents (Stangl 2008). The main reasons of this low efficiency are mainly the aerosol generation rates are too high, the increasing breathing frequencies due to the animal stress that lead to decrease dose deposition, particles deposition in the upper airways and large quantities of the aerosol remaining onto the coat or getting swallowed. For all these reasons, the preclinical efficacy studies of pulmonary administration of NCE in a mouse model of chronic lung infection was carried out taking advantage of the commercial MicroSprayer ^{TM} aerolizer (Penn Century). This is a non-invasive pulmonary aerosol delivery system that makes possible repeated administration

ensuring a good attainment and spread of molecules into the airways (Bivas-Benita, Zwier et al. 2005). This technique has been previously used in a pre-clinical study reporting administration of a new formulation of levofloxacin, the MP-376, in comparison with tobramycin and aztreonam, (Sabet 2009) making possible the passage from the MP-376 preclinical studies to the clinical phases (Geller, Flume et al. 2011). In a chronic pneumonia model repeated s.c. administration of NCE showed an improved weight gain of mice and a significant decrease of the bacterial load, compared with the vehicle, against MDR-RP73 strain. However a better efficacy results regarding improved weight gain and decrease in bacterial load was achieve after the treatment with NCE when administered repeatedly by MicroSprayer ^{TM} aerolizer emphasizing the potential therapeutic effect of the molecule in chronic airways infections. Moreover, pulmonary administration of NCE attenuated the inflammatory response to chronic lung infection. In particular the concentrations of inflammatory mediators, including CXCL2/MIP-2, CXCL1/KC and IL-1β, were decreased in NCE-treated mice, compared to mice treated with the vehicle. The efficacy of NCE was also demonstrated in a mouse model of CF. CF mice treated with NCE showed a significantly improved weight gain and lower bacterial load in the lung compared to animals treated with the vehicle. In this study long-term *P. aeruginosa* chronic infection and sustained inflammation has been achieved similarly in CF mice and non-CF. Repeated local treatment with NCE showed a similar beneficial effect in mice with different genetic background indicating that in this case CF environment does not represent an obstacle for this antibiotic treatment. Overall these data obtained in the first part of my PhD thesis support the further evaluation and clinical testing of NCE as a novel therapeutic for the treatment of *P. aeruginosa* infection.

A suitable alternative aimed to limit the use of antibiotics and focused in the prevention of pulmonary colonization is represented by immunotherapy. Many efforts have been made in this direction, but clinical efficacy has, to date, been disappointing (Holder 2004). Several *P. aeruginosa* vaccine candidates have been assessed in experimental animals and humans, including sub-cellular fractions, capsule components, purified proteins and recombinant proteins (Sharma, Krause et al. 2011). However, at present, no vaccine is available for clinical use against *P. aeruginosa* infections demonstrating that the strategies adopted for vaccine development right now are ineffective (Doring and Pier 2008). *P. aeruginosa* virulence factors like LPS, flagella or pili, have been widely taken under consideration as targets against immunotherapy. Despite the capacity to induce an immune responses of most of these vaccines has been demonstrated in rodent models of *P. aeruginosa* infection, only partial protection was achieved, underlining the need of more effective targets. A big limitation in the vaccine development against *P. aeruginosa* has been probably the limited

knowledge of reasonable targets. *P. aeruginosa* reference strain PAO1 encodes 5,570 predicted protein coding sequences. Notably only the 6.7% of the genome (372 ORFs) are known genes with demonstrated functions while a large proportion of the genome (45.8% of ORFs) is still not associated with a known function. Among the latter one third (769 ORFs) shares homology to genes with unknown function predicted in other bacterial genomes and the remaining 32% of ORFs does not have strong homology with any previously reported sequence and consequently their relevance in the course of establishing in vivo infection remain unknown (Stover, Pham et al. 2000). To overcome this obstacle genomic approaches have been recently exploited for the design of novel vaccines. They allow the identification of a large amount of antigens *in silico*, independently of their abundance and without the need to grow the microorganism in vitro (Grandi 2006). It has been shown that the ideal protective antigens are those particularly expressed during the infection process and those easily accessible for the host immune system, such as proteins localized on the bacterial cell surface (Rappuoli and Covacci 2003). The appropriate choice of the genomic approaches and the selection criteria consent the identification of novel antigens for the development of an efficacious vaccine. In this context the reverse vaccinology has revolutionized the approach of vaccine research (Scarselli, Giuliani et al. 2005). The process starts from genomic sequence and involves the following steps: bioinformatic software to screen genomes for surface-expressed proteins, high-throughput expression of these proteins and in vitro confirmation of their surface location, animal-based immunogenicity testing and finally conventional human vaccine trials (Capecchi, Serruto et al. 2004; Kelly and Rappuoli 2005). The reverse vaccinology has been successfully applied in the last few years. The first example of its potential has been the identification of novel antigens of Meningococcus B as potential candidates for a novel and effective vaccine and it has been successfully applied to other important human pathogens, demonstrating the feasibility to develop vaccines against any infectious disease (Mora, Veggi et al. 2003). Based on these advances in vaccine development novel vaccine candidates against *P. aeruginosa* were selected by "reverse vaccinology" and by a combination of advanced whole genomic approaches. These activities were started before my PhD as part of the activities of the lab. Starting from the *in silico* analysis or PAO1 reference strain a final short list of 53 proteins were selected for the expression and 32 of them were successfully expressed obtaining a sufficient amount for mice immunization.

Criteria for the elaboration of the short list of antigens were: accessibility for the immune system, conservation of the proteins among seven sequenced *P. aeruginosa* strains, expression profile under aerobic and anaerobic growth conditions, exclusion of epitopes similar to those present in human and mice, reported immunogenicity and relevance for in vivo infection by other

databases (Potvin, Lehoux et al. 2003; Montor, Huang et al. 2009). During my PhD work the efficacy of 32 vaccine candidates were tested in a mouse model of *P. aeruginosa* acute pneumonia. 10 proteins (PSE10, PSE11, PSE21, PSE27, PSE21, PSE44, PSE47, PSE52, PSE53 and PSE54) showed an increase in the survival curves compared with a negative control group. Mice antisera were used to characterized *in vitro* the 10 vaccine candidates for immunogenicity and cellular localization. Bands at expected weight were observed by western blot analysis against all the recombinant proteins indicating that the vaccine candidates were immunogenic. Expected bands were also observed in western blot against whole cell extract of homologous *P. aeruginosa* strain PAO1 and MDR-RP73 suggesting that proteins selected were effectively express in bacteria. Results of a double immunofluorescence staining, using murine antisera and a specific antibody for *P. aeruginosa* anti-cell wall, suggested that the proteins were presented in the bacterial cell surface. Together results of the antigens supported the robustness of the screening method used in this study. Combining together two by two the most promising vaccine candidates an increase in the protection was observed with some combinations reaching up to 50% of protection. In particular five combinations showed a statistically significant increase both in the survival curves and mean survival time compared with a negative control group. Surprisingly, in the most promising combinations the PSE54 was always present. Right now, this protein appear to be present only in *P. aeruginosa* and its function remain unknown. The combination that achieved the highest protection rate (50%) is composed by the unknown proteins PSE54 and PSE44; this indicated that approaches for the identification of vaccines candidates used in this study favor to pick out both targets with known and unknown functions. It has been widely described that *P. aeruginosa* adaptation to the airways include different expression of a large variety of virulence factors, modification of several surface antigens and an adapted metabolism (Sousa and Pereira 2014). Genetic variability among different *P. aeruginosa* strains, especially in clinical isolates, could affect the success of the response to vaccination preventing the clearance of the bacteria from the lung of patients (Tummler 2006; Doring and Pier 2008). For these reason an important purpose reside in comparing multiple gene sequence in different *P. aeruginosa* strains. Amino acid identity among a collection of clinical isolates suggested that our vaccine candidates could be suitable for the prevention of a large number of *P. aeruginosa* infections including those due to early and late CF isolates, but also those phenotypes more able to escape to the current antibiotics treatments like mucoidi, hypermutable and multidrug resistant strains. Overall these results suggest that the combination of comparative genome analysis and innovative methods in vaccine design successfully applied in other organism, like reverse vaccinology, are valid tools for the identification of novel vaccine candidates in *P. aeruginosa* and could contribute for the new vaccines against *P. aeruginosa*. Now novel proteins

identified should be most extensively studied and characterized in order to provide a better understanding not only with regarding to the prevention of infection but also to learn about unknown factors that make this bacterium one of major causes of infection in humans.

The validation of an antibiotic with new mechanism of action and novel vaccine candidates in murine model of airways infection demonstrated that both approaches could become powerful tools for the prevention/eradication of *P. aeruginosa* human infections.

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Antibiotic pressure compensates the biological cost associated with *Pseudomonas aeruginosa* hypermutable phenotypes *in vitro* and in a murine model of chronic airways infection

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Objectives: Hypermutable strains of *Pseudomonas aeruginosa* frequently emerge during chronic airways infection in cystic fibrosis (CF) patients. While the increased accumulation of mutations by hypermutable strains determines a biological cost for the colonization of secondary environments, the mutator phenotypes might confer a selective advantage under antibiotic treatment in a CF airways environment.

Methods: To test this hypothesis, the reference strain PAO1 and clonal pairs of CF clinical hypermutable and wild-type *P. aeruginosa* strains belonging to different genotypes were subjected to competition experiments *in vitro* and in a mouse model of chronic infection.

Results: Both *in vitro* and *in vivo*, under antibiotic selection pressure, clinical hypermutable *P. aeruginosa* strains and the reference PAO1Δ*mutS* outcompeted their wild-type strains, promoting *P. aeruginosa* hypermutable strains in the airways colonization. This advantage for the hypermutable strain did not occur in the absence of antibiotic treatments. Severe histopathological lesions were detected during chronic murine airways infection after antibiotic pressure, indicating that the advantage of the hypermutable population in the lungs may contribute to disease progression.

Conclusions: Overall, these results showed that *P. aeruginosa* hypermutability, previously associated with a biological cost, increases colonization potential under selection pressure in a context of CF chronic airways infection and can contribute to lung damage during long-term persistence.

Keywords: *P. aeruginosa*, hypermutators, cystic fibrosis, chronic airway infection, mouse model

Introduction

Pseudomonas aeruginosa chronic airways infection is recognized as being the most important contributor to the morbidity, mortality and premature death in many cases of patients with cystic fibrosis (CF).[1] Despite intensive antibiotic treatments, *P. aeruginosa* infections are difficult to eradicate.[2] The antibiotic treatment may actually favour the emergence of antimicrobial drug resistance and selects pathogenic variants.[3] One of the most striking characteristics of *P. aeruginosa* in the lung of CF patients is the diversification of the bacterial population and the presence of multiple phenotypes, including those that are highly resistant to any chemotherapy treatment.[4] These clones can arise from a number of different mechanisms involving mutation of the chromosomal genes linked only in part to the consumption of antibiotics.[5] Such mechanisms usually involve mutations in the genes encoding drug targets or in the systems that affect drug accumulation.[6] In addition, defects in the methyl-directed mismatch repair (MMR), a post-replicative repair system that corrects errors on newly synthesized DNA strands to ensure the fidelity of chromosome replication, are present in naturally occurring strains.[7] Elevated mutation rates displayed by hypermutators are likely to benefit the organisms by increasing the frequency with which antibiotic resistance arises and enhancing the opportunity for compensatory mutations to reduce fitness costs sometimes associated with the acquisition of endogenous antibiotic resistance.[8] There is alarming evidence of naturally occurring hypermutable strains with

antibiotic multiresistant phenotypes in CF patients. Hypermutable strains were detected in up to 37% of the CF patients with *P. aeruginosa* chronic infection in the first report, in which a strong link between hypermutation and antibiotic resistance was also observed.[9] The high prevalence of chronic infection by hypermutable *P. aeruginosa* strains in patients with CF and other chronic underlying respiratory diseases was confirmed in subsequent studies.[10–14] The causal relationship between colonization by hypermutable bacteria and deterioration of lung function has not been demonstrated directly. However, recent clinical studies suggest that the presence of hypermutable strains is linked to the poorer respiratory function of CF patients compared with patients colonized by non-hypermutable bacteria.[15,16] Given the effect of antibiotic failure on the prognosis of CF patient lung function, it is likely that colonization with hypermutable bacteria leads first to antibiotic treatment failure and consequently to lung deterioration. Furthermore, the intensive genetic adaptation process catalysed by hypermutable strains may also favour their long-term persistence in CF airways.[12] Our work investigates the response of several clonal pairs of *P. aeruginosa* hypermutable and wild-type CF strains to antibiotic pressure *in vitro* and in a murine model, and provides key results on lung pathogenicity during long-term chronic infection.

Methods

Ethics statement

Animal studies were conducted according to protocols approved by the San Raffaele Scientific Institute (Milan, Italy) Institutional Animal Care and Use Committee (IACUC) and adhered strictly to the Italian Ministry of Health guidelines for the use and care of experimental animals.

Research on the bacterial isolates from the individuals with CF was approved by the responsible physician at the CF centre at Hannover Medical School, Germany. All patients gave informed consent before sample collection. Approval for storing of biological materials was obtained by the Hannover Medical School, Germany.

Bacterial strains

A total of 12 *P. aeruginosa* strains of clinical origin (BT1, BT2, BST2, BST44, SG2, NN84, NN2, NN83, RP73, RP74, MF1 and MF2) obtained from sputum samples or throat swabs from CF patients were used in this study. Clinical *P. aeruginosa* strains were genotyped as described previously.[17] *P. aeruginosa* laboratory strains PAO1[18] and PAO1Δ*mutS*[19] were used in this study as reference strains. Hypermutable strains (BT1, BST44, SG2, NN84, NN83, RP74 and MF2) had been previously characterized through the determination of rifampicin resistance mutation frequencies,[9,13] and were complemented with a wild-type copy of the MMR gene (BT1*mutS*$^+$, BST44*mutS*$^+$, SG2*mutS*$^+$, NN84*mutS*$^+$, NN83*mutL*$^+$, RP74*mutL*$^+$, MF2*uvrD*$^+$ and PAO1Δ*mutS*/*mutS*$^+$) as described previously.[13]

Antibiotic susceptibility testing

MICs were determined for amikacin, ceftazidime, imipenem, meropenem, ciprofloxacin, piperacillin/tazobactam, ticarcillin/clavulanic acid, streptomycin and nalidixic acid. MICs were determined by the broth microdilution method in Mueller–Hinton cation-adjusted broth (MHB) according to the CLSI guidelines.

In vitro competition experiments

Competition between *P. aeruginosa* wild-type and hypermutable clonal strains was performed *in vitro*. Strains were grown overnight in tryptic soy broth, and each culture was diluted to a starting 600 nm optical density (OD_{600}) of 0.025 and subcultured for an additional 24 h into fresh medium in the presence of a competitor. Competition experiments were performed in the presence or absence of 50 mg/L streptomycin or 0.5 mg/L ciprofloxacin added to the fresh medium used for the subcultures. The starting ratios of hypermutable to wild-type were 1:1 and the ratio of the mixed inoculum after 24 h was determined on medium-selective tryptic soy agar (TSA) plates, taking advantage of different antibiotic resistance patterns identified within the pairs. Therefore, susceptible strains BST2 and NN2, and resistant strains BST44 (10 mg/L amikacin) and NN83 (30 mg/L amikacin) were tested in competition. For PAO1Δ*mutS*, resistance to kanamycin (250 mg/L) was used. The plating efficiency on antibiotic medium, i.e. cfu enumeration for cultures of the resistant strain, yielded the same numbers in plates with and without antibiotic. The competition index (CI) was calculated as the ratio of hypermutable to wild-type bacteria determined from TSA plates with and without antibiotic after 24 h adjusted by the input ratio (*in vitro* CI).[13,20]

Mouse model

The agar beads *P. aeruginosa* mouse model was used.[4,21,22] A starting amount of 5×10^9 hypermutable/wild-type bacteria, mixed at a ratio of 1:1, was used for inclusion in the agar beads.[13] C57BL/6 male mice (Charles River; 22–24 g) were infected with 2×10^6 cfu. Competitions in the presence of antibiotic treatment were performed by adding streptomycin (0.001 mg/L) to the drinking water[8,23] or treating mice subcutaneously once a day with ciprofloxacin (20 mg/kg).[24] Fourteen days after infection the murine lungs were excised, homogenized and plated onto TSA plates in the presence and absence of antibiotics in different indicator plates. The CI was calculated as the ratio of hypermutable to wild-type bacteria recovered from the murine lungs after 14 days from infection adjusted by the input ratio that were inoculated in each animal (*in vivo* CI).[13,20]

Histopathology and immunofluorescence

After 14 days of infection, the lungs were removed en bloc, fixed in 10% buffered formalin for at least 24 h and embedded in paraffin. Consecutive 2 μm sections from the middle of the five lung lobes were used for the histological and immunofluorescence examination of each mouse. Sections for histological analysis were stained with haematoxylin and eosin and examined blindly. Localization of *P. aeruginosa* was performed in de-paraffinized lung sections by employing a rabbit antiserum specific for *P. aeruginosa* and Texas Red-labelled goat anti-rabbit IgG as described previously.[4,25] Immunofluorescence images were recorded with an EM-CCD Hamamatsu C9100 camera (Hamamatsu Photonics, Hamamatsu City, Japan) mounted on an UltraVIEW Spinning Disk Confocal Microscope (Perkin Elmer, Waltham, MA, USA). Slides stained with haematoxylin and eosin were visualized with Axioplan2 (Zeiss, Jena, Germany) with AxioCam equipped with the CCD MRc5 (Zeiss).

Statistical analysis

Data were analysed for statistical significance using unpaired two-tailed non-parametric Student's *t*-test or χ^2. A *P* value of $\leq$0.05 was considered significant. The GraphPad statistical package was used for the statistical analysis.

Results

Effect on antibiotic resistance of complementation of P. aeruginosa hypermutable strains with MMR genes

Seven clinical *P. aeruginosa* hypermutable strains and their clonal pairs, previously identified and characterized from CF patients,[13] were tested for resistance to seven antibiotics (amikacin, ceftazidime, imipenem, meropenem, ciprofloxacin, piperacillin/tazobactam and ticarcillin/clavulanic acid) routinely used in chemotherapy and two additional antibiotics (streptomycin and nalidixic acid) by measuring their MICs (Table 1). The presence of endogenous resistance or development of resistance *in vitro* following exposure to antibiotics of hypermutable *P. aeruginosa* clinical strains was distinguished by complementation of hypermutable strains with the wild-type copy of MMR genes *mutS*, *mutL* or *uvrD*.

As shown in Table 1, both the *P. aeruginosa* laboratory strain PAO1Δ*mutS* and the clinical hypermutable strains were generally more resistant to antibiotics when compared with the non-hypermutable strains. Interestingly, the complementation of the clinical *P. aeruginosa* hypermutable strains with the wild-type copy of the MMR genes decreased the MICs, changing the antibiotic profile of the strain. In particular, a reduction in the MICs of nine antibiotics was observed for hypermutable strain BST44, eight for NN83, seven for MF2, five for RP74, four for BT1, two for NN84 and one for SG2 (Table 1). The MICs for PAO1Δ*mutS*, used as a control, were reduced for all antibiotics tested when complemented with *mutS* (PAO1Δ*mutS*/*mutS*$^+$), indicating an absence of endogenous antibiotic resistance. On the other hand, the complementation with the MMR genes only partially restored wild-type antibiotic susceptibility in the mutator CF isolates, in agreement with previous work showing, through Etest susceptibility testing, that the documented resistance in clinical *P. aeruginosa* hypermutable strains is partially a consequence of both antibiotic exposure *in vitro* and endogenous resistance acquired during previous exposures in the CF patient's lung.[26]

Costs versus benefits of P. aeruginosa hypermutable strains in the absence or presence of selection pressure in vitro

In a previous work we showed that in the absence of selection pressure *P. aeruginosa* hypermutable strains might be generally

Table 1. MICs (mg/L) of various antimicrobial agents for *P. aeruginosa* isolates: wild-type, hypermutable and complemented with the MMR genes

Strain	AMK	CAZ	IPM	MEM	CIP	TZP	TIM	STR	NAL
PAO1	2	1	1	1	0.125	4	16	64	128
PAO1Δ*mutS*[a]	**16**	**32**	**8**	**4**	**0.25**	**128**	**128**	**256**	**512**
PAO1Δ*mutS*/*mutS*$^+$	**8**	**4**	**1**	**2**	**0.125**	**16**	**32**	**128**	**256**
BT2	4	2	1	0.5	0.5	4	2	32	512
BT1[a]	256	**4**	0.5	0.5	0.0625	**2**	**2**	**512**	32
BT1*mutS*$^+$	256	**2**	0.5	0.5	0.0625	**1**	**1**	**256**	32
BST2	0.5	2	0.5	0.5	0.125	2	16	8	64
BST44[a]	**32**	**16**	**4**	**16**	**0.25**	**16**	**128**	**256**	**256**
BST44*mutS*$^+$	**8**	**4**	**2**	**4**	**0.125**	**4**	**32**	**128**	**128**
SG2[a]	32	64	1	0.125	0.0625	128	**128**	64	8
SG2*mutS*$^+$	32	64	1	0.125	0.0625	128	**64**	64	8
NN84[a]	8	64	8	32	**8**	**128**	128	256	512
NN84*mutS*$^+$	16	64	16	32	**4**	**64**	128	256	512
NN2	8	16	1	4	0.5	128	128	128	512
NN83[a]	**256**	**16**	**16**	**8**	**1**	**128**	**128**	**128**	128
NN83*mutL*$^+$	**8**	**4**	**1**	**1**	**0.125**	**16**	**64**	**16**	256
RP73	64	32	16	16	1	64	256	128	128
RP74[a]	8	**64**	**32**	16	4	**256**	256	**128**	**256**
RP74*mutL*$^+$	8	**32**	**16**	16	4	**64**	256	**64**	**128**
MF1	2	4	1	0.5	0.0625	8	32	4	128
MF2[a]	8	8	**4**	**2**	**1**	**32**	**64**	**64**	**512**
MF2*uvrD*$^+$	8	16	**0.5**	**1**	**0.0625**	**4**	**32**	**8**	**128**

AMK, amikacin; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; TZP, piperacillin/tazobactam; TIM, ticarcillin/clavulanic acid; STR, streptomycin; NAL, nalidixic acid.
Bold type indicates a decrease in the antibiotic resistance between a *P. aeruginosa* hypermutable strain and its complemented strain.
[a]*P. aeruginosa* hypermutable strains.

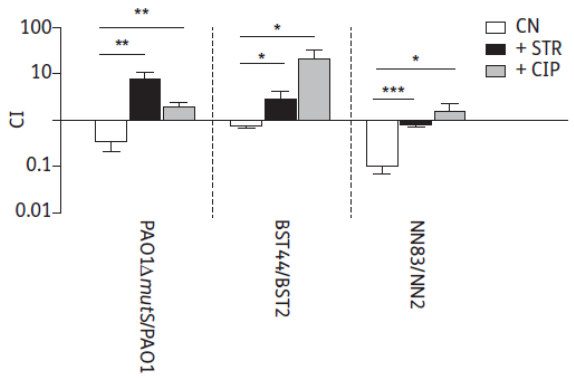

Figure 1. *In vitro* competition between clonal pairs of hypermutable and wild-type *P. aeruginosa* strains. Competitions were performed between hypermutable and wild-type strains in the absence (CN) or presence of streptomycin (STR) and ciprofloxacin (CIP). Each strain from the co-cultures was distinguished on different antibiotic plates, taking advantage of resistance patterns that were originally present in each lineage or carried by plasmids. The value reported is the mean CI of at least three separate experiments (log scale). A CI <1 indicates a defect of the hypermutable strain. Asterisks indicate statistically significant differences (*t*-test): $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$.

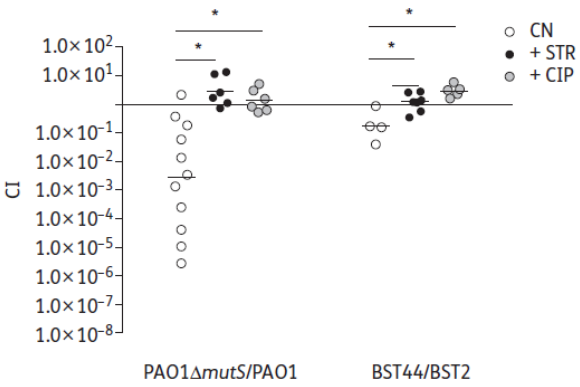

Figure 2. Competition between clonal pairs of hypermutable and wild-type *P. aeruginosa* strains in C57BL/6 mice in the presence or absence of antibiotic treatment. Wild-type and hypermutable strains were embedded in agar beads and used to infect C57BL/6 mice. The chronic infection continued for 2 weeks and the number of cfu per lung evaluated on selective plates. Each circle represents the CI for a single animal in each group. White circles indicate the untreated group of mice (CN), black circles indicate the group treated with streptomycin (STR) and grey circles indicate the group treated with ciprofloxacin (CIP). A CI <1 indicates a disadvantage for the hypermutable strain in being maintained *in vivo* compared with the wild-type clonal strain. The geometric mean of the CIs for all mice is shown as a solid line (log scale). Asterisks indicate statistically significant differences (*t*-test): $^{*}P \leq 0.05$.

disadvantaged when grown in the same environment with clonally related wild-type strains.[13] However, the hypermutable phenotype might reasonably advantage the *P. aeruginosa* strain in the presence of selection pressure such as an antimicrobial agent. To verify this hypothesis, pairs of clinical hypermutable/wild-type clonally related *P. aeruginosa* strains, selected for their high propensity to develop antibiotic resistance after exposure *in vitro*, and the pair of reference strains PAO1Δ*mutS*/PAO1 were co-cultured at a starting ratio of 1:1 (Figure 1). The final ratio was determined after 24 h of growth in the presence or absence of two different antibiotics—streptomycin or ciprofloxacin. In the presence of streptomycin, the hypermutable strain outcompeted the wild-type strain(s) in the case of the pairs PAO1Δ*mutS*/PAO1 and BST44/BST2 (PAO1Δ*mutS*/PAO1 treated versus untreated CI 7.02 versus 0.33, $P=0.0059$; BST44/BST2 treated versus untreated CI 2.74 versus 0.75, $P=0.0218$). In the lineage NN, the presence of streptomycin determined a statistically significant increase in fitness for the hypermutable strain when compared with the absence of antibiotic, even if no outcompetition for the hypermutable strain was observed (NN83/NN2 treated versus untreated CI 0.82 versus 0.10, $P<0.0001$). In the presence of ciprofloxacin, the hypermutable significantly outcompeted the wild-type strain(s) in the reference and clinical pairs (PAO1Δ*mutS*/PAO1 treated versus untreated CI 1.91 versus 0.33, $P=0.0013$; BST44/BST2 treated versus untreated CI 20.63 versus 0.75, $P=0.015$; NN83/NN2 1.51 versus 0.10, $P=0.025$).

Virulence of hypermutable P. aeruginosa strains in the absence or presence of selection pressure in a murine model of chronic infection

We previously demonstrated that the *P. aeruginosa* hypermutable strains are generally disadvantaged and less efficient in colonizing the murine airways when compared with wild-type under conditions of infection similar to those provided by the CF mucus.[13] To assess the virulence of a mixed population of *P. aeruginosa* hypermutable and wild-type strains during the course of chronic airways infection under selection pressure, competition experiments between reference PAO1Δ*mutS*/PAO1 and clinical BST44/BST2 pairs of hypermutable/wild-type strains were performed in the agar beads murine model (Figure 2 and Table 2). After treatment with streptomycin or ciprofloxacin for 14 days, mortality, chronic infection, and the total population size of the bacteria present in lung samples were measured. Antibiotic treatment significantly reduced the mortality of mice infected with PAO1Δ*mutS*/PAO1 compared with placebo (streptomycin or ciprofloxacin treated 0% versus untreated 27%; χ^2, $P=0.03$), while the percentage of mice with chronic infection after 14 days was no different after treatment. The total cfu/lung was unchanged when we compared untreated or antibiotic-treated mice, indicating the therapeutic failure to eradicate chronic infection under these experimental conditions. However, under streptomycin or ciprofloxacin treatment the PAO1Δ*mutS* population outcompeted the wild-type population, while in the absence of antibiotic treatment the results were the opposite (PAO1Δ*mutS*/PAO1 treated versus untreated CI 2.83 and 1.38 for streptomycin and ciprofloxacin, respectively, versus 0.003; *t*-test, $P=0.011$). As shown in Figure 2, the *P. aeruginosa* clinical lineage BST followed the laboratory strain and the ratio of BST44 hypermutable to BST2 wild-type strains recovered from treated mice increased when compared with the absence of streptomycin or ciprofloxacin (BST44/BST2 untreated versus treated CI 0.17 versus 1.26 or 2.90 for

Table 2. Colonization of murine lung with wild-type and hypermutable clonally related *P. aeruginosa* strains in competition experiments

	PAO1Δ*mutS*/PAO1			BST44/BST2		
	untreated ($n=30$)[a]	+STR ($n=14$)[a]	+CIP ($n=15$)[a]	untreated ($n=17$)[a]	+STR ($n=12$)[a]	+CIP ($n=10$)[a]
Mortality, % (no. of dead/total mice)	27 (8/30)	0 (0/14)*	0 (0/15)*	12 (2/17)	0 (0/12)	0 (0/10)
Chronic infection, %[b] (no. of infected/surviving mice)	50 (11/22)	57 (8/14)	60 (9/15)	27 (4/15)	67 (8/12)*	50 (5/10)
Hypermutable, %[c] (no. of hypermutable infected/total infected)	45 (5/11)	100 (8/8)**	100 (9/9)**	100 (4/4)	100 (8/8)	100 (5/5)
Wild-type, %[c] (no. of wild-type infected/total infected)	100 (11/11)	88 (7/8)	78 (7/9)	100 (4/4)	100 (8/8)	100 (5/5)
Total cfu/lung[d]	3.25×10^4	6.83×10^5	1.77×10^4	6.32×10^4	1.02×10^5	1.13×10^5
Hypermutable cfu/lung[d]	1.12×10^3	3.85×10^5	9.47×10^3	6.75×10^3	8.25×10^4	7.34×10^4
Wild-type cfu/lung[d]	3.25×10^4	9.21×10^4	8.26×10^3	5.64×10^4	2.10×10^4	3.53×10^4
CI[e]	0.003	2.8*	1.4*	0.17	1.26*	2.90*

CIP, ciprofloxacin; STR, streptomycin.
[a]Pooled mice, analysed in two to three independent experiments.
[b]Infected mice, surviving 14 days after challenge.
[c]Number of pooled mice infected with *P. aeruginosa* hypermutable or wild-type/total infected at 14 days.
[d]Median values are reported.
[e]Geometric mean in 4–14 mice for each clonal lineage.
*Statistical significance of treated versus untreated groups by two-tailed Student's *t*-test or χ^2 is indicated ($P<0.05$).
**Statistical significance of treated versus untreated groups by two-tailed Student's *t*-test of χ^2 is indicated ($P<0.01$).

streptomycin or ciprofloxacin, respectively; *t*-test, $P<0.02$). In addition, antibiotic treatment increased the percentage of mice infected with BST44/BST2, a figure that was significant in the case of streptomycin (streptomycin treated 67% versus untreated 27%; χ^2, $P=0.03$). Mortality was lower for mice infected with BST44/BST2 and treated with antibiotic compared with untreated, but the differences did not reach statistical significance as in the case of *P. aeruginosa* reference strains.

Overall, these results show a positive selection of the *P. aeruginosa* hypermutable strain during long-term airway colonization of mice administered antibiotic treatment compared with untreated mice.

Histopathological lesions of P. aeruginosa chronic pneumonia in the absence or presence of antibiotic treatment

The histopathological analysis of mice infected with the pair of *P. aeruginosa* hypermutable/wild-type clinical strains BST44/BST2 indicated that the severity of lesions in the lung was similar in untreated (Figure 3a) or antibiotic-treated mice (Figure 3b). Bronchi of untreated mice or ciprofloxacin-treated mice were filled with agar beads containing bacterial macrocolonies (Figure 3c) or single cells (Figure 3d) surrounded by a massive neutrophilic infiltration with the parenchyma principally infiltrated by macrophages, lymphocytes and neutrophils. Immunofluorescence staining showed that the infected bronchi contained agar beads with *P. aeruginosa* macrocolonies for untreated mice (Figure 3e), while single bacterial cells (Figure 3g) were present in the beads of ciprofloxacin-treated mice, indicating that the 14 days of antibiotic treatment had affected the bacterial growth in the beads. In addition, compared with the mice treated with ciprofloxacin, the infected bronchi of untreated mice contained fewer and smaller agar beads, which were partially destroyed by extensive bacterial growth and degraded by neutrophils. The parenchyma showed persisting bacterial cells in untreated mice (Figure 3f), but not in the ciprofloxacin-treated mice (Figure 3h). Severe histopathological lesions were detected during chronic murine airway infection under antibiotic treatment. Indeed, antibiotic treatment did not lead to significant improvement in the lung pathology scores, though it encouraged selection of the hypermutable population. It can be assumed that multiple phenotypes of *P. aeruginosa* may also contribute to lung deterioration and disease progression in the CF lung habitat.

Discussion

One of the most striking characteristics of *P. aeruginosa* chronic lung infection in CF patients is the intense genetic adaptation and diversification of the bacterial population, leading to the co-existence of multiple phenotypes that are highly resistant to any chemotherapy treatment.[4,27] Whole genome sequence analysis of *P. aeruginosa* sequential strains indicated rapid adaptation following colonization of the CF airways, followed by a long period (which also included the exacerbation period[28]) with limited phenotypic change.[29] It has been shown that the accumulation of these so-called adaptive mutations is catalysed in part by the high prevalence of hypermutable strains in the CF setting that may emerge after the onset of colonization[30] or at a later stage.[12] If no hypermutators evolved, the point mutations remained amazingly low.[30] These observations are consistent with previous work on the CF mouse model showing that hypermutation favours long-term colonization.[31] Nevertheless, in

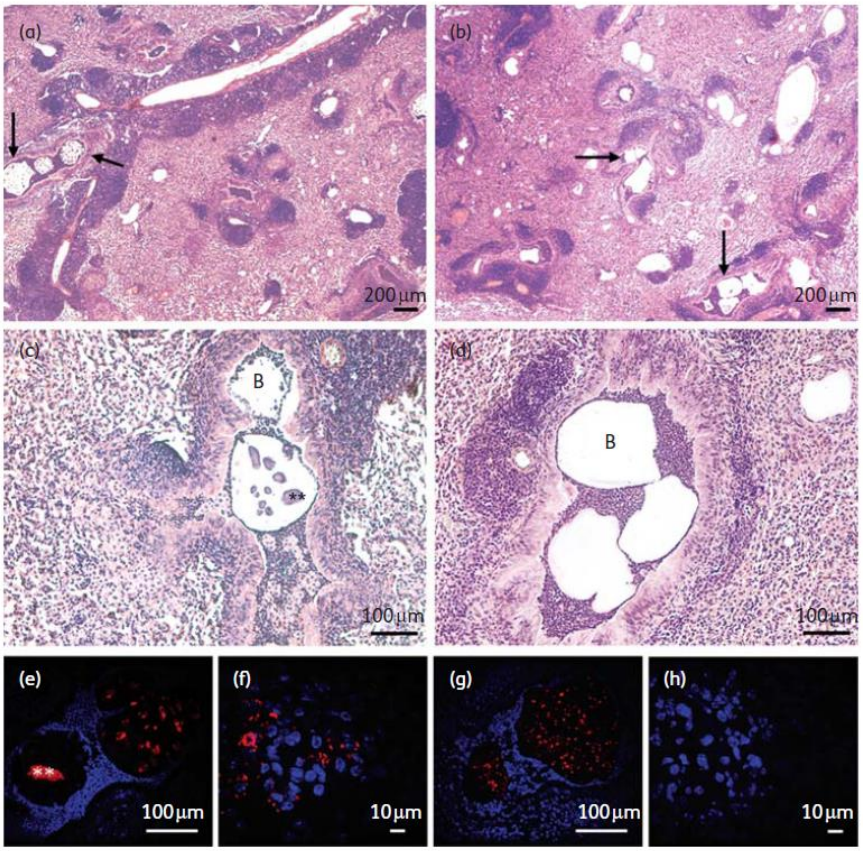

Figure 3. Histological lesions and localization of *P. aeruginosa* cells in the presence or absence of antibiotic treatment. Lung involvement in (a) untreated and (b) ciprofloxacin-treated mice after 14 days of infection with BST2/BST44. Agar beads (arrow) are localized in the bronchi characterized by acute and chronic lesions; the pulmonary parenchyma is infiltrated by macrophages and lymphocytes. (c) Bronchi of untreated mice are characterized by the presence of inflammatory cells and bacterial macrocolonies (**) in the agar beads (B). (d) Bronchi of ciprofloxacin-treated mice are characterized by the presence of inflammatory cells and single bacterial cells in the agar beads (B). (e) and (g) Immunofluorescence staining observed with confocal microscopy shows fluorescent bacterial macrocolonies (**) localized in beads in untreated (e) and single colonies in treated (g) mice. (f) and (h) Immunofluorescence staining showing bacteria in the parenchyma of untreated mice (f) and the absence of bacteria in the parenchyma in treated mice (h). 4′-6-Diamidino-2-phenylindole dihydrochloride ('DAPI') was used to stain the nuclei.

those experiments, adapted mutator lineages showed a reduced capacity for establishing new colonizations (i.e. reduced transmissibility), probably due to the accumulation of deleterious mutations for secondary enviroments.[29] Consistent with these findings, recent studies suggest that hypermutation might be counter-selected in late stages of microevolution of highly adapted and transmissible CF clones, such as clone C, DK2 or LES-1.[27–29] Likewise, we have previously reported a decreased fitness and virulence of *P. aeruginosa* CF mutator populations in the absence of selection pressure when tested in competition experiments either *in vitro* or in a murine model of chronic infection.[13]

However, in this study we showed that the disadvantage of hypermutability can become an advantage in the presence of selection pressure from the antimicrobial agent both *in vitro* and *in vivo*. During 2 weeks of administration of the antimicrobial agent to infected mice, the hypermutable PAO1Δ*mutS* strain outcompeted its isogenic wild-type PAO1 strain. These results confirmed and expanded previous findings in short-term evaluations (e.g. 3 days) of the therapeutic efficacy of antipseudomonal agents against the reference PAO1Δ*mutS*.[24,32] The capacity of the hypermutable population to generate adaptive mutations faster than the wild-type enables bacteria to rapidly and efficiently cope with the drastic environmental changes encountered during selection pressure with the antimicrobial agent when grown *in vitro* and in infected murine lung.

Although the PAO1 strain has been highly informative, this laboratory-adapted strain, recovered from a wound infection more than five decades ago and sequenced many years ago,[18] does not represent the *P. aeruginosa* diversity observed in the natural population that belongs to the same species and that colonizes CF patients.[33] Therefore we tested additional pairs of clonally related *P. aeruginosa* hypermutable and wild-type strains. These *P. aeruginosa* strains belonged to CF patients

that had been subjected to various antibiotic treatments during the course of the chronic infection, with the generation of antibiotic-resistant phenotypes as determined by their MICs. In several lineages the clinical hypermutable strains were more resistant to antibiotics when compared with non-mutator strains. To distinguish between endogenous resistance or the development of resistance *in vitro* following exposure to antibiotics of hypermutable *P. aeruginosa* clinical strains, MICs were compared with strains complemented with the wild-type copy of MMR genes. The complementation with the MMR genes only partially restored wild-type antibiotic susceptibility in the mutator CF isolates, in agreement with previous work. Through Etest susceptibility testing, this demonstrated that the documented resistance in clinical *P. aeruginosa* hypermutable strains is partially a consequence of both antibiotic exposure *in vitro* and endogenous resistance acquired during previous exposures in the CF patient's lung.[26]

Streptomycin and ciprofloxacin were introduced as selection pressure to the natural environment in which wild-type and hypermutable competition takes place. Streptomycin was chosen as an antibiotic not routinely used in the clinical situation, but a drug that can reach the lung tissue,[24,34] and ciprofloxacin was chosen as a commonly used treatment in CF patients. When tested *in vitro*, the BST and NN lineages followed the PAO1 strain and show an advantage of the hypermutable strain when compared with the wild-type under streptomycin or ciprofloxacin antibiotic treatment. This advantage for the hypermutable strain did not occur in the absence of antibiotic treatment. Next, pairs of wild-type/hypermutable clinical strains were included in the agar beads and used to infect mice under antibiotic treatment. The cfu counts of clinical hypermutable strains in the lung were increased when compared with the wild-type strains in the process of establishing colonization under antibiotic pressure. Thus the advantage demonstrated by the *P. aeruginosa* hypermutable PAO1Δ*mutS* strain *in vivo* was also confirmed in the clinical strain. Severe histopathological lesions were detected during chronic murine airways infection under antibiotic treatment. Indeed, antibiotic treatment did not lead to significant improvement in the lung pathology scores while simultaneously selecting the hypermutable population. Obviously, multiple phenotypes of *P. aeruginosa* may also contribute to lung deterioration and disease progression in the CF lung habitat. Overall, these results showed that *P. aeruginosa* hypermutability, associated with an important biological cost in the absence of selection pressure, can be considered an advantage in the presence of selection pressure such as antibiotic treatments in the PAO1 laboratory strain and clinical strains. These findings emphasize the need for further studies to determine the generalizability of this mechanism with additional *P. aeruginosa* clonal lineages and to establish whether and how *P. aeruginosa* long-term chronic infection with hypermutable strains can determine the progression of lung disease.

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Transparency declarations

None to declare.

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Evaluation of efficacy of P0L7001 against *Pseudomonas aeruginosa* in lung infection models

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The development and clinical exploitation of antibiotics with new modes of action are a top priority in fighting untreatable chronic infections in CF patients. POL7001 is a Protein Epitope Mimetic (PEM) antibiotic with potent activity against Pseudomonas aeruginosa (PA) (Srinivas et al, Science 2010).

To evaluate CF lung infections as potential clinical application of this new antibiotic, the activity of POL7001 was tested in vitro against PA isolated from the onset of infection up to 16 years or until death/lung transplantation in CF patients, and in murine models of acute and chronic lung infection. Comparison to clinically approved antibiotics was included.

MICs for POL7001 ranged between 0.015−0.5mg/mL with a median of 0.125 mg/mL for all solates with no difference against mucoid, non-mucoid orhypermutable isolates. Over time, many of the CF PA isolates became resistant to antibiotics while remaining sensitive to POL7001. Mice were infected with a multi-drug resistant PA isolate and treated with POL7001 or ciprofloxacin. Subcutaneous administration showed a comparable efficacy of both antibiotics, with more than 1.5 log10 CFU/mL reduction after 24 hrs, while intratracheal administration showed faster killing and better efficacy of POL7001 than ciprofloxacin, with 3 log10 and 1 log10 CFU/mL reduction after 24 hrs, respectively. In a model of chronic lung infection daily subcutaneous treatment showed that POL7001 is more efficaciousthan ciprofloxacin, with 1 log10 CFU/mL reduction in the BAL. These results represent a step forward in the pre-clinical development of POL7001 to treat CF lung infections.

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Pre -clinical evaluation of novel antibiotic POL7001 against *Pseudomonas aeruginosa* in lung infection models

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Objectives: The discovery, development, and clinical exploitation of antibiotics with a new mode of action combined with efficient pulmonary drug delivery systems is a top priority in the battle against untreatable chronic infections in cystic fibrosis (CF) patients. POL7001 is a novel Protein Epitope Mimetic (PEM) antibiotic with potent activity against Pseudomonas aeruginosa (Pa) (Srinivas et al, Science 2010). POL7001 showed a potent in vitro activity against a large panel of Pa CF multi-drug resistant strains. To evaluate CF lung infections as potential clinical application, the therapeutic efficacy of POL7001 in mouse models was investigated.

Methods: Both Pa acute and chronic airway infection were established, and mice were treated by subcutaneous (s.c.) or pulmonary administrations (i.t.). Body weight, bacterial count and inflammation in lungs were evaluated at different time points. Comparison to clinically approved antibiotics was included.

Results: High antibacterial activity of POL7001, in particular after i.t. administration, was demonstrated. Leukocyte recruitment (in particular neutrophils) in the airways was reduced after POL7001 i.t.administration. Pharmacokinetic studies confirmed that POL7001 reached favorable concentrations in the lung after i.t. administration, with rather low systemic exposure.

Conclusion: The efficacy of POL7001 was superior to ciprofloxacin, one of the most effective clinically-approved antibiotics and used as an internal positive control in our pre-clinical studies. Based on these promising results, POL7001 was selected for further pre-clinical profiling.

Submitted paper

Comparative genomics and biological characterization of sequential *Pseudomonas aeruginosa* isolates from persistent airways infection.

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No competing interests to declare.

ABSTRACT

Background. *Pseudomonas aeruginosa* establishes life-long chronic airway infections in cystic fibrosis (CF) patients. As the disease progresses, *P. aeruginosa* pathoadaptive variants are distinguished from the initially acquired strain. However, the genetic basis and the biology of host-bacteria interactions leading to a persistent lifestyle of *P. aeruginosa* are not understood. As a model system to study long term and persistent CF infections, the *P. aeruginosa* RP73, isolated 16.9 years after the onset of airways colonization from a CF patient, was investigated. Comparisons with strains RP1, isolated at the onset of the colonization, and clonal RP45, isolated 7 years before RP73 were carried out to better characterize genomic evolution of *P. aeruginosa* in the context of CF pathogenicity.

Results. Virulence assessments in disease animal model, genome sequencing and comparative genomics analysis were performed for clinical RP73, RP45, RP1 and prototype strains. In murine model, RP73 showed lower lethality and a remarkable capability of long-term persistence in chronic airways infection when compared to other strains. Pathological analysis of murine lungs confirmed advanced chronic pulmonary disease, inflammation and mucus secretory cells hyperplasia. Genomic analysis predicted twelve genomic islands in the RP73 genome, some of which distinguished RP73 from other prototype strains and corresponded to regions of genome plasticity. Further, comparative genomic analyses with sequential RP isolates showed signatures of pathoadaptive mutations in virulence factors potentially linked to the development of chronic infections in CF.

Conclusions. The genome plasticity of *P. aeruginosa* particularly in the RP73 strain strongly indicated that these alterations may form the genetic basis defining host- bacteria interactions leading to a persistent lifestyle in human lungs.

Running title: *P. aeruginosa* sequential isolates from persistent infection.

Keywords: cystic fibrosis, *P. aeruginosa*, genome, adaptation, chronic infection, mouse model

BACKGROUND

The opportunistic pathogen *Pseudomonas aeruginosa* has broad capabilities to thrive in diverse ecological niches and to establish serious human infections [1]. Poor clinical outcome of *P. aeruginosa*-associated infection was described in immune compromised patients and those in intensive care units, connected to mechanical ventilation or other invasive devices. *P. aeruginosa* is also the leading cause of chronic lung infections and death in patients with cystic fibrosis (CF), as well as a frequent cause of exacerbations in individuals with advanced chronic obstructive pulmonary disease (COPD) [2].

The genetic basis of *P. aeruginosa* leading to acute or chronic infection is not yet understood [3]. Genome sequencing projects are underway with the aim of providing new data to dissect the molecular basis of *P. aeruginosa* infections. Seventeen completely sequenced and assembled genomes are currently available and draft genomes exist for 561 additional genomes. The genome size of *P. aeruginosa* is larger than those of most sequenced bacteria and varies between 5.2 and 7 Mbp, with ~5500 ORFs [4]. A significant number (8,4%) of *P. aeruginosa* genes are predicted to be involved in regulation, which at the time of publication was the largest fraction of regulators among sequenced bacterial genomes. Irrespective of their origin, *P. aeruginosa* isolates share a remarkable amount of similarity in their genome content and in virulence traits (core genome). The extent of divergence between strains is determined by extra-chromosomal elements like plasmids or blocks of DNA inserted into the chromosome at various loci [5]. These genetic features are likely to be acquired by horizontal gene transfer from different sources including other species or genera and can be present in subgroups of the *P. aeruginosa* population but may also be unique to single strains, accounting for most of intra- and inter-clonal *P. aeruginosa* genome diversity. These strain-specific segments of the genome are not scattered randomly through the core genome; rather, they tend to be clustered in certain loci, referred to as regions of genome plasticity (RGPs) [6]. The genetic sequences occupying many RGPs are often referred to as genomic islands (GIs) and islets. Therefore, the *P. aeruginosa* chromosome presents a picture of a mosaic, consisting of a conserved core component, interrupted in each strain by the inserted parts of the accessory genome. Genetic elements within the accessory genome may encode properties that contribute to niche-specific adaptation of the particular strains that harbor them.

Furthermore, mutations of single nucleotides also confer specific *P. aeruginosa* phenotypes that are advantageous under certain conditions [7-10]. Long-term colonization of the CF host is maintained

by *P. aeruginosa* pathoadaptive lineages, which are clonal with the initially acquired strain and carry phenotypic variants. Pathoadaptive mutations are frequent in virulence genes, essential for acute infection but no longer compatible with the novel lifestyle of the *P. aeruginosa* in CF airways. However, little is known about the genetic basis and the biology of host-bacteria interactions leading to a persistent lifestyle of *P. aeruginosa*.

To define the genetic basis of *P. aeruginosa* persistent lifestyle, longitudinal isolates from CF patient were selected. In particular, *P. aeruginosa* RP73 was isolated after long-term chronic infection and compared with the preceding RP1 and clonal RP45, as well as prototype PAO1 and PA14 strains. When murine model of chronic lung infection was used, RP73 showed a marked persistent lifestyle. Thus, genome sequencing and comparative genomics analysis were carried out. Our results show the links between genomic properties and pathogenic potential of RP73 that may define the basis of long-term chronic infection by *P. aeruginosa*. The significance of these results is discussed in the context of understanding disease pathogenesis.

RESULTS AND DISCUSSION

Chronic colonization of a CF patient's airways with the P. aeruginosa RP isolates

CF was suspected in the exocrine-insufficient patient index case RP (*CFTR* genotype: F508del/R1162X) by a positive meconium test at birth and was confirmed by pathological sweat tests at the age of 4 months. The RP patient's airways became colonized with *P. aeruginosa* by the age of 7 years (**Figure 1**). The CF clinic in Hannover has collected sequential isolates from this patient since the onset of colonization for up to 28 years [11]. The patient was chronically carrying *P. aeruginosa* isolates of clone type OC2E, for the first eleven years. During this time period strains of the clone type OC4A were sporadically isolated, but thereafter OC4A has become the dominant clone type until today. The RP patient received one to four annual 2-week courses of intravenous (iv) antipseudomonal chemotherapy since onset of colonization and was administered aerosolized colistin on a daily basis during the last 17 years. The last clone type OC2E strain was isolated from the patient's sputum four months after the start of colistin inhalation. The patient's clinical status remained stable during the 28 years of chronic airway infection. Lung function parameters fluctuated between 70–90% predicted for forced vital capacity (FVC) and 60–

80% forced expiratory volume (FEV_1) during the last 20 years with no tendency to irreversible decline.

In this study three *P. aeruginosa* isolates from RP patient were selected for genetic and biological characterization. RP1 was the first *P. aeruginosa* isolate and belongs to clone type OC2E, while RP45 and RP73, isolated after 10 and 16.9 years from the onset of colonization, belong to clone type OC4A (**Additional File 1** and Cramer et al. [12]). Thus, RP73 *P. aeruginosa* isolate was able to establish long-term infection replacing the initially RP1 acquired isolate and likely adapting within CF airways respect to RP45.

Pathogenicity of P. aeruginosa RP isolates in murine model of airways infection.

To translate data from CF patients into disease models, *P. aeruginosa* clinical isolates RP1, RP45 and RP73 were tested in the agar beads mouse model of chronic airways infection in comparison to prototype PAO1 and PA14 strains [13, 14]. Bacteria embedded in the immobilizing agents appear to grow in the microaerobic/anaerobic conditions in form of microcolonies, similarly to the growth in the mucus of patients with CF [15, 16]. RP1 isolate, as well as prototype PA14 strain, caused death in all mice (100%) within the first three days of *P. aeruginosa* infection (**Figure 2** and **Additional File 2**). Lower incidence of mortality (50%) was recorded after infection with RP45 strain, while RP73 were not lethal (0%). Thus, despite their clonality, RP45 and RP73 were significantly different in the risk of death. As previously reported, prototype PAO1 showed 24% of acute mortality [17]. Next, the capacity to establish chronic infection in the surviving mice was assessed at 14 days. Nearly all mice had chronic airways colonization by RP45 (80%) and RP73 (90%), demonstrating the persistent lifestyle of this lineage among surviving mice. PAO1 strain showed less capacity to establish chronic infection (24%). The ability of the clinical isolate RP73 to achieve long-term chronic infection associated with no risk of mortality in mice was superior to all other *P. aeruginosa* clinical strains tested in previous studies [17].

To assess clinical trait of chronic infection, lung histopathology was performed after 14 days from *P. aeruginosa* challenge with the persistent RP73 isolate. Chronic pulmonary disease, including inflammation and mucus secretory cells, was detected. The bronchi were filled by a massive neutrophil inflammation, whereas the parenchyma was infiltrated by macrophages, lymphocytes and some neutrophils (**Figure 3A)**. Agar beads were observed in bronchial lumina (**Figure 3B**). Mucous secretory cells hyperplasia (**Figure 3C**) was found. These features resembled lesions found in CF patients with advanced chronic pulmonary disease [18].

Genome sequences of RP isolates and comparative genomics analyses.

To link the persistent lifestyle with a genetic basis, we sequenced the genome of RP73[19], in addition to those of preceding RP1 and clonal RP45 isolates, and performed comparative genomic analysis. The fully assembled RP73 genome consists of a single circular chromosome of 6,342,034 base pairs (**Additional File 3** for genome description). Twelve genomic islands were predicted in this genome (**Table 1**); three of them distinguished RP73 from other prototype strains and corresponded to regions of genome plasticity (**Figure 4**) [5]. They include known genomic islands PAGI-9,which is similar to rearrangement hot spots (*Rhs*) [20], and plasmid pKLC102, which carries the *pil* gene cluster and *chvB* glucan synthetase [21]. Nucleotide blast search on NCBI limited to *P. aeruginosa* showed that the former can be found in multiple clinical isolates, while the latter is identical to RP73 only in strain 8380, isolated from the human gut. However, plasmid pKLC102 is often partially present [22]. A SMC4389 CRISPR repeat sequence also differentiates RP73 from most prototype strains [6]. In fact, blast search for this sequence resulted in a single hit from soil strain *Azotobacter chroococcum* NCIMB 8003. The RP73 genome also contains full length LESGI-4, which was identified in the Liverpool epidemic strain (LES) [23].Genomic islands predicted in RP73 were investigated in the draft genomes of RP1 and RP45. While RP45 carries all 12, RP1 lacks full-length plasmid pKLC102 and an ABC transporter protein. A circular map comparing the 3 sequenced RP genomes clearly shows the genomic similarity between RP45 and RP73 on one hand, and between RP1 and strain PA14, which showed similar results in the murine infection model, on the other (**Figure 4**).

In order to study the evolution of chronic infection and determine which genetic determinants are involved in this process, further comparisons were made among RP isolates. First, analyses were carried out to study the relationship between these three strains. A core genome phylogeny was performed using 53 sequences from a previous study [24] representing an extensive sampling of *P. aeruginosa*'s diversity. In the resulting tree (**Figure 5**), RP73 and RP45 cluster together while RP1 is found in a different and independent branch. A multi-locus sequence typing (MLST) analysis was also performed. RP73 and RP45 shared the same MLST profile while RP1 showed a different one (**Table 2**). All these analyses suggest that RP73 and RP45 are close to each other from a genetic point of view, while RP1 is more distantly related.

In order to explain the molecular bases of the development of chronic infection in a CF context, SNPs were determined between PA14 and RP1 compared to RP45 and RP73. A genomic comparison between PAO1 and RP1 was already performed by Hilker *et al.* [22]. A total of 54,621 SNPs was found by taking PA14 as reference strain and searching for positions at which at least one among RP1, RP45 and RP73 strains showed a different nucleotide compared to PA14. About one

fifth of these SNPs (10,819) were non-synonymous substitutions (**Additional File 4)** that are likely to have an effect on protein function and structure. Several of these SNPs were located in virulence genes representing good candidates to explain the diversity in patterns of mortality and chronicity we observed.

Virulence factors of RP isolates and their putative role in pathogenicity .

The genome of RP isolates contains most of the virulence factors, described for other P. a eruginosa genomes and identified in the Virulence Factor (VF) database (http://www.mgc.ac.cn/VFs/) [25], with few exceptions. Several VFs of RP73 isolate shows signatures of pathoadaptive mutations within the genome when compared to the preceding clonal RP45 isolate as reported in Additional File 4. Phenotypic characterization of RP isolates is reported (Table 4) and their putative role in pathogenicity discussed.

Motility, adherence and cell interaction. Pili, flagella and outer membrane proteins promote motility, attach to epithelial or endothelial cells, activate or inactivate host cellular pathways and immune responses [26]. These *P. aeruginosa* VFs play a key role in acute infection and are present in RP1 isolate. Variations in the twitching and swimming motility are common in *P. aeruginosa* isolates from CF patients and described to be hallmarks of bacterial adaptation to the airways [7, 27]. Both RP73 and its clonal RP45 isolate did not encode *pilA*, *pilV*, *pilW*, *pilY2* and *fimT*, carried a deletion at the N-terminal in *pilC* and a premature stop mutation in *pilO* (**Table 3**). The RP73 and RP45 phenotypes are consistent with the absence of twitching and swimming motility (**Table 4**). Lack of motility was associated with decreased virulence in models of acute infection [28, 29] and increased risk of chronic infection [17]. Our results obtained in RP73 and RP45-infected mice (**Figure 2**) are consistent with the observation that unlike strains RP1, PA14 and PAO1, favor long-term persistence.

Lipopolysaccharides (LPSs) are potent immune stimulants through their interactions with Toll-like receptor 4 (TLR4). *P. aeruginosa* strains isolated from CF patients evolved the capacity to reduce host immuno-detection by modulating LPS structure [30]. Biochemical and biological characterization of RP73 LPS showed to possess an under-acylated lipid A leading to a lower pro-inflammatory capacity in a murine model of intranasal instillation when compared to the LPS from the prototype strain PAO1 [31]. This structure is distinguished by the absence of hexa- or hepta-acylated lipid A species that are typical phenotypic changes that can occur on the LPS molecule of

a *P. aeruginosa* chronic strain. Furthermore, RP73 carries an R-type LPS without O-antigen in which the lipid A is covalently linked to the core oligosaccharide region. Absence of LPS O-antigen in RP73 suggests an adaptation of this strain to persistent lifestyle.

Among genes responsible for lipid A modification (*lpxO1, lpxO2, phoP, phoQ, pagL*and *OprH*) [30], our genetic characterization showed that RP73 isolate carries a stop mutation and lacks of the C-terminus of the protein in *lpxO2* compared to the preceding clonal RP45, as well as RP1 isolate and prototype PAO1 and PA14 strains (**Table 3**). These data suggests that an adaptive process has occurred to the LPS structure of the RP lineage in the period of time between the isolation of RP45 and RP73.

Secretion systems and toxins. In *P. aeruginosa* genome, the genes (*psc, pcr, exs* and*pop*) encoding the type III secretion system (T3SS) are clustered together. The *psc*and *prc* genes primarily encode components of the bacterial secretion apparatus whereas the *exs* gene products are involved in regulation of TTSS. Two *pop* genes encode proteins (PopB and PopD) essential for the translocation of the effectors into host cells. Remarkably, RP73 isolate lost the entire 32-gene cluster encoding the T3SS which was present in the preceding clonal RP45, as well as RP1 and prototype PAO1 and PA14 strains (**Table 3**) [25]. However, the *exoS, exoT,* and *exoY* genes encoding for the ‘‘T3SS translocated effectors’’ are still present both in RP73 and RP45 genome with two non-synonymous SNPs recorded in the *exoT* when compared with RP1 and PA14 (**Table 3** and **Additional File 4**). T3SS is an important virulence determinant of *P. aeruginosa* which may act at the site of infection and contribute to subversion of the host immune response. In contrast to acute infection, small proportion of isolates infecting CF patients secrete T3SS proteins and this proportion decreases with duration of infection [32]. We speculate that the differences in the risk of mortality associated to RP73 and RP45 isolates may be linked to absence or presence of the T3SS that changed during the progression of CF chronic infection.

Genes encoding for *toxA, hcnA, hcnB* and *hcnC* are present. However, if we consider the exotoxin A, we observed several non-synonymous SNPs (**Table 3** and **Additional File 4**).

Finally, *pldA* coding for the periplasmic phospholipase D (**Table 3**), one of effectors of the type VI secretion system [33], is absent in RP73 genome. However *pldA* is reported to be not conserved among *P. aeruginosa* genomes [34].

Iron uptake and pigment. The ability to produce siderophores, like pyochelin and pyoverdine, has been linked to the bacterial pathogenic potential. Phenotypic assay showed that RP isolates secreted lower (RP73 and RP1) or no (RP45) levels of siderophores when compared to PAO1 and PA14 (**Table 4**). When we look at the genome sequence, RP73 and RP45 carries stop mutations both in *pchD* and *pvdD* involved in the synthesis of the two principal siderophores while *pvdD* is deleted in RP1 (**Table 3**). PvdD production was shown to be required for airways bacterial colonization in rat, lethal virulence in burned and immunosuppressed mouse models[35, 36]. Pyoverdine was detected in the sputa of CF patients [37], while in a larger study, one-third of sputa positive for *P. aeruginosa* contained no detectable pyoverdine [38]. These data suggests that pyoverdine-mediated iron uptake may not always be essential for chronic infection and other mechanisms are active in CF [39].

Phenotypic tests showed that RP73 and RP45 are able to secrete less pyocyanin when compared to RP1, PAO1 and PA14 strains (**Table 4**). However, no changes in genes involved in pyocyanin biosynthesis were found. Pyocyanin is required for full virulence in animal models and has been detected in patients' airway secretions, promoting virulence by interfering with several cellular functions in host cells including electron transport, cellular respiration, energy metabolism, gene expression, and innate immune mechanisms [40].

Antiphagocytosis. RP isolates are phenotypically non-mucoid (**Table 4**) with absence of mutations in *mucABD* locus. Other key regulators of alginate pathway are present with the exception of *algP* (**Table 3**), required for transcription of the key biosynthetic gene *algD* and necessary for exopolysaccharide production [41].Absence of functional AlgP, has been associated with non-mucoid phenotype in strains from CF patients.

The phenotypic switch to mucoidy in *P. aeruginosa* infections is a well-established paradigm in CF. Infection with the mucoid phenotype, which produces large amounts of the exopolysaccharide alginate, has been associated with a more rapid decline in pulmonary function than infection with non-mucoid bacteria [42]. However, some but not all *P. aeruginosa* isolates became mucoid in the CF lung suggesting that a mucoid phenotype did not always confer a selective advantage to bacterial cells in persistence [43]. In mouse model, CF clonal strains, displaying a mucoid and a non-mucoid phenotype, showed a similar capacity of persistence [17]. Our data obtained in mouse model with RP73 and RP45 isolates support the notion that non-mucoid *P. aeruginosa* strains are able of long-term persistence.

Regulation. In *P. aeruginosa*, many virulence determinants and secondary metabolites are regulated in a cell population density-dependent manner via cell-to-cell communication or "quorum sensing" (QS) [44]. *P. aeruginosa* possesses two *N*-acylhomoserine lactone (AHL)-dependent QS systems. These are termed the *las* and*rhl* systems, consisting of the LuxRI homologues, LasRI and RhlRI, respectively. RP isolates have no mutations in *lasI*, *rhlR* and *rhlI* genes. However, inactivation of the transcriptional regulator LasR, lacking 20 aminoacidic residues at the C-terminus (**Table 3**), was found both in RP73 and RP45, while the RP1 has no changes. The distinctive *lasR* mutant phenotype was confirmed by colony morphology that includes surface iridescent sheen and colony flattening exclusively in RP73 and RP45 (**Table 4**) [45].

Mutations in *lasR* lead to several phenotypic changes of potential clinical significance, including a growth advantage in amino acid abundant CF airway secretions. LasR regulates the production of virulence factors (elastase, protease, alkaline protease and exotoxin A) affecting the immune response and antibiotic resistance [46]. Most importantly, *lasR* mutations are often associated with the progression of CF lung disease and may serve as a marker of early CF adaptive change of prognostic significance. [47].

Antibiotic resistance. RP73 showed remarkable resistance to most of the antibiotic classes while the preceding RP45 and RP1 isolates were not, indicating an increased treatment-refractory during the course of the chronic infection in this CF patient (**Table 4**). A strong link between antibiotic resistance and hypermutation was observed in patients with CF [48]. However, RP73 strain does not have mutations in*mutS*, *mutL* and *uvrD*, described previously as responsible for the hypermutable phenotype [49]. Regarding efflux pumps, the RP73 strain did not show mutation in*mexEF-oprN*, *mexCD-oprJ* and *mexXY*. No insertions or deletions in *ampC*, *ampR*,*mexR*, *mexZ* and *oprD* were detected. An insertion at the N-terminal of MexA and anon-synonymous SNP was found in RP73 and RP45 (**Table 3** and **Additional File 4**). MexA belongs to the efflux pump complex MexAB-OprM, which is anchored to the inner membrane via N-terminal fatty acids. Adaptive mutations in *mexA* have been reported in CF isolates [7].

Additional modifications were detected at the N-terminal of *mexT*, which is not conserved in RP73 and RP45 (**Table 3**). An additional non-synonymous aa change at position 128 was found in RP73 when compared to RP45 (**Additional File 4).** MexT plays a pleiotropic role in modulating *P. aeruginosa* virulence such as TTSS, pyocyanin production and early surface attachment [50]. Similarly to MexA, also MexT is an hallmark of *P. aeruginosa* adaptation in CF

patients [7]. Among the additional 58 PAO1 coding sequence annotated as "antibiotic resistance and susceptibility", only *arr*, a putative aminoglycoside response regulator, is absent in RP73 while present in RP45 genome.

CONCLUSIONS

Taken together, our study combined clinical data, whole-genome analysis and animal models to link the persistent lifestyle of *P. aeruginosa* in CF lungs with the bacterial genetic basis. Starting from a clinical case of CF, *P. aeruginosa* RP73 was isolated after long-term chronic infection and compared with the preceding RP1 and clonal RP45, as well as prototype PAO1 and PA14 strains. When tested in the animal model, *P. aeruginosa* RP73 isolate, but not other strains, mimics most of the traits of airways infection and inflammation observed in CF patients. These results suggested that key features of RP73 isolate may contribute to its pathogenesis. The genome sequence of RP73 and comparative genomics analysis with other *P. aeruginosa* genomes, pointed clearly to signatures of pathoadaptive mutations within the genome. This in turn correlated with the major impact on the *in vitro* phenotypes and *in vivo* maintenance observed and described here. Our findings support and better define the hypothesis that genes encoding major virulence factors are deleted and/or contain beneficial mutations when *P. aeruginosa* establishes long-term chronic infection. The results presented in this study provide important information with respect to the *P. aeruginosa* mosaic genome structure and chronic infections found in CF patients.

METHODS

Bacterial strains and CF patient. CF clinical *P. aeruginosa* RP1, RP45 and RP73 isolates were chosen from the collection of the CF clinic Medizinische Hochschule Hannover, Germany. Genotypic analysis by multimarker array and phenotypic data of P. aeruginosa strains were partly published [51]. P. aeruginosa was cultured in Pseudomonas isolation agar (PIA) or Trypticase Soy Broth (TSB) at 37°C. CF patient gave informed consent before the sample collection. Approval for storing of biological materials was obtained by the Hannover Medical School, Germany.

Phenotypic characterization. Swimming and twitching capacities protease, siderophore and pyocyanin secretion, hemolytic activity, and LasR mutant phenotypic analysis were assayed as described in the online data supplement.

Genome sequencing. The genome of RP73 was previously published [19]. Genomic DNA from strains RP1 and RP45 was isolated from overnight cultures using the DNeasy Blood and Tissue Kit (QIAGEN). Genomic DNA (500 ng) was mechanically fragmented for 40 sec using a Covaris M220 (Covaris, Woburn MA, USA) with default settings. Fragmented DNA was transferred to PCR tubes and library synthesis was performed with the Kapa Hyperprep kit (Kapa biosystems, Wilmington MA, USA) according to manufacturer's instructions. TruSeq HT adapters (Illumina,SanDiego, CA, USA) were used to barcode the samples and each library wassequenced in 1/48 of an Illumina MiSeq 300 bp paired-end run at the Plateforme d'Analyses Génomiques of the Institut de Biologie Intégrative et des Systèmes (Laval University, Quebec, Canada). Sequencing data for each genome was assembled with the A5 pipeline [52].

Genomic analyses. Blast (NCBI) was used to compare the genome of RP73 to prototype strains and to the complete *Pseudomonas aeruginosa* content in Genbank. Genomic islands were predicted with Island Viewer [53] and annotated with xBase [54]. The core genome phylogeny was determined using the Harvest suite [52]. The data set of sequences we used to generate the core phylogeny includes 53 strains representative of P. aeruginosa diversity [55]. MLST profiles were determined combining the results obtained from the pubmlst database (http://pubmlst.org) and the SRST2 software package [56]. SNPs between PA14 and RP1, RP45 and RP73 were detected with the Samtools software package [57] (samtools mpileup options: -C 50, SNPs with quality score of less than 30 were discarded).

Mouse model of P. aeruginosa acute and chronic lung infection. For chronic infection, C57BL/6NCrlBR male mice (20-22g, Charles River) were infected with 1-2x106 CFUs of *P. aeruginosa* strains, embedded in agar beads [15, 17]. Fourteen days post-challenge lungs were recovered, homogenized and plated for CFUs counting. In additional group of mice, the lungs were excised for histopathology. Additional details are reported in the Online data supplement. Student's t-test and the $\chi 2$ test onsidering $p<0.05$ as the limit of statistical significance was performed.

Animal studies were conducted according to protocols approved by the San Raffaele Scientific Institute (Milan, Italy) Institutional Animal Care and Use Committee.

AVAILABILITY OF SUPPORTING DATA

The data sets supporting the results of this article are included within the article and its additional files.

LIST OF ABBREVIATIONS

N-acylhomoserine lactone (AHL); coding sequences (CDSs); cystic fibrosis (CF) colony forming units (CFUs); chronic obstructive pulmonary disease (COPD); forced expiratory volume (FEV); forced vital capacity (FVC); genomic islands (GIs); Liverpool epidemic strain (LES); Lipopolysaccharides (LPSs); multidrug-resistant(MDR); open reading frames (ORFs); *Pseudomonas* isolation agar (PIA); Pulsed Field Gel Electrophoresis (PFGE); Toll-like receptor 4 (TLR4); Trypticase Soy Broth (TSB); type III secretion system (T3SS); Virulence Factor (VF)

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

IB, JJ, LF, BAF, MF, BB and IKI performed research; IB, AM, BT, RL, and AB designed research; BT, AM, RL and AB contributed new reagents/analytic tools; IB, JJ, LF, BAF, MF, BB, AM and AB analyzed data; IB, JJ, LF, BAF, BT, RCL and AB wrote the paper.

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LEGENDS

Figure 1. *P. aeruginosa* sequential isolates from patient RP. Two clone types (OC2E and OC4A) of *P. aeruginosa* strains were isolated from patient RP who is heterozygous for F508del and R1162X mutations in the *CFTR* gene. OC2E was isolated at the onset of chronic colonization for the first eleven years. Thereafter OC4A became the dominant clone. Strain RP1 belongs to the clone type OC2E and was the first *P .aeruginosa* strains isolated. Strains RP45 and RP7 belong to the clone type OC4A and were isolated after 10 and 16.9 years respectively after the onset of chronic colonization of the patient's airways with *P. aeruginosa* (**Additional File 1**and Cramer et al. [12]). Lung function parameters at the time of *P. aeruginosa* isolation are indicated.

Figure 2. Virulence of *P. aeruginosa* RP isolates in comparison with prototype strains in murine model. C57Bl/6NCrlBR mice were infected with 1-2*10_6 CFU/lung *P. aeruginosa* RP1, RP45, RP73, PAO1 and PA14 strains embedded in agar beads. Mortality induced by bacteremia (black) and survival (light gray) was evaluated on challenged mice. Clearance (white) and capacity to establish chronic infection (dark gray) were determined on surviving mice after 14 days. The data show the percentage of mice infected with single *P. aeruginosa* strains analyzed in two to three independent experiments. Statistical significance by Chi-square test is indicated: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure 3. Histological lesions after chronic *P. aeruginosa* infection in mice. Mice were infected with 2x10_6 cfu/lung of *P. aeruginosa* RP73 strain embedded in agar beads and lung harvested after 14 days from challenge. Histopathological analysis of lungs chronically infected by RP73 strain are characterised by acute and chronic lesions; the pulmonary parenchyma is infiltrated by macrophages, lymphocytes and some neutrophils **(A)**. Agar beads (arrow) containing bacteria macrocolonies (*) are localised in the bronchia and surrounded by a massive neutrophils inflammation **(B)**. Alcian blue staining shows mucus secretory cells hyperplasia (circle) **(C)**.

Figure 4: Circular map of *P. aeruginosa* RP isolates and prototype strain PA14.
Circular map constructed with the CGView Comparison Tool [58]. Starting from the outside: genomic islands predicted with Island Viewer (see **Table 1** for details) [53],RP73, RP45, RP1, PA14 and GC content. Colored regions are shared with RP73 according to blast search. Dotted lines: known genomic islands (GIs) that distinguish RP73 because they are incomplete or absent in

the 12 complete *P. aeruginosa*genomes available at pseudomonas.com. RP isolates also carry LESGI-4, identified in the Liverpool epidemic strain.

Figure 5: Core genome phylogeny for RP isolates and strains representative of *P. aeruginosa* diversity. The figure represents a partial view of the tree to show the relationships between RP1, RP45 and RP73. The position of RP1 is indicated in blue, while the position of RP45 and RP73 is indicated in red. PA14 is distantly related to all these strains.

TABLES

Table 1: Predicted genomic islands in the genome of *P. aeruginosa* RP73 and comparison with other RP isolates and prototype strains.

RP73 genomic island annotation						Other strains from patient RP		Prototype strains			
Predicted Island	**Start position**	**End position**	**Size (bp)**	**Annotation**	**Region of genome plasticity**	**RP45**	**RP1**	**PA14**	**PAO1**	**LESB58**	**PA7**
1-3	1023578	1126198	102620	*Pseudomonas aeruginosa* strain C plasmid pKLC102	41	P	*	*	-	-	*
4	1372342	1385723	13381	Major facilitator transporter, 2-isopropylmalate synthase, putative membrane-bound lytic murein transglycolase A, hypothetical proteins	na	P	P	P	P	P	-
5	1408824	1421034	12210	*Pseudomonas aeruginosa* strain SMC4389 CRISPR repeat sequence	12	P	P	-	-	-	-
6	2576892	2587628	10736	Non-ribosomal peptide synthetases, hypothetical proteins	na	P	P	P	P	P	-
7	3092401	3101220	8819	Type II secretion system proteins, ABC transporter permease, hypothetical proteins	na	P	P	P	P	P	-
8	3764047	3768307	4260	Hypothetical proteins	na	P	P	P	P	P	-

9	3818768	3828628	9860	Non-ribosomal peptide synthetase, FAD-dependent monooxygenase, short chain dehydrogenase, cytochrome P450, 3-oxoacyl-(acyl carrier protein) synthase III, acyl carrier protein, major facilitator transporter	na	P	P	P	P	P	-
10	4356197	4363888	7691	*Pseudomonas aeruginosa* PAGI-9 genomic island sequence	89	P	P	-	-	-	-
11	5866390	5877731	11341	Putative short-chain dehydrogenase, ABC transporter ATP-binding protein, hypothetical proteins	na	P	*	*	*	*	*
12	6002572	6007043	4471	Hypothetical proteins	na	P	P	-	P	P	-

Genomic islands were predicted using Island Viewer [53] and described based on annotation with xBase [54] using *P. aeruginosa* PAO1 as a reference genome.

Co-localization with regions of genome plasticity previously described by Klockgether et al. (2011) [5], P: present, *: partially present (20-90%coverage), -: absent.

Table 2: MLST typing of RP isolates.

Strain	Profile	acs	aro	gua	mut	nuo	pps	trp
RP1	395	6	5	1	1	1	12	1
RP45	198	11	5	11	11	3	27	7
RP73	198	11	5	11	11	3	27	7

In silico MLST typing was performed using Pubmlst (http://pubmlst.org) and Srst2 [59].

Table 3: Comparative pathogenomics of mutations present in the major virulence factors of RP isolates respect to the prototype strain PAO1 and evaluation in others *P. aeruginosa* sequenced strains.

Virulence factors	ORFs	PAO1*	PA14*	LESB58*	PA7*	RP1	RP45	Mutation in RP45	RP73	Mutation in RP73
Type IV Pili biosynthesis	*pilA*	PA4525	PA14_58730	PLES_49071	PSPA7_5161	RP1_3294	deleted		deleted	
	pilC	PA4527	PA14_58760	PLES_49101	PSPA7_5163	RP1_3296	RP45_2662	-271 aa at N-ter	RP73_4913	-271 aa at N-ter
	pilO	PA5042	PA14_66640	PLES_54321	PSPA7_5779	RP1_0159	RP45_2283-RP45_2284	stop mutation after 138aa	RP73_5487-RP73_5486	stop mutation after 138aa
	pilV	PA4551	deleted	PLES_49341	PSPA7_5191	RP1_3324	deleted		deleted	
	pilW	PA4552	PA14_60290	PLES_49351	PSPA7_5192	RP1_3325	deleted		deleted	
	pilY2	PA4555	deleted	PLES_49381	PSPA7_5195	RP1_3328	deleted		deleted	
	fimT	PA4549	deleted	PLES_49321	PSPA7_5189	deleted	deleted		deleted	
Alginate regulation	*algP/algR3*	PA5253	PA14_69370	PLES_56471	PSPA7_5998	deleted	deleted		deleted	
Pyochelin	*pchD*	PA4228	PA14_09240	PLES_06991	PSPA7_0872	RP1_5714	RP45_5410-RP45_5411	stop mutation after 282aa	RP73_4595-RP73_4596	stop mutation after 282aa
Pyoverdin	*pvdD*	PA2399	PA14_33650	PLES_28971	deleted	deleted	RP45_5541	-1072 aa at N-ter	RP73_2604-RP73_2605	stop mutation after 607aa
Phospholipase D	*pldA*	PA3487	PA14_18970	deleted	deleted	deleted	deleted	deleted	deleted	
N-(3-oxo-dodecanoyl)-L-homoserine lactone QS system	*lasR*	PA1430	PA14_45960	PLES_39841	PSPA7_3898	RP1_6176	RP45_5017	-20 aa at C-ter	RP73_1573	-20 aa at C-ter
Type III Secretion System	*pscQ*	PA1694	PA14_42610	PLES_36321	deleted	RP1_4653	RP45_5298		deleted	
	pscP	PA1695	PA14_42600	deleted	deleted	RP1_4654	RP45_5297		deleted	
	pscO	PA1696	PA14_42580	PLES_36311	deleted	RP1_4655	RP45_5167		deleted	
	pscN	PA1697	PA14_42570	PLES_36301	deleted	RP1_4656	RP45_5166		deleted	
	popN	PA1698	PA14_42550	PLES_36291	deleted	RP1_4657	RP45_5165		deleted	

pcr1	PA1699	PA14_42540	PLES_36281	deleted	RP1_4658	RP45_5164	deleted
pcr2	PA1700	PA14_42530	PLES_36271	deleted	RP1_4659	RP45_5163	deleted
pcr3	PA1701	PA14_42520	PLES_36261	deleted	RP1_4660	RP45_5162	deleted
pcr4	PA1702	PA14_42510	PLES_36251	deleted	RP1_4661	RP45_5161	deleted
pcrD	PA1703	PA14_42500	PLES_36241	deleted	RP1_4662	RP45_5160	deleted
pcrR	PA1704	PA14_42490	PLES_36231	deleted	RP1_4663	RP45_5159	deleted
pcrG	PA1705	PA14_42480	PLES_36221	deleted	RP1_4664	RP45_5158	deleted
pcrV	PA1706	PA14_42470	PLES_36211	deleted	RP1_4665	RP45_5157	deleted
pcrH	PA1707	PA14_42460	deleted	deleted	RP1_4666	RP45_5156	deleted
popB	PA1708	PA14_42450	PLES_36201	deleted	RP1_4667	RP45_5155	deleted
popD	PA1709	PA14_42440	PLES_36191	deleted	RP1_4668	RP45_5154	deleted
exsC	PA1710	PA14_42430	PLES_36181	deleted	RP1_4669	RP45_5153	deleted
exsE	PA1711	PA14_42410	deleted	deleted	RP1_4670	RP45_5152	deleted
exsB	PA1712	PA14_42400	PLES_36171	deleted	RP1_4671	RP45_5151	deleted
exsA	PA1713	PA14_42390	PLES_36161	deleted	RP1_4673	RP45_5150	deleted
exsD	PA1714	PA14_42380	PLES_36151	deleted	RP1_4675	RP45_5148	deleted
pscB	PA1715	PA14_42360	PLES_36141	deleted	RP1_4676	RP45_5147	deleted
pscC	PA1716	PA14_42350	PLES_36131	deleted	RP1_4677	RP45_5146	deleted
pscD	PA1717	PA14_42340	PLES_36121	deleted	RP1_6486	RP45_5145	deleted
pscE	PA1718	PA14_42320	PLES_36111	deleted	deleted	deleted	deleted
pscF	PA1719	PA14_42310	PLES_36101	deleted	RP1_6485	RP45_5144	deleted
pscG	PA1720	PA14_42300	PLES_36091	deleted	RP1_6484	RP45_5143	deleted
pscH	PA1721	PA14_42290	PLES_36081	deleted	RP1_6483	RP45_5142	deleted
pscI	PA1722	PA14_42280	PLES_36071	deleted	RP1_6482	RP45_5141	deleted
pscJ	PA1723	PA14_42270	PLES_36061	deleted	RP1_6481	RP45_5140	deleted
pscK	PA1724	PA14_42260	PLES_36051	deleted	RP1_6480	RP45_5139	deleted

	pscL	PA1725	PA14_42250	PLES_36041	deleted	RP1_6479	RP45_5138		deleted	
LPS	*lpxO2*	PA0936	PA14_52150	PLES_43801	PSPA7_4774	RP1_2395	RP45_1632		RP73_0952	-200 aa at N-ter
	oprH	PA1178	PA14_49200	PLES_41431	PSPA7_4201	RP1_2675	RP45_4510		RP73_1306	+45 aa at C-ter
Efflux pumps	*mexA*	PA0425	PA14_05530	PLES_04231	PSPA7_0525	RP1_1913	RP45_1414	+29 aa at N-ter	RP73_0441	+29 aa at N-ter
	mexT	PA2492	PA14_32410	pseudogene	PSPA7_2746	RP1_5791	RP45_4636	N-ter not conserved	RP73_2696	N-ter not conserved
Antibiotic resistance	*arr*	PA2818	deleted	deleted	PSPA7_2339	RP1_6537	RP45_5061		deleted	

* Sources for PAO1, PA14, LESB58 and PA7 information are the Virulence Factor Database (http:/ /www.mgc.ac.cn/cgi-bin/VFs/compvfs.cgi?Genus=Pseudomonas&type=1) and Pseudomonas Database (www.pseudomonas.com).

Table 4: Phenotypic characterization of *P. aeruginosa* RP isolates and prototype strains.

Strain	Mucoidy	Twitching* (ø cm)	Swarming* (ø cm)	Protease (ø cm)	Siderophore (ø cm)	LasR phenotype§	Pyocyanin #	Biofilm°	Antibiotic resistance
RP1	-	2.3	3.4	2.1	1.2	-	0.094±0.016	0.332±0.141	-
RP45	-	-	-	-	-	+	0.051±0.015	0.493±0.146	GEN
RP73	-	-	-	-	1.3	+	0.05±0.013	1.289±0.596	AMK; CAZ; GEN; IMP; MER
PAO1	-	1.2	2.6	1.8	2.0	-	0.127±0.015	1.719±0.217	-
PA14	-	1.2	5	2.1	2.0	-	0.09±0	3.553±0.457	-

* Indicates twitching and swarming motility zone diameter, as measured by subsurface stab assay.

§ Isolates with iridescent and metallic sheen of the colony surface, that is typical for a *lasR* mutant, are indicated (+).

# Indicates mean value ± SD at 26h. Values ≤ 0.05 indicate no production of pyocyanin.

° Indicates mean value ± SD at 24h.

AMK= amikacin; CAZ= ceftazidime; GEN= gentamicin; IMP= imipenem; MER = meropenem

ADDITIONAL MATERIAL

Additional File 1. PFGE of *P. aeruginosa* RP isolates.

Additional File 2. Virulence of *P. aeruginosa* RP isolates, and prototype strains in a murine model of chronic airways infection.

Additional File 3: Genomic features of RP strains compared to others complete *P. aeruginosa* genomes.

Additional File 4: Non-synonymous SNPs between prototype strain PA14 and strains RP73, RP45 and RP1

Figure 1

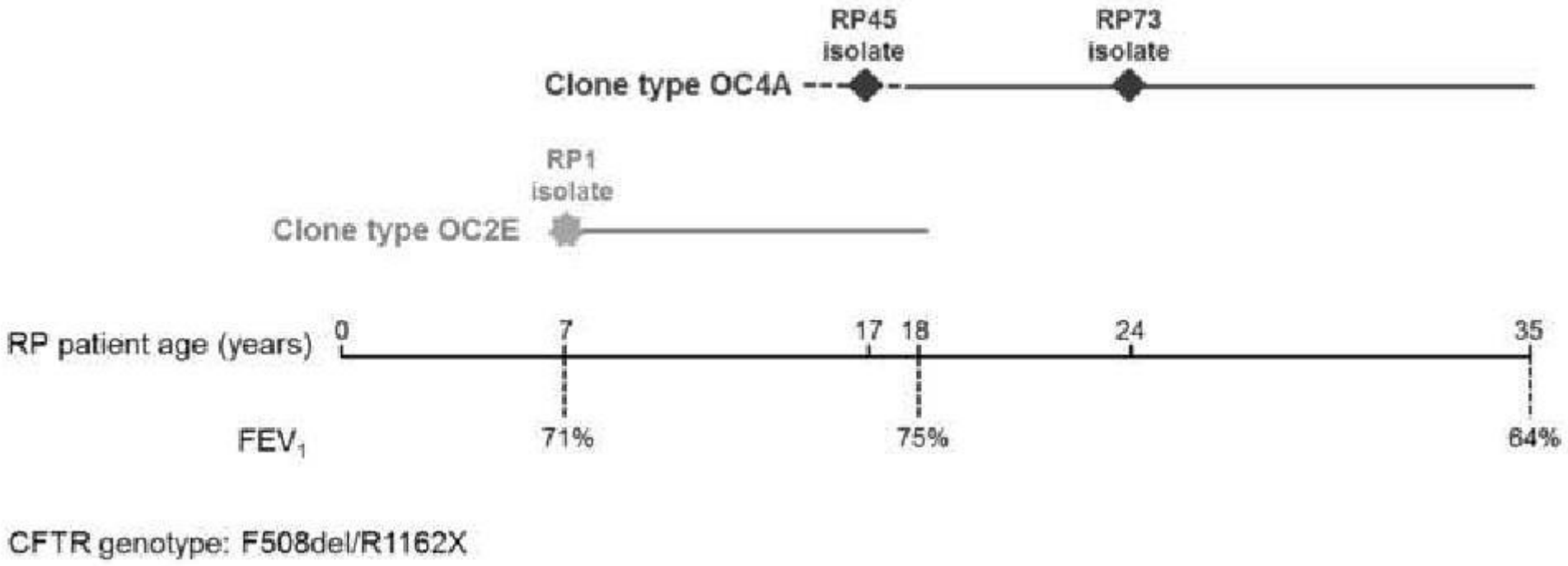

RP45
isolate
RP73
isolate
Clone type OC4A
RP1
isolate
Clone type OC2E
RP patient age (years)
0
7
17
18
24
35
FEV1
71%
75%
64%
CFTR genotype: F508del/R1162X

Figure 2

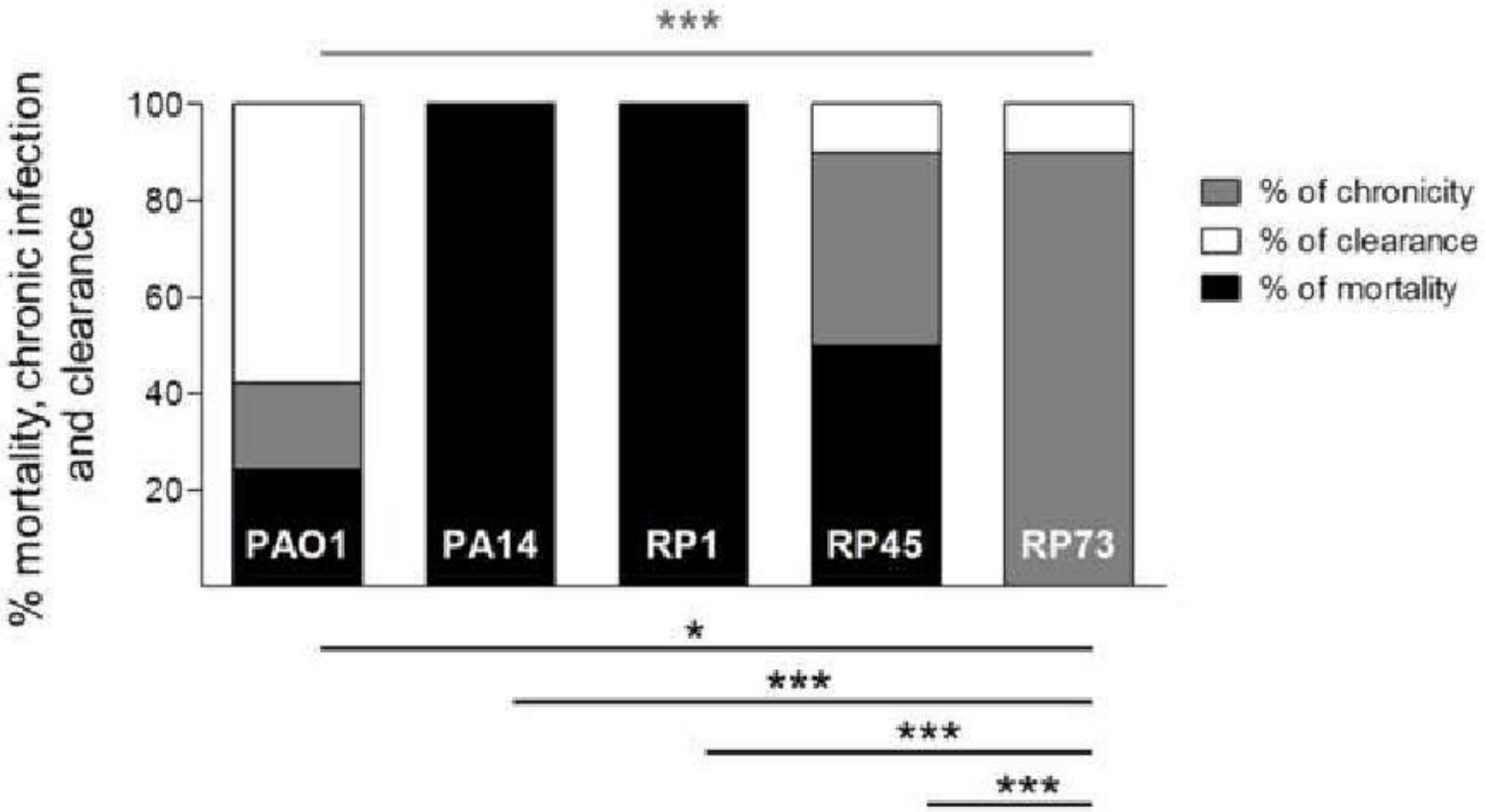

***
% mortality, chronic infection and clearance
100
80
60
40
20
PAO1
PA14
RP1
RP45
RP73
% of chronicity
% of clearance
% of mortality
*
***
***
***

Figure 3

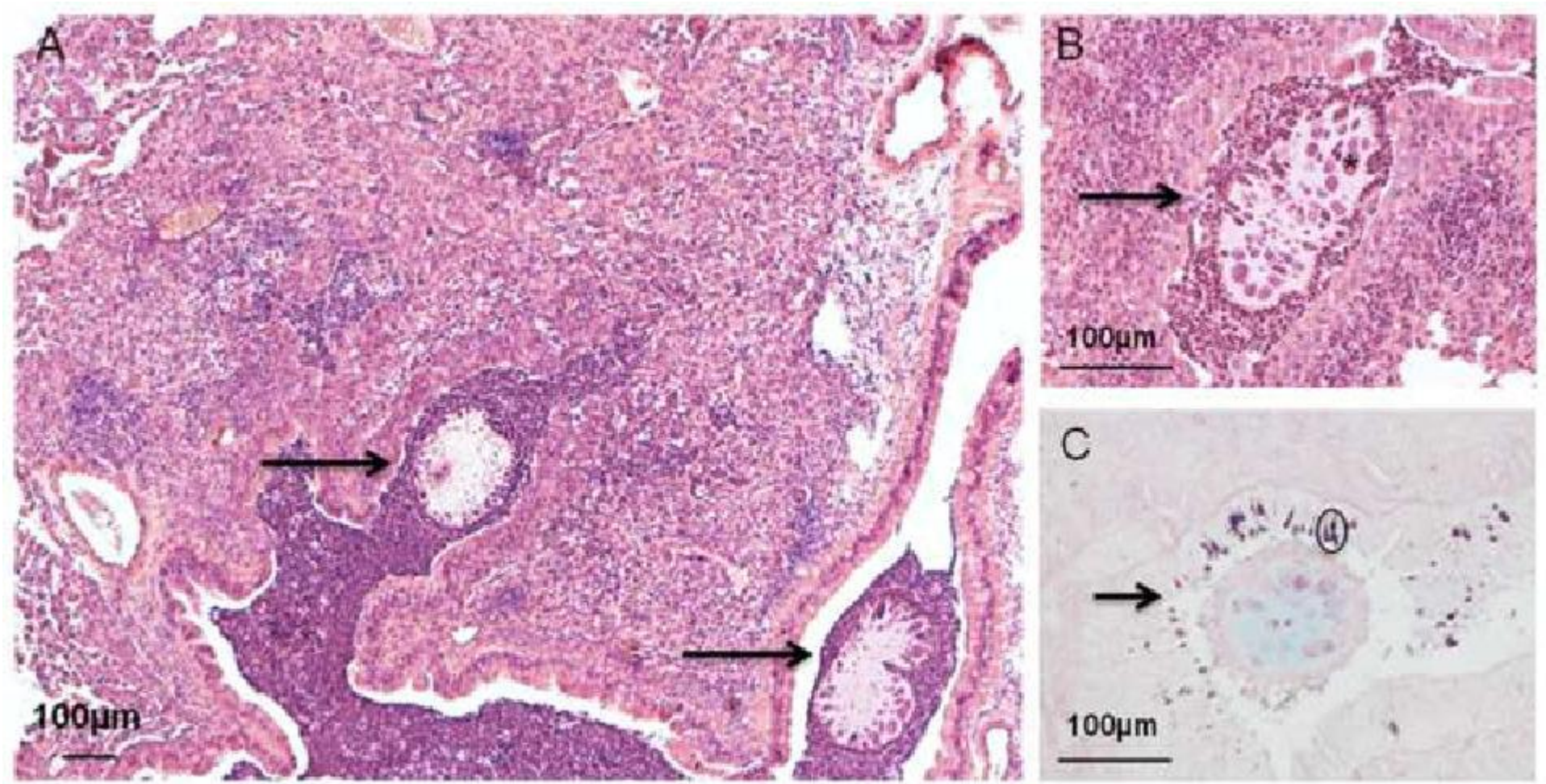


Figure 4

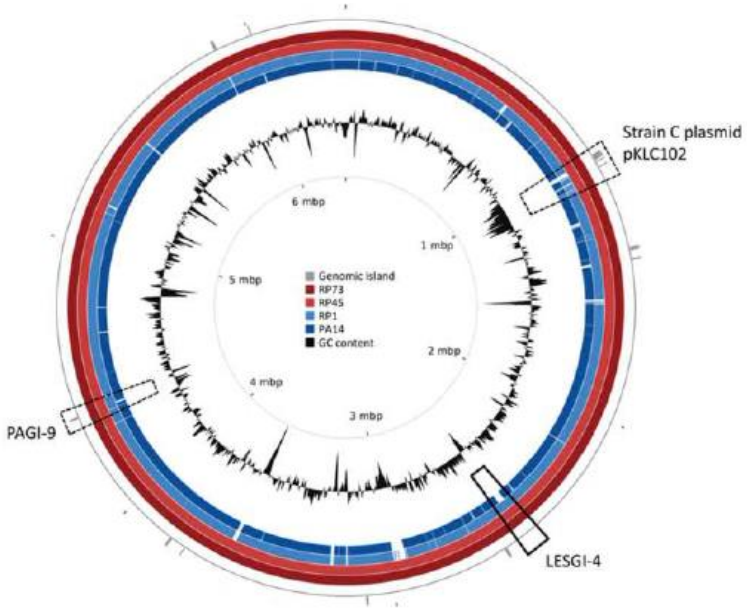
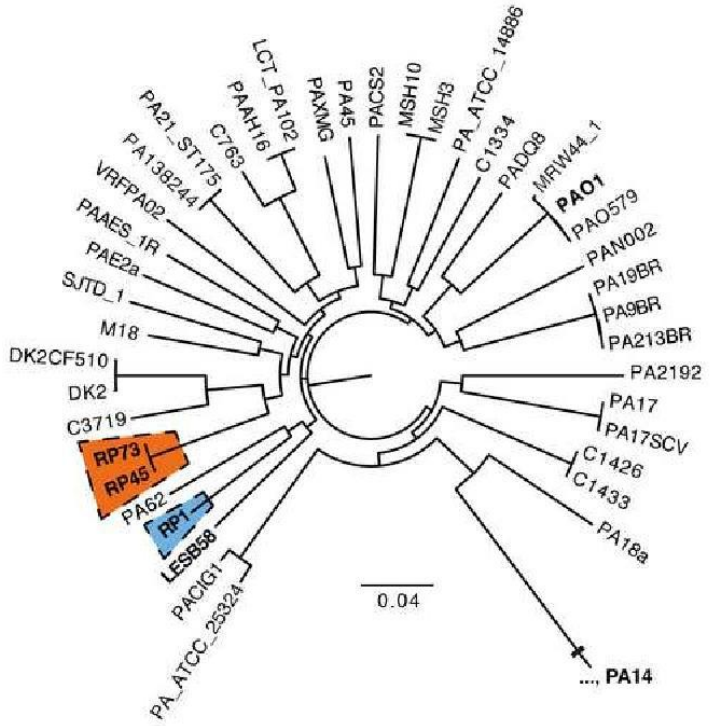


Figure 5



Submitted paper and manuscript in preparation

The submitted paper “Efficacy of the novel antibiotic NCE in pre-clinical models of *Pseudomonas aeruginosa* pneumonia” (European Respiratory Journal) and the manuscript in preparation ”Genome-based approach deliver vaccine candidates against *Pseudomonas aeruginosa*” cannot be attached due to current patent rights.