Host-Pathogen Interactions in Pseudomonas aeruginosa Invasive and Respiratory Tract Infection

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Host-Pathogen Interactions in *Pseudomonas aeruginosa* Invasive and Respiratory Tract Infection

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LUND
UNIVERSITY

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Abstract

Pseudomonas aeruginosa is an opportunistic bacterium that causes debilitating infections when the immune defence is compromised. It possesses an arsenal of virulence traits to colonize most compartments of the body and is often highly resistant against commonly used antimicrobial drugs.

Outer membrane vesicles (OMV) are spheres released from Gram-negative bacteria. They are packed with proteins, including beta-lactamase. By exploring OMV from a pathogen related to *P. aeruginosa* (*Moraxella catarrhalis*) we discovered that beta-lactamase inside OMV was protected from neutralization by IgG and could protect bacteria from amoxicillin.

Many pathogens avoid killing by the complement system by capturing complement regulators at their bacterial surface. We found that *P. aeruginosa* from the airways bound more vitronectin than other clinical isolates. By using a proteomic approach, we identified vitronectin-binding adhesins and found that *Pseudomonas* uses Porin D to capture vitronectin on its surface.

To investigate the *in vivo* importance of vitronectin-binding, we analysed vitronectin concentrations in bronchoalveolar lavage fluid (BALF). Patients with pneumonia had significantly higher concentrations than control subjects. This vitronectin increase was confirmed by pulmonary exposure of endotoxins to healthy volunteers. We also found that bacteria could capture vitronectin from BALF and subsequently survive challenge with serum. Immunocytochemistry indicated that epithelial cells produced vitronectin. This production was confirmed *in vitro* and was triggered by OMV-dependent stimulation of epithelial cells.

Finally, after collecting data on bacteraemic *P. aeruginosa* episodes, we investigated the effect of comorbidities, treatment, and microbiological characteristics on the outcome of bacteraemia. The choice of treatment was critical, particularly choosing a combination-treatment including ciprofloxacin was beneficial. Moreover, respiratory origin of the infection correlated to high mortality, indicating that compartment-specific factors impacted the outcome.

In conclusion, interactions between the host and bacteria are multifaceted and *P. aeruginosa* utilizes outer membrane proteins and vesicles to protect against the attacks of the human immune system.

Key words: Bacterial colonization, respiratory tract infections, *Pseudomonas aeruginosa*, Outer membrane vesicles, Outer membrane proteins, Complement system, Innate immunity, Vitronectin, Blood stream infections, Ciprofloxacin, Antimicrobial therapy

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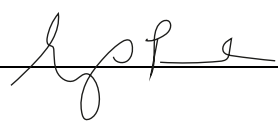
Signature  Date 2017-04-10

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To the nerds, the geeks and the late bloomers

(Well, who else would read this anyway?)

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Paper I

Schaar V, **Paulsson M**, Mörgelin M, Riesbeck K. Outer membrane vesicles shield *Moraxella catarrhalis* beta-lactamase from neutralization by serum IgG. Journal of Antimicrobial Chemotherapy. 2013 Mar;68(3):593–600.

Paper II

Paulsson M, Singh B, Al-Jubair T, Su Y-C, Høiby N, Riesbeck K. Identification of outer membrane Porin D as a vitronectin-binding factor in cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*. Journal of Cystic Fibrosis. 2015 Sep;14(5):600–7.

Paper III

Paulsson M, Che KH, Ahl J, Smith M, Qvarfordt I, Su YC, Lindén A, Riesbeck K. Vitronectin is produced in the lung upon infection by respiratory pathogens, and is utilized to conquer the innate immunity. Manuscript in preparation.

Paper IV

Paulsson M, Granrot A, Ahl J, Tham J, Resman F, Riesbeck K, Månsson F. Antimicrobial combination treatment including ciprofloxacin decreased the mortality rate of *Pseudomonas aeruginosa* bacteraemia: a retrospective cohort study. European Journal of Clinical Microbiology & Infectious Diseases. 2017 Jan 21;68:2697.

Abbreviations

AMP	Antimicrobial polypeptides
BALF	Bronchoalveolar lavage fluid
BSI	Blood stream infection
C4BP	C4b binding protein
CAP	Community-acquired pneumonia
CCP	Complement control protein
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ESBL	Extended spectrum beta-lactamases
EVs	Extracellular vesicles
HAP	Hospital-acquired pneumonia
Ig	Immunoglobulin
IL	Interleukin
kDa	kilo Dalton
LOS	Lipooligosaccharides
LPS	Lipopolysaccharides
MAC	Membrane attack complex
MBL	Mannose binding protein

MDR	Multi-drug resistance
MIC	Minimal inhibitory concentration
MID	Moraxella IgD-binding protein
MLST	Multi-locus sequence typing
mRNA	Messenger ribonucleic acid
NTHi	Non-typeable *Haemophilus influenzae*
OMP	Outer membrane protein
OMV	Outer membrane vesicles
OR	Odds ratio
pAbs	Polyclonal antibodies
PAMP	Pathogen-associated molecular patterns
PBP	Penicillin binding protein
PRR	Pattern recognition receptor
PQS	*Pseudomonas* quinolone signal
RCA	Regulators of complement activation
RT-PCR	Reverse transcription polymerase chain reaction
TEM	Transmission electron microscopy
T3SS	Type III secretion system
T4SS	Type IV secretion system
T6SS	Type VI secretion system
TLR	Toll like receptor
Usp	Ubiquitous surface protein
UTI	Urinary tract infection
VAP	Ventilator associated pneumonia

Populärvetenskaplig sammanfattning

Det är samspelet mellan bakterie och människa som avgör vilka infektioner som gör oss sjuka och vilka som till och med kan vara av nytta för oss. På vår hud och i våra tarmar finns miljarder bakterier av tusentals olika arter och även i friska luftvägar finns bakterier. Men det finns bakterier som genom sitt sätt att växa och använda näringsämnen skadar kroppen, sätter igång immunförsvarets skyddsmekanismer och orsakar sjukdom. Även om *Pseudomonas aeruginosa* är en mycket vanlig bakterie i naturen, tillhör den denna grupp av sjukdomsalstrande bakterier.

Trots det tillhör inte *P. aeruginosa* de bakterier som oftast ger upphov till lunginflammation, urinvägsinfektion eller sårinfektioner hos personer som är i övrigt friska. Istället är det individer med nedsatt immunförsvar, kroniska sjukdomar eller akuta skador som drabbas. Patienter som får cellgifter drabbas av spridd bakterieinfektion i blodet, patienter med kroniskt obstruktiv lungsjukdom eller cystisk fibros av lunginflammation, urinvägskateterbärare av urinvägsinfektion och brännskadade av sårinfektion. När väl bakterien fått fäste kan den ge upphov till livshotande infektioner som är mycket svårbehandlade. *Pseudomonas* är nästan alltid resistent mot våra vanligaste antibiotika. Dessutom har stammar med motståndskraft mot mer ovanliga antibiotika börjat spridas. Syftet med denna avhandling är att undersöka samspelet mellan strukturer på bakteriens yta, vårt immunförsvar och sjukvårdens antibiotika, så att vi bättre kan rikta kommande vacciner, antibiotika eller andra behandlingar dit där de är mest effektiva.

Många bakterier bildar runda blåsor (vesiklar) från sitt yttre hölje. Dessa klotformade partiklar avknoppas från bakteriernas yta när de växer. De är mycket små, ca 100 nm, men innehåller större delen av de proteiner, sockerarter och membran som omger bakterierna. Även DNA och en del av innehållet i bakterierna följer med vesikeln. Det var känt att *P. aeruginosa* tillverkade penicillin-nedbrytande enzymer, beta-laktamas, och utsöndrade dessa i vesiklar. Vi utnyttjade vesiklar från en besläktad bakterieart, *Moraxella catarrhalis*, för att studera om de hindrade immunförsvaret från att bryta ner beta-laktamas, vilket de gjorde. Konsekvensen av detta fynd är att vi nu vet att bakterierna kan packa olika ämnen i vesiklar och skicka iväg dem i kroppen utan att ämnena bryts ner. Genom denna långdistansleverans förhindrar beta-laktamas från *Moraxella* att andra bakterier i omgivningen dödas av penicillin, antingen på platsen där vesikeln skapades, eller någon annanstans i kroppen.

På utsidan är vesiklarna mycket lika hela bakterier och orsakar ett kraftigt svar från immunförsvaret. Detta utnyttjade vi experimentellt efter att vi hade upptäckt att patienter med lunginflammation hade mycket vitronektin i lungorna. Vitronektin är ett protein som reglerar immunförsvaret och skyddar våra celler från nedbrytning. För att visa att denna ökning är ett svar på bakterieinfektionen återskapade vi lunginflammationen i modeller. Först såg vi en ökning av vitronektin hos friska försökspersoner efter att de fått ytämnen från bakterier (LPS) i sina lungor. Sedan utsatte vi celler i provrör för vesiklar från *Pseudomonas*. Vi såg då att cellerna svarade med en ökning i tillverkningen av vitronektin. Det var känt att vissa sjukdomsalstrande bakterier utnyttjar vitronektin på så sätt att de skyddas mot immunförsvaret genom att de binder proteinet till sin yta. Vi visar att detta kan ske i lungorna. Sammantaget beskriver vi en fascinerande mekanism där bakterierna orsakar ökningen av vitronektin i lungan genom att de signalerar till kroppens celler med sina ytstrukturer, de binder sedan till sig vitronektinet på sin yta och stoppar därmed upp immunförsvarets attack.

För att hålla fast vitronektin på ytan behöver bakterien en bindande molekyl. På bakteriecellens yta finns mängder av olika proteiner och genom att rena fram dessa och dela upp dem efter storlek och laddning kunde vi hitta ett protein som band till vitronektin. Detta var ett vanligt förekommande ytprotein, Porin D. Vi tillverkade sedan Porin D i stora mängder genom att flytta genen för Porin D in i en muterad bakterie. Då kunde vi undersöka hur dessa två proteiner reagerade med varandra. Vi upptäckte även att en bakterie som från början inte kunde binda vitronektin på sin yta började göra det om vi satte in genen för Porin D.

Vid de allvarligaste infektionerna med *P. aeruginosa* växer bakterier i blodet hos patienten. Vi samlade in information om 292 fall av blodinfektioner orsakade av *P. aeruginosa* från de skånska sjukhusen. Här såg vi att de som drabbas av blodinfektion är personer med dåligt immunförsvar orsakat av hög ålder eller andra sjukdomar. Valet av antibiotika var mycket viktigt för att patienten skulle överleva infektionen. En kombination av två antibiotika visade sig vara bäst, där det ena skulle vara ciprofloxacin. Vi fann att de flesta som fick blodinfektion antingen hade *P. aeruginosa* i lungan eller i urinvägarna först. Om infektionens ursprung var i lungorna var dödligheten högre. Samspelet mellan bakterie och immunförsvar i lungorna är därför mycket intressant och utgör fokus för övriga arbeten i denna avhandling.

Våra studier har pekat på svårigheterna med att behandla *P. aeruginosa* och visat att man behöver använda två sorters antibiotika samtidigt. I takt med att bakteriernas motståndskraft mot antibiotika ökar kommer vi behöva nya behandlingsalternativ. Ett sätt att förhindra infektion är genom vaccinering, men traditionella vacciner har inte varit framgångsrika mot *Pseudomonas*. Det finns ett vaccin riktat mot en besläktad bakterie, meningokocker, som utnyttjar vesiklar tillsammans med viktiga proteiner från bakteriens yta. Vi har genom att studera samspelet mellan *Pseudomonas*

ytstrukturer och kroppens immunförsvar visat att Porin D skulle kunna vara en del av ett sådant kombinerat vaccin. Ett annat sätt att förhindra att bakterierna drar det längsta strået i kampen mot immunförsvaret kan vara att påverka bildningen av vesiklar, men eftersom forskningen på bakterievesiklar fortfarande är i sin linda vet vi ännu inte vad som styr deras uppkomst. Det är rimligt att anta att mycket forskning kommer riktas mot detta spännande område framöver.

Introduction

Pseudomonas aeruginosa is an extraordinary bacterium. It has been portrayed as a harmless saprophyte thriving in soil and water, as a commensal and member of the human microbiome and as one of our deadliest pathogens, resistant to all currently available antibiotics. It inhabits a wide variety of ecological niches and can grow in the drain of a sink, in the rinsing channels of a bronchoscope or in insufficiently concentrated ethanol used to disinfect a hospital bed. This adaptability makes it one of the most important nosocomial infectious agents. It can cause infections in most organs of the human body, but also in other mammals, fish, nematodes, insects, plants, and amoeba. One explanation for this remarkable flexibility is the large genome that *P. aeruginosa* carries. When it was sequenced in year 2000, it was the largest known bacterial genome comprising 6.26 million base pairs [1]. By comparison, the genomes of the strictly human pathogens *Moraxella catarrhalis* and *Haemophilus influenzae* contain 1.86 and 1.83 million base pairs, respectively. The genome of *P. aeruginosa* has likely evolved to favour adaptability to many ecological niches. The strict human pathogens, on the other hand, evolved to become highly specialized organisms that can not survive for long outside the human body [1,2].

The topic of this thesis is how *P. aeruginosa* uses its arsenal of traits to change from an environmental colonizer to establish and invade the human respiratory tract. By using vesicles from *M. catarrhalis*, we investigate how excreted bacterial products are protected from neutralization when they are contained inside extracellular membrane vesicles (Paper I). The roles of vesicles in colonization are further investigated with focus on the interactions between the innate immunity and *P. aeruginosa* that precede the establishment of infection (Papers II and III). Finally, invasive infections are explored epidemiologically to find host and treatment factors that affect the outcome, to enable us to give the patients the best possible available treatment and to give clues as to where future research should be directed (Paper IV).

The Bacteria

Pseudomonas aeruginosa is a Gram-negative rod-shaped bacterium in the phylum *Proteobacteria* and class *Gamma-proteobacteria*. "*Aeruginosa*" was derived from Latin, meaning copper rust and refers to the resemblance of the typical blue-green of the bacterial pigments to oxidized copper. The German botanist Joseph Schroeter suggested the name *Bacterium aeruginosum* based on experiments with pigmented microbes during 1870-1872. Although his descriptions in "Ueber einige durch Bacterien gebildete Pigmente" are not methodologically crystal clear, he used boiled potatoes in a growth plate to find one recurring species that formed green dots on the surface of the potato and coloured the pulp blue. He was never able to see any growing bacteria in his microscope, but he could cultivate new colonies from the original, which also secreted the pigments [3]. The first pure culture was isolated almost a decade later by Carle Gessard in Paris, which he described in his dissertation "De la pyocyanine et de Son Microbe" [4]. Gessard also linked the bacteria to clinical infections and called the bacteria *Bacillus pyocyaneus,* after "blue pus".

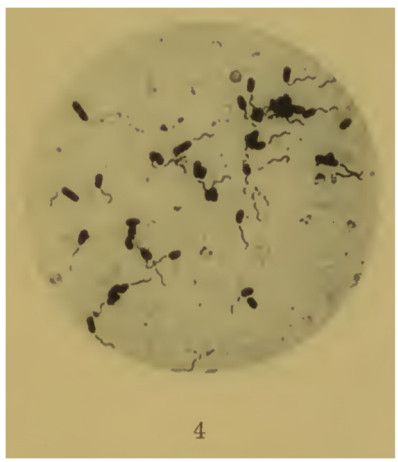

Figure 1:
Drawing of *Pseudomonas pyocyanea* in by Migula in "System der Bakterien" 1892 as seen in his microscope at 1000x [5]. The species was renamed *P. aeruginosa* a few years later.

His peer at the Karlsruhe University, Walter Migula, continued to characterize the bacterial species (Figure 1). He changed its name, first to *Pseudomonas pyocyanera* in his "System der Bakterien" and later to *P. aeruginosa* [5]. The name "*Pseudomonas*" stems from the Greek *pseudo*, meaning false, and *monas*, meaning unit. It is not entirely clear how this name was derived, but "*mons*" was an alternative name for bacteria in general at the time. Eventually, the order, family, and genus all adopted this name (*Pseudomonadales*; *Pseudomonadaceae*; *Pseudomonas*) [6].

In addition to *P. aeruginosa*, both *M. catarrhalis* (Paper I) and *H. influenzae* (Paper III) were studied in this compilation thesis. Like *Pseudomonas*, they are Gram-negative *Gamma-proteobacteria* that cause disease in the human respiratory tract. *Moraxella* belongs to the order *Pseudomonadales*, while *Haemophilus* belongs to the *Pasteurellaceae*.

Moraxella catarrhalis is a diplococcus that was first isolated in 1890 from patients with influenzae-like disease [7]. As it was found to be a part of the microbiome of the upper respiratory tract, a debate followed about the pathogenicity of the species. It has since been moved between different genus, from *Micrococcus*, to *Neisseria*, *Branhamella*, and, finally, *Moraxellacae* [8].

Haemophilus influenzae is an aerobic coccobacilli and a human-specific pathogen. The species is typed using capsular antigens (type a-f) or by their lack of capsule (non-typeable *Haemophilus influenzae*; NTHi). This bacterium was the first free living organism to have its genome fully sequenced [2]. Historically, *H. influenzae* type b has been the most clinically important type as the cause of invasive disease, and was a main cause of epiglottitis, meningitis and severe pneumonia before general vaccination programs were introduced [9]. NTHi, on the other hand, is a common commensal of the human nasopharynx. Similarly to the other two pathogens in this thesis, it can cause opportunistic infection when the respiratory tract is compromized by infection or disease [10].

Microbiological characteristics of *Pseudomonas aeruginosa*

In contrast to the *Enterobacteriaceae*, *P. aeruginosa* is a non-fermenter and derives energy from carbohydrates by oxidation. During aerobic incubation, it is non-fastidious and grows on most agar plates and in all common liquid growth media without any special supplements, although it will grow more readily at the oxygen rich air-liquid interphase. It is a fast-growing bacteria with a doubling time in rich media of 24-27 minutes and of about 66 minutes when grown in sputum [11].

Although *P. aeruginosa* is an oxygen loving bacteria, it can grow in an anaerobic atmosphere but requires the addition of an alternative terminal electron acceptor, for instance nitrate or L-arginine. It can use a wide variety of carbon sources for energy production, for instance sugars and amino acids, but also alcohols (including ethanol), detergents, polycyclic aromatic hydrocarbons from petroleum, crude oil and some plastics [12,13]. This property is likely beneficial for bacterial growth in soil and elsewhere in the environment, but devastating from an infection control perspective in a hospital setting [14].

On agar plates with rich growth media the colonies vary between small dwarf-like to large mucoid types. The colonies, and sometimes the surrounding media, are

coloured green from soluble pigments and develop a characteristic smell of grapes or apples due to secretion of 2-aminoacetophenone [15]. *Pseudomonads* contain cytochrome c oxidases that render the colonies positive in oxidase test, allowing for rapid differentiation from fermenting bacteria.

Most experimental reports about *P. aeruginosa* are from studies on the laboratory strains PAO1 or PA14. PAO1 was originally isolated in the 1950s in Australia from a wound infection and is considered less virulent than the burn wound isolate PA14 [16]. Both reference strains are widely used, the respective genomes are sequenced and transposon mutant libraries are publicly available for the two to facilitate further investigations [17]. The genome of these strains overlap largely and 92% of the PA14 genome is present in PAO1 and 96% of PAO1 in PA14 [18]. The PAO1 genome was annotated by the Pseudomonas aeruginosa Community Annotation Project, PseudoCAP, after the complete sequence was available (available at www.pseudomonas.com) [1]. Based on PAO1, each locus tag was given named PA- and a four digit number, for instance PA0958 for Porin D (Paper II). The database is continuously updated as new data is generated and whenever known, the locus tag annotations are used in this introduction in addition to the protein names. For genes not found in the PAO1 genome, the PA14 counterpart is used instead (PA14_five digit number).

Both reference strains and most clinical *Pseudomonas* strains produce the fluorescent pigments pyocyanin and pyoverdine. Pyocyanin is an antimicrobial pigment that is blue in its oxidized state [19]. Pyoverdine is a yellow-green siderophore that is secreted from the cell to bind iron in the extracellular space. It is then reimported into the periplasm where the iron is harvested. This iron transport system is crucial for biofilm development [20]. Together these pigments are responsible for the distinctive blue-green colour of *P. aeruginosa* colonies and blue pus, sometimes seen in superficial *Pseudomonas* infections.

Biofilm

A signature characteristic of *P. aeruginosa* growth is the development of biofilm. This property has been extensively studied and of paramount importance for the character of clinical *Pseudomonas* infections. Biofilms are bacterial populations enclosed in a self-produced matrix containing polysaccharides, deoxyribonucleic acid (DNA), cell debris and outer membrane vesicles (OMV or extracellular vesicles [EVs], introduced in detail below) [21]. The bacteria are adherent to the matrix, to each other and sometimes, but not necessarily, to a solid surface.

The structure of the biofilm varies with the maturity and the components of the matrix (Figure 2). As one example, rhamnolipids influence how bacteria move by decreasing surface tension and therefore the three dimensional structure of biofilm

[22]. Rhamnolipids are amphipathic molecules from *P. aeruginosa* that are composed of a hydrophobic lipid and a hydrophilic sugar part (rhamnose). These biosurfactants were discovered in 1949 and have since been used industrially for many purposes, for instance as a detergents, in cosmetics and as biodegradants [23].

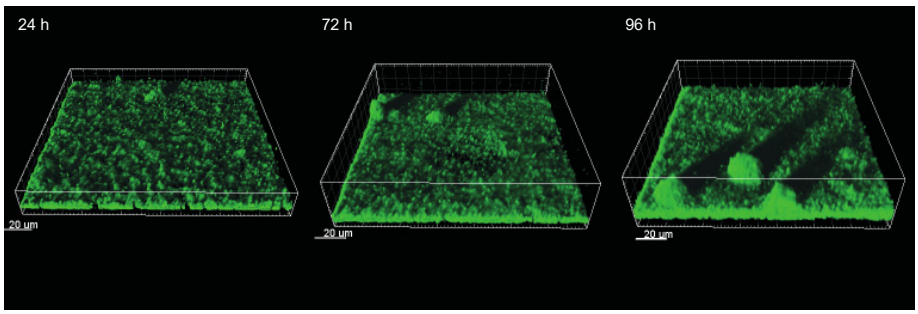

Figure 2: Biofilm
Biofilm development of *P .aeruginosa* PAO1 after 24, 72 and 96 h grown in a flow cell chamber. After one day, a tight "lawn" of bacteria is visible. After three days a mature biofilm is formed with development of typical mushroom shaped multicellular structures, which then continues to grow to the following day. Picture by Dr. Kasper Nørskov Kragh, University of Copenhagen (Paulsson *et al.*, manuscript in preparation).

Bacteria growing in biofilm have a different gene expression and phenotype than their planktonic counterparts. This change is mediated by intercellular signalling systems known as quorum sensing systems. *Pseudomonas aeruginosa* uses the two major quorum-sensing regulatory systems *las* and *rhl* to modulate the expression of many of its virulence factors [24]. These two population density-dependent systems concert the expression of transcriptional through self-generated extracellular signal molecules, *e.g.*, *Pseudomonas* quinolone signal (PQS). The sensing systems regulate the expression of about 10% of the *P. aeruginosa* genome, including rhamnolipids, proteases, exotoxins and pyocyanin [25,26]. PQS is a prerequisite for biofilm formation. Deletion mutants lacking PQS do not form biofilm, unless they are co-incubated with PQS secreting counterparts [27]. This also exemplifies that cells in a biofilm function as a collective, as any excreted molecules are available to all surrounding cells.

Enclosing bacteria in biofilms facilitate bacterial persistence for long periods of time in the respiratory tract or paranasal sinuses, partly due to increased antibiotic resistance. The antibiotic resistance is mediated by several factors. For instance, the polysaccharides thwart penetration of beta-lactam antibiotics through the structure, while oxygen limitation and low metabolic activity of cells in the centre of the biofilm reduces susceptibility to ciprofloxacin and tobramycin [28,29]. The encapsulation in a biofilm matrix also shields against phagocytosis and the effector molecules of the innate immunity [30].

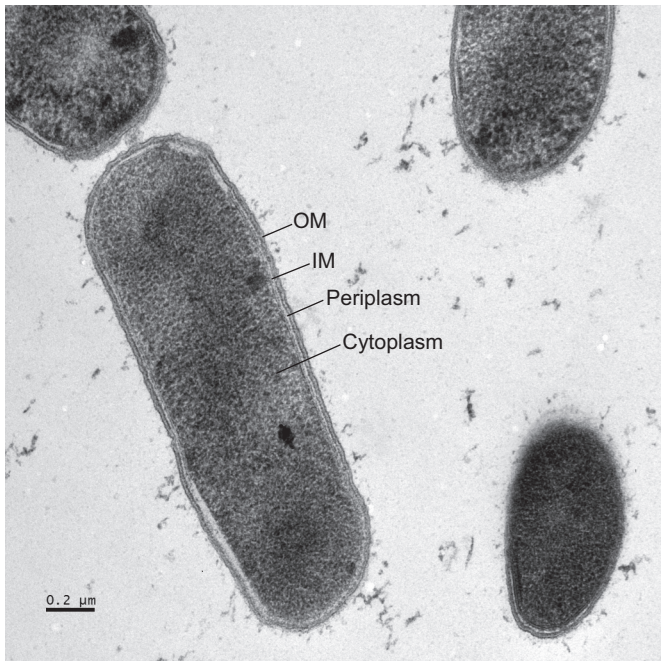

Figure 3: *Pseudomonas aeruginosa* cell
Transmission electron micrograph of *P. aeruginosa* PAO1. The outer membrane (OM), inner membrane (IM), the periplasm and the cytoplasm are marked on the picture. Picture kindly provided by Dr. Linda Sandblad, Umeå Core Facility for Electron Microscopy.

Morphology of the cell

Pseudomonas aeruginosa cells are shaped as thin and elongated rods (0.5-1 X 1.5-5 µm), arranged singly or in pairs (Figure 3). The cells are motile through movement of the flagella. In a study from 1964, Jessen and Lautrop found that 97% of the studied *P. aeruginosa* had one polar flagellum, the remaining 3% had two flagella [6]. As in other Gram-negative bacteria, the cell envelope consists of an outer membrane situated above a peptidoglycan layer (which together form the cell wall), and an inner membrane, also known as the plasma or cytoplasmic membrane [31]. The inner membrane encloses the cytoplasm, but unlike in eukaryotic cells, bacteria have no membrane-bound nucleus. The DNA in the cytoplasm is arranged as a supercoiled double-stranded DNA in a single chromosome without any surrounding membrane. Protein synthesis takes place in the ribosomes, using messenger ribonucleic acid (mRNA) as template. The bacterial ribosomes differ from the eukaryotic ribosome in that they consist of a 50S and a 30S subunit. These differences are important for the action of antimicrobial drugs and will be discussed below.

General structure of the cell wall

Nutritional versatility and low permeability of the outer membrane are two key factors for the ecological success of *P. aeruginosa* in the environment and as a pathogen. Coupled with a vast range of virulence traits and antimicrobial resistance mechanisms, this pathogen causes great difficulties in the nosocomial setting. Compared to *Escherichia coli*, the outer membrane is approximately 15-fold less permeable [32]. In addition to the low permeability of the membrane itself, *P. aeruginosa* has fewer large porins (water filled channels), which allow for passage over the membrane. There are, however, a number of specific influx channels that transfer nutrients into the periplasm and efflux pumps that secrete unwanted molecules out of the cell (Figure 4). In contrast, the Gram-positive cell wall lacks the outer membrane and Gram-positive bacteria are hence less protected against harmful compounds such as oxygen radicals, lysozymes and some antimicrobial drugs.

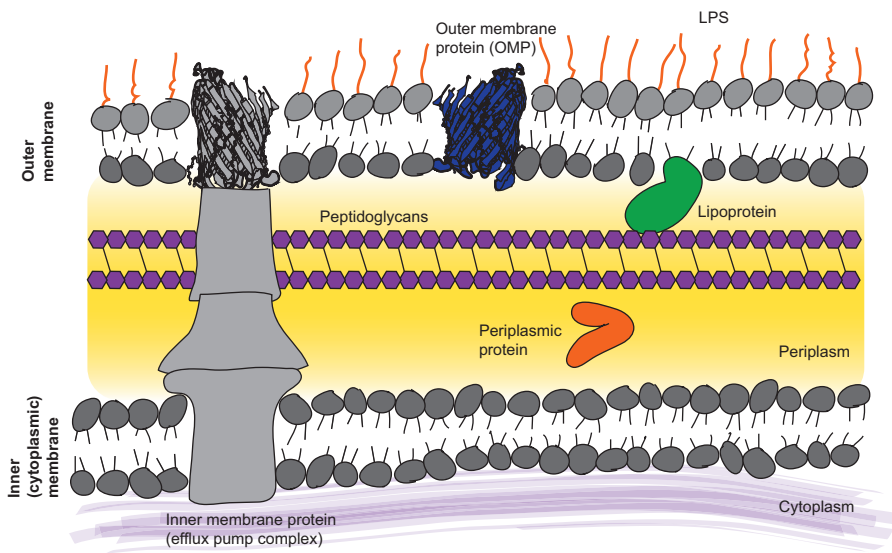

Figure 4: The Gram-negative cell wall
The cell wall of *P. aeruginosa* is less permeable than most other Gram-negative bacteria, but the general structure is similar. The drawn efflux pump is a generalized model representing that of for instance OprM/MexXY.

The Gram-negative cell wall is strong enough to withstand 3-5 atm of turgor pressure [31]. The outer membrane is asymmetrical with an inner phospholipid leaflet and an outside made of lipopolysaccharides (LPS). The outer membrane is the anchoring point for flagella, pili, outer membrane proteins (OMP) as well as lipoproteins.

The periplasm is the space between the outer and the inner membrane. It contains peptidoglycans and soluble periplasmic proteins and forms a protected space where many proteins are cleaved or folded to their final structure. The peptidoglycan layer is a polymeric matrix with a web-like structure that encloses the entire cell. It is non-stretchable, but bendable, and is unique for prokaryotic cells. It is composed of alternating N-acetylglucosamine and N-acetylmuramic acid residues that are crosslinked with peptide side-chains. It is by impairing the cross-linking enzymes that beta-lactam antibiotics cause damage to the bacterial cell.

Proteins in the outer membrane

Two types of proteins are directly associated with the outer membrane: the barrel shaped OMP and lipoproteins. Both types are synthesized in the cytoplasm as precursors with a specific signal sequence and translocated into the periplasm where the signal sequences are removed. OMP are assembled into the outer membrane through the beta-barrel assembly machinery (Bam-pathway) and lipoproteins are attached to lipids at an N-terminal cysteine residue and delivered to the outer membrane by the lipoprotein outer membrane localization machinery (Lol-pathway) [33,34]. Proteins can also be indirectly attached to structures in the outer membrane without being integrated in the membrane itself, for instance by attaching to other proteins or polysaccharides on the bacterial surface. This includes moonlighting proteins, which are proteins with tasks in several cellular compartments.

Lipoproteins are soluble lipid-anchored proteins that are tethered to the outer membrane. An *in silico* analysis has deduced that there are 175 lipoproteins in total in *P. aeruginosa*. Most of these are predicted to be associated with the outer membrane and only about 20% with the inner membrane [35].

In 1990, Hancock *et al*. suggested a unified nomenclature for the proteins in the outer membrane of *P. aeruginosa*. He proposed the use of Opr, for outer membrane protein, to prevent confusion with Omp, the designation used in *E. coli*. The known proteins were named starting with OprC and organized in alphabetical order according to the distance the protein migrated in gel electrophoresis, *i.e.,* the smaller the protein, the higher the letter [36]. Both lipoproteins and barrel-shaped outer membrane proteins were included in this nomenclature. The designation is no longer used when annotating proteins and today, in total, several hundred proteins are known to be located in the outer membrane, many of them with unknown functions (Figure 5).

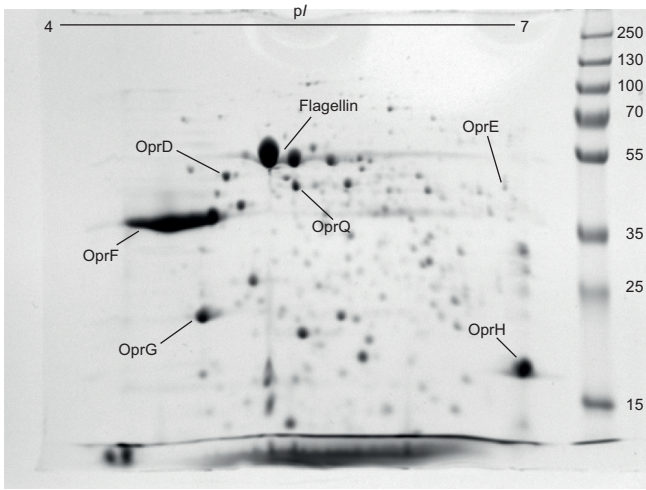

Figure 5: Proteins in the outer membrane
2D-gel of all proteins in the outer membrane of *P. aeruginosa* PAO1 separated by gel electrophoresis and isoelectric focusing. Selected major OMP are marked on the picture. 2D-gel courtesy by Dr. Yu Ching Su, Lund University (Paulsson *et al*., manuscript in preparation).

Barrel-shaped OMP

The integral proteins of the OMP family are transmembrane proteins that share a common core structure, with few exceptions. This functionally diverse group of proteins have 8 to 24 strands of β-sheets, forming a barrel shape surrounding a central pore. In contrast, inner membrane proteins consist of α-helices. The β-barrel strands contain both hydrophobic and hydrophilic regions and are linked to each other with loops that protrude both into the periplasm and towards the extracellular space, forming exterior antigenic epitopes that are exploited in vaccine development [37]. OMPs are involved in a broad range of functions, for instance: transport of molecules across the membrane, enzymatic activity, cell adhesion and structural anchoring to the underlying peptidoglycan layer. Because of the large homology between β-sheets in OMPs, regions coding for OMPs can be easily identified in genomic analysis to find proteins that are with high certainty destined for the outer membrane [38].

The most abundant OMP in *P. aeruginosa* is OprF (PA1777). This 38 kDa protein shares much of its structure with *E. coli* OmpA and has two possible conformations, a larger open and a closed form. Out of the suggested 200,000 copies of the protein on each cell, only 0.2% have the larger channel that allow passage of water and smaller sugars. The vast majority form a closed form, a narrow channel that is water impermeable, in part explaining the low permeability of the outer membrane and the high intrinsic antimicrobial resistance of those bacteria [36]. OprF is involved in the structural integrity of the bacteria, possibly through tethering the outer membrane to

the peptidoglycan layer, a property attributed to other OmpA-like proteins [36,39]. In addition, OprL (PA0973) and OprI (PA2853) connect the outer membrane to the peptidoglycans, but it should be noted that only OprL shares the OmpA structure, while OprI is a lipoprotein similar to the *E. coli* Braun's lipoprotein [39].

Moonlighting proteins

The third group of proteins that is present in the bacterial outer membrane are the moonlighting proteins. These are a heterogenous group of proteins with one function in the cytoplasmic cellular machinery and a different function on the cell surface. The first described bacterial moonlighting protein was glyceraldehyde-3-phosphate dehydrogenase from *Streptococcus pyogenes*. While a glycolytic enzyme in the cytoplasm, on the surface of the cell it enables the cell to adhere to fibronectin and hence to adhere to the extracellular matrix in the respiratory tract [40]. The ortholog protein in *E. coli,* and many other species, has since been shown to have similar functions [41]. Two examples of moonlighting proteins in *P. aeruginosa* are the translational elongation factor (TufB, PA4277) and the dihydrolipoamide dehydrogenase (Lpd, PA1587), both of which are involved in binding of complement inhibitors and plasminogen [42,43]. It is yet unclear how microbial moonlighting proteins are exported to or anchored to the bacterial outer membrane.

Lipopolysaccharides

At least four general kinds of sugar molecules surround *P. aeruginosa*: alginate, Psl and two types of LPS. The secreted exopolysaccharides alginate and Psl form a gelatinous structure around the bacterial cell and are important for biofilm formation, intracellular survival and, in the case of alginate, for the mucoid appearance of some isolates and strains.

Pseudomonas aeruginosa LPS is structurally similar to LPS from other Gram-negative bacteria and are composed of three units: "lipid A", which forms the outer leaflet of the outer membrane, linked to the nine-sugar, branched "core oligosaccharide" which in turn is linked to the "O-antigen" (also known as O-chain) that extends into the extracellular space. Strains originating from chronically infected patients may have LPS that consist of only lipid A and core and are referred to as "rough". Strains expressing the full LPS including O-antigen are referred to as "smooth" [44].

The O-antigens are the outermost part of the LPS molecule and are strongly connected to bacterial virulence. They are composed of polysaccharides of varying length and composition, and extend about 40 nm outside the lipid outer membrane [31]. Unlike for instance *E. coli*, *P. aeruginosa* has two general types of LPS: type A (alternative names used elsewhere: A-band, common polysaccharide) and type B (alternative names: B-band, O-antigen). These are structurally different in the sugar

compositions of the O-antigen (Figure 6) [45]. Type B LPS are similar to LPS from *E. coli* and form the structural basis of antigenic typing by which the International Antigenic Typing Scheme classifies *P. aeruginosa* into 20 major serotypes, O1 to O20 [46].

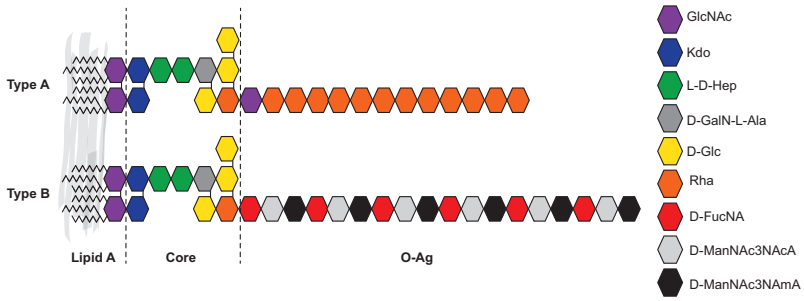

Figure 6: Lipopolysaccharide (LPS)
Pseudomonas aeruginosa has two kinds of LPS, type A and B. They differ in the O-antigen composition and in the immunoreactivity. Modified from [45].

Most *P. aeruginosa* express both the type A and type B LPS. Type A LPS are composed of repeated rhamnan polysaccharides and synthesized in the inner membrane via an ABC transporter-dependent pathway, whereas the type B O-antigen synthesis follows the 'Wzy-dependent' pathway [47]. The lipopolysaccharide transport (Lpt) system is responsible for transporting LPS from the inner membrane, to the cell surface, where it is then inserted in the OM. This system has been extensively studied in *E. coli*, where it consists of seven essential proteins located in the inner membrane, in the periplasm and in the OM. Recent data indicate that *P. aeruginosa* has a similar mechanism [48]. LPS from *E. coli* was used to experimentally mimic Gram-negative pneumonia in Paper III. In the clinical setting, it is common to refer to LPS as "endotoxins".

Outer membrane vesicles

The outer membrane is the origin of outer membrane vesicles (OMV), bacterial extracellular vesicles that are produced by all Gram-negative bacteria during growth. In Papers I and III, different aspects of vesicles originating from *M. catarrhalis*, *P. aeruginosa* and NTHi are studied.

The spherical particles were first observed by transmission electron microscopy (TEM) in studies of bacterial structures in the 1960s. They were described as extracellular globules containing LPS surrounding *E. coli* and as extracellular particles pinched off from *Vibrio cholerae* [49,50]. The interest for these particles in the research community was initially modest as it was thought that the vesicles were

formed during lysis of the bacterial cell. The interest has, however, increased markedly during the last decades. This is illustrated by a search at www.pubmed.gov for "Outer membrane vesicle" OR "Outer membrane vesicles". The search results in 1062 hits that were published up until the 31th of December 2016. Of those, 122 where published in 2016, 40 in 2006, 14 in 1996, 5 in 1986 and 0 in 1976.

The increase in interest was boosted following some key findings. In 1980, Gankema *et al*. demonstrated that the vesicles were not a result of cell lysis as the mass of OMV were 30- to 50-fold higher than what could be explained by debris from lysis [51]. Another important finding was that vesiculation could be increased by gentamicin, but that the protein content of these artificial vesicles differed from naturally produced vesicles. This indicated that the budding was regulated and that the composition of the vesicles was not random [52]. Finally, the realization that OMV induce an immune response spurred interest in their role in pathogenesis as well as the use of OMV in vaccine development. This resulted in the addition of OMV as one of the components in a now routinely used vaccine against *Neisseria meningitides* serogroup B [53]. The focus of current research is on the formation of vesicles, the proteomic composition and the immunologic properties [54].

Outer membrane vesicles are readily produced during growth *in vitro*, but also *in vivo* by pathogenic bacteria during infections. The first case report of OMV found in a human came from Nashville, USA in 1982, where "outer membrane blebs" were found in cerebrospinal fluid of a 6 months old boy with *N. meningitides* infection after one day of fever and lethargy [55]. Large amounts of OMV associated with meningococci has since repeatedly been shown [56]. More recently, OMV from *M. catarrhalis* have been reported in the nasopharynx of a patient with sinusitis [57].

OMV composition

The composition, genesis and biological functions of OMV originating from different Gram-negative bacteria are likely to share a high degree of similarity, although the specific components reflect the bacteria it originated from.

The 50-250 nm vesicles are pinched off from the outer membrane and are mainly composed of proteins, lipids, and carbohydrates from the outer membrane, including large amounts of LPS. The interior contains mainly periplasmic proteins and peptidoglycans, but also cytoplasmic proteins, DNA, and mRNA (Figure 7). DNA from lysed cells is found on the outside of the vesicle, but at least plasmid DNA is protected from degradation by DNase and is likely to be inside the vesicle [57,58].

The phospholipid composition of OMV generally resembles that of the outer membrane it originated from. However, OMV from *P. aeruginosa* differ in lipid composition from the outer membrane. The highly immunogenic type B LPS is enriched, whereas the type A LPS is not [52,59]

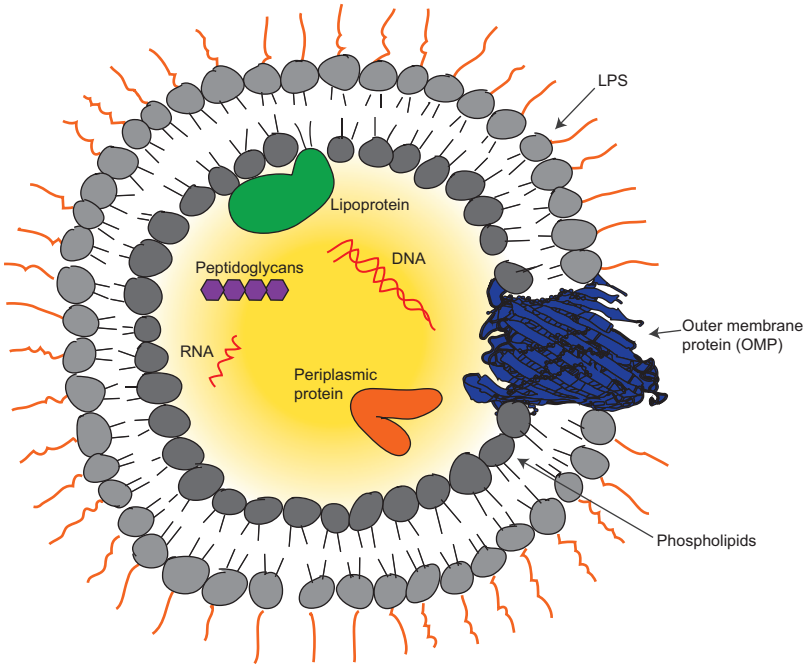

Figure 7: Structure of an outer membrane vesicle (OMV)
The OMV structure largely reflects the composition of the bacterial outer membrane. In addition, some periplasmic and cytoplasmic constituents follow into the vesicle. DNA is found on the outside of the vesicle, but, at least, plasmid DNA is found inside the sphere.

Early studies used gel electrophoresis and immunoblotting to characterize the protein content of OMV. Recent mass spectrometry-based proteomic studies have identified thousands of OMV-associated proteins [54]. The composition varies depending on growth conditions. Lipid and protein analysis of OMV from *M. catarrhalis* suggest that the OMV are composed mainly of outer membrane structures including outer membrane proteins [60]. The most abundant proteins in OMV from *P. aeruginosa* are also membrane proteins, exemplified by OprD, OprI and OprF. These are concentrated in the OMV compared to in the outer membrane, suggesting a selective packaging of the vesicles. The presence of a selection mechanism is supported by that proteins of the Bam-family (involved in the OMP assembly) are consistently underrepresented although they are also integrated in the outer membrane [61,62].

The outer membrane vesicles carry multiple virulence factors, as for instance toxins and enzymes. Elastase, phospholipase C, beta-lactamase and CFTR inhibitory factor (Cif, PA2934) have all been found in OMV from *P. aeruginosa* [52,63]. A similar composition has been found in OMV from NTHi [64]. These effector molecules may also be concentrated and are protected from proteases by the enveloping membrane.

As some proteins are concentrated and others are withheld from passage into the vesicles, the formation is, if not fully regulated, at least selective. This suggests that the vesicles can also be directed to certain cells or tissues, as some OMPs function as specific adhesins [65]. Hence, the OMV may act as targeted long distance delivery systems packed with multiple and concentrated virulence factors.

OMV biogenesis

Detailed studies of the composition are important as they provide clues on the vesiculation process as well as the immunogenic and pathogenic components of OMV. The selectivity of OMV protein composition further supports the notion that OMV biogenesis is a controlled process, rather than a random event.

It is not fully clear what triggers vesicle formation or how the process is regulated (Figure 8). Several explanatory models exist, which are not mutually exclusive, as reviewed in [54,59,65]. Membrane instability alone does not correlate with increased vesiculation levels [66].

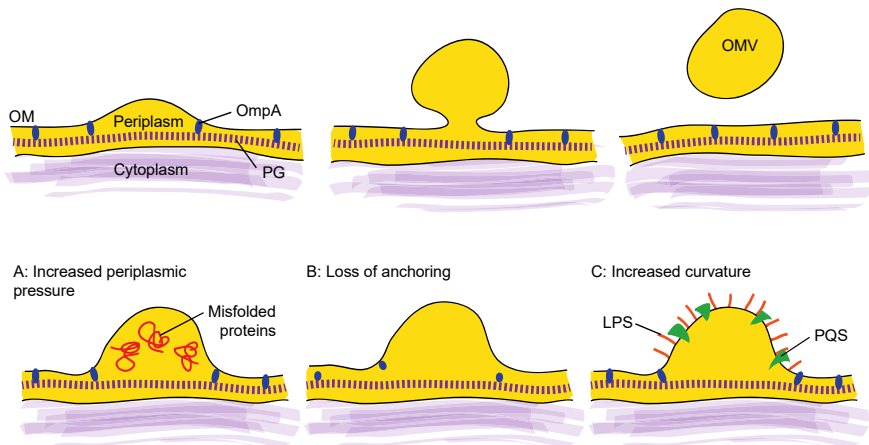

Figure 8: Biogenesis of OMV
The spherical vesicle is pinched of from the outer membrane of Gram-negative bacteria (upper panel). Three examples of increased vesiculation (lower panel, from left to right): (A) High turgoid pressure in the periplasm caused by, *e.g.*, accumulation of misfolded proteins. (B) Decreased tethering of the outer membrane to the peptidoglycan layer caused by few or defective OMPs, often belonging to the OmpA family. (C) Increased curvature of the outer membrane caused by insertion of additional molecules, e.g. PQS, or by change of the LPS composition [65].

The first model focuses on the turgor pressure in the periplasm. Accumulation of proteins or peptidoglycans causes increased osmotic pressure and subsequent bulging of the outer membrane, eventually releasing a vesicle to the surrounding space. The activation of bacterial stress response systems following for instance heat shock or fluoroquinolone treatment increases the budding, probably through accumulation of misfolded proteins in the periplasm [65,67].

A second model focuses on the bonds between the outer membrane and the peptidoglycans. The outer membrane is anchored to the peptidoglycan by several OMPs, for instance OprF and OprI. Mutant bacterial isolates lacking these proteins produce more vesicles, probably because the outer membrane more easily let go from the rest of the bacteria. Similarly, an increase in the turnover rate of the outer membrane would result in relaxed bonds between the outer membrane and the rest of the cell.

Finally, a third model derived from studies on *P. aeruginosa* focuses on the curvature of the outer membrane. The curvature can be increased by including molecules as wedges in the membrane, as for instance the quorum sensing molecule PQS. The curvature may also be affected by the LPS composition. While the outer membrane contains both type A and B LPS, the OMV mostly contain type B. As the type B LPS is longer and more negatively charged than the type A, this may lead to a repelling force that causes a bulging of the membrane [39,68].

OMV protect bacteria from environmental factors

The OMV can protect bacteria against hostile environmental factors mechanically by physical shielding or acting as decoys and chemically by carrying degradative enzymes. These mechanisms may be of high biological importance as bacterial stress further stimulates OMV production [69].

Outer membrane vesicles are integral parts of biofilms, where they consolidate matrix surrounding the bacterial cells. The vesicles can act as decoys and bind antimicrobial phages as well as toxins and antimicrobial drugs, for instance polymyxin B and colistin [70].

Vesicles from *P. aeruginosa* and *M. catarrhalis* contain beta-lactamase that hydrolyze penicillins [71]. Excreted vesicles are available not only to the producing bacteria or even species, but are common goods in the extracellular milieu. Because of this, OMV shedded from *M. catarrhalis* protect both *H. influenzae* and *Streptococcus pneumoniae* from killing by amoxicillin [72]. Moreover, just like *P. aeruginosa*, *Acinetobacter baumannii* produces catalase [73]. OMV from this species contain catalase that neutralizes reactive oxygen species to resist the attack from neutrophils [74].

Finally, the vesiculation itself can be protective when the bacterial cell is compromized by toxins. For instance, during gentamicin-induced hypervesiculation, OMV contain gentamicin and by excreting the drug in vesicles, the intracellular concentration is decreased. Similarly, hypervesiculation in cells stressed by oxidation helps the bacterium to excrete degraded proteins that accumulate in the periplasm [52,67]. In this sense, vesiculation may be a protective “waste disposal system”.

Bacterial interactions with the innate immunity

The rich amount of nutrients, the humidity and the constant temperature in the human body fulfil most requirements for bacterial growth and persistence. Fortunately, the elaborate immune system keeps clear of most invading microbes. The innate immunity comprises the combined efforts of the immune response that are ever-ready to keep clear of invading bacteria, viruses, fungi and debris. All living organisms have an innate immunity, whereas vertebrates in addition have an adaptive immune system. The adaptive immunity is introduced in detail elsewhere. The effector processes of the immune system cause many of the symptoms that are recognized by us as symptoms of infection.

Until recently, it was believed that the lower respiratory tract was normally sterile. This truth has been challenged by studies of the microbiome and it seems as if a diverse flora of commensals inhabits this compartment [75]. The innate immunity that keep clear of pathogens in the respiratory tract consists of mechanical barriers, secreted products and pathogen-responsive cells.

The innate immunity is promptly activated upon invasion, and for successful colonization and persistence, it is necessary for the pathogen to withstand all of its parts [76,77]. Owing to its large genome, *P. aeruginosa* isolates have an extensive arsenal of virulence traits to do this and facilitate colonization, promote robust growth and prevail despite the efforts of the human immune system, competition with other bacterial strains and the use of antibiotic drugs. The most studied mechanisms will be introduced, but it should be noted that the importance of each trait depends on the location and character of the infection.

Mechanical barriers, adhesion and invasion

Mucus and cilia

Microbes enter the airway with inhaled air or by aspiration. The inhaled air contains 10^2-10^4 colony forming units of bacteria per m^3, including *Pseudomonads* [78]. Some inhaled bacteria are trapped in the upper respiratory tract by filtration through narrow passages and turbulent airflow, but a fraction follows the air into the lower respiratory tract. To minimize aspirated bacteria, the epiglottis and cough reflex prevent oral flora from descending to the lungs.

In the bronchi, the epithelium is an important antibacterial defence. The epithelium is constituted by ciliated epithelial cells, mucin-producing goblet cells and the underlying basal cells. The ciliated epithelial cells are covered by a fluid layer that is 5-20 μm thick. The inner part of this layer is the periciliary liquid, which is thin and

allows for ciliary movement. The tips of the cilia reach into the thicker and sticky covering mucus layer, propelling the mucus and any trapped bacteria or particles towards the oropharynx with their beats, where it is swallowed or expectorated (Figure 9) [79,80].

To prevent from being washed out by this "mucociliary escalator", many pathogens adhere to the epithelial cells or the extracellular matrix (ECM) during colonization. Adherence is usually achieved by a combination of surface structures such as pili, fimbriae, flagella, and surface-exposed proteins. For *P. aeruginosa*, both the flagella, pili and outer membrane proteins are involved in this adhesion. For instance, the absence of OprF results in impaired adhesion and loss of flagellar structures cause both reduced adherence and internalization into epithelial cells [81]. The species also has laminin receptors on its surface that can bind and adhere to exposed basal lamina [82].

Similarly, *Moraxella catarrhalis* expresses several surface exposed proteins (Moraxella IgD-binding protein: MID, Ubiquitous surface protein: UspA1, UspA2) that bind to epithelial cells, and the ECM proteins laminin, vitronectin and fibronectin [83,84].

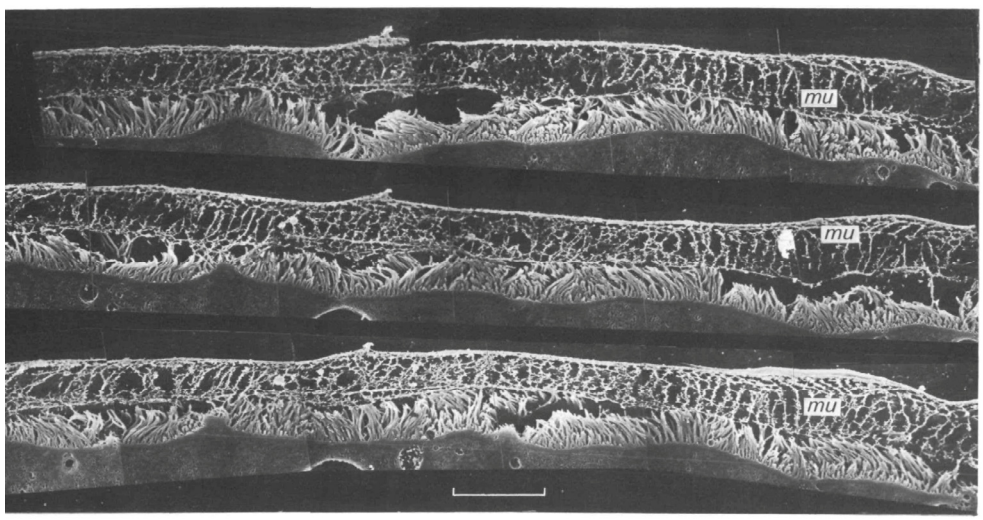

Figure 9: Cilia moving thick and fibrous mucus
Rabbit tracheal epithelium examined by scanning electron microscopy illustrating the mucociliary escalator as the cilia extend into the mucus (*mu*) to move the thick, fibrous mucus layer towards the right [80]. Picture reprinted with permission from Company of Biologists.

Pseudomonas aeruginosa is one of a limited number of organisms that can synthesise hydrogen cyanide. Cyanide is a highly toxic volatile compound that in higher concentrations inhibit eukaryotic cytochrome c oxidase and therefore paralyze the eukaryotic cell by interrupting electron transfer in the mitochondria. The ciliary

beating that propel the mucociliary escalator is inhibited, the bacteria avoids expulsion and can persist in the airway [85]. In experimental models using the nematode *Caenorhabditis elegans* to investigate *P. aeruginosa* virulence factors, the worms were killed by cyanide poisoning [86]. As cyanide can be measured in sputum and exhaled breath samples, it has been under consideration as a biomarker of *Pseudomonas* infection in the lung [87,88].

Defects in the protective structures of the epithelia lead to increased susceptibility to infections. One example of this is the dysfunctional ciliary movement and the thickened mucosal secretion in patients with cystic fibrosis (CF). These patients are frequently colonized with bacteria, often *P. aeruginosa*, during childhood. Following this, chronic infection can persist for decades despite that other parts of the immunity are intact and despite administration of antimicrobial drugs for extended periods of time [89]. Another example is ventilator associated pneumonia (VAP), which will be introduced later. The mechanical ventilation normally requires tracheal intubation. The endotracheal tube causes recurrent micro-aspirations as well as an obstructed mucociliary escalator. Moreover, biofilms develop on the plastic tube that is out of reach for antibiotic drugs, resulting in adherent bacteria that form a bacterial reservoir [90]. As a consequence, the risk of pneumonia is very high among mechanically ventilated patients.

Basal membrane and extracellular matrix

The mechanical barriers also include the tight cell-cell junctions of the epithelial cells that protect against bacterial invasion into the underlying tissues. If this barrier is damaged, the extracellular matrix and the basal membrane are exposed. Like other pathogens, *P. aeruginosa* can bind and adhere to several of the extracellular matrix proteins, including fibronectin, laminin, and collagen [91]. Once below these linings, the invasive bacteria cause further damage by tissue degrading enzymes.

By degrading host tissues, the pathogen harvest nutrients. *Pseudomonas aeruginosa* secretes at least 7 proteases and one phospholipase into the extracellular space. The most studied of the proteases are elastase (LasB protease, pseudolysin, PA3724), staphylolysin (staphylolytic endopeptidase, LasA, PA1871), protease IV (lysyl endopeptidase, PrpL, PA4175) and alkaline proteinase (aeruginolysin, PA1249) [92]. Proteases are enzymes that can hydrolyze peptide bonds within proteins. They are classified by their active residues into subgroups. The most abundant proteases belong to the metallo- and serine-proteases [93].

A main component of the basal membrane is laminin, which is often exposed during chronic infections. Both alkaline protease and elastase cleave laminin and may therefore be of importance during tissue invasion [94]. Elastase and alkaline protease are metalloproteases with several important substrates in the human body [95]. The substrates for elastase include several other human proteins found in the respiratory

tract, for instance elastin and collagen [96-99]. Elastin is a protein-biopolymer in human organs and tissues with elastic properties. Elastase cause decreased levels of elastin in the lung of patients with CF, ultimately resulting in lung fibrosis [100].

Staphylolysin is a metalloprotease with some elastolytic properties. It is known as staphylolysin because it causes rapid lysis of *Staphylococcus aureus* by disrupting the peptidoglycan layer in the cell wall. It enhances the effect of other proteases and has been associated with invasive infections [101,102].

Phospholipases hydrolyze phospholipids into fatty acids and can destruct cellular membranes. Phospholipase C (PA0844) has been reported to be strongly involved in the virulence of *P. aeruginosa* through tissue destruction and intraperitoneal injection of phospholipase C in mice caused a marked accumulation of inflammatory cells [103].

The innate cellular response

PAMPs and PRRs

The innate response is activated by pathogen-associated molecular patterns (PAMPs) in the extracellular space, on the surface of the bacteria, or on OMV. Respiratory epithelial cells, alveolar macrophages and neutrophils have pattern recognition receptors (PRRs) that respond directly to the PAMPs with phagocytosis or production of an inflammasome, the combined complex of proteins related to host stimuli. The PAMPs are usually molecules that are essential for survival of the bacteria. Hence, they are difficult to alter for the bacteria and are highly conserved.

The pathogen-responsive cells phagocytose, lyse or counteract further bacterial colonization by reactive oxygen radicals, excreted proteins or DNA-containing neutrophil extracellular traps (Figure 10) [104]. Distant of the large bronchi where the effect of physical expulsion is less effective, the response by alveolar macrophages may be the most important defence [105].

Respiratory epithelial cells can internalize bacteria. When this is followed by apoptosis, desquamation and expulsion, it may be an efficient method for reducing bacterial loads in the lung. However, *P. aeruginosa* can under the right circumstances survive and replicate as intracellular bacteria [106]. Hence, internalization may be beneficial for the host, but also a mean for the bacteria to invade further down into the tissues by crossing the epithelial barrier.

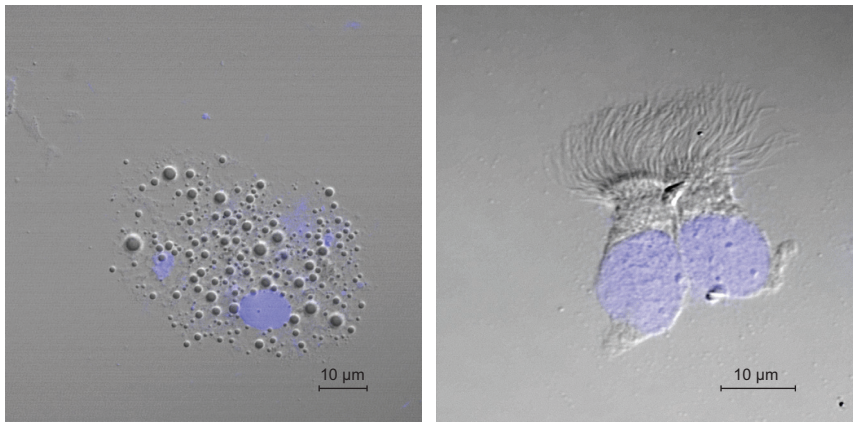

Figure 10: The most abundant cells in the lower respiratory tract
Confocal micrographs picturing alveolar macrophages with multiple vacuoles (left) and ciliated bronchial epithelial cells (right). Nuclei stained blue with DAPI.

LPS is a typical example of a bacterial product that induces a strong immune response and its cellular receptor, the toll like receptor (TLR) 4 is a typical example of a PRR [107]. The TLRs are the largest known group of PRRs and consist of both surface exposed receptors and receptors that are located intracellularly in the lysosome. They are expressed in both immune cells such as macrophages and non-immune cells as epithelial cells. All of these receptors induce signalling pathways resulting in pro-inflammatory responses through release of active NF-κB (nuclear factor kappa beta), a DNA transcription regulation factor [108].

TLR 4 is surface-exposed and so are TLR 2 that binds lipopeptides and TLR 5 that attracts flagellin. The intracellular TLR 9 binds unmethylated cytosine-guanine motifs in bacterial DNA (CpG-DNA). Also NOD1 and 2 are intracellular PRRs. These are peptidoglycan-receptors that respond to intracellular bacteria [109,110].

The interaction between LPS and eukaryotic cells was unveiled after intense research during the 1990s and finally confirmed by Bruce Beutler's group when mice with a missense mutation in the gene coding for TLR 4 did not respond to LPS [111]. Bruce Beutler was awarded the Nobel Prize in Physiology or Medicine 2011 for this important discovery. It is now known that the TLR 4 – lipid A interaction require presence of the cofactors CD14 and MD2 and is responsible for a large extent of the reaction leading to the septic cascade in Gram-negative infections [112].

Compared to lipid A from *E. coli*, lipid A from *P. aeruginosa* is less antigenic and induce a lower inflammatory response by the cells of the innate immunity. This difference is attributed to a conformational variation of the disaccharide backbone of lipid A, *i.e.*, the base of the structure with attached fatty acids that integrate LPS in

the outer membrane. These fatty acids are shorter in *P. aeruginosa* (10-12 instead of 12-14 as in *E. coli*) and are less optimal ligands for TLR 4 [47,113].

As briefly introduced earlier, LPS has been used in experimental studies to simulate Gram-negative infection. For instance, endotracheally instilled LPS causes an inflammatory process in the lung with a predictable chain of events (Paper III). In an early phase (2-6h), neutrophils accumulate and pro-inflammatory cytokines are elevated. In a later phase (24-48 h), neutrophils, macrophages, monocytes, and lymphocytes are elevated and many pro-inflammatory markers are still elevated, but the earliest mediators, as for instance, interleukin (IL) 6 have returned to normal levels [114].

Just like the bacterial cell, OMV contain PAMPs including LPS, lipoproteins, peptidoglycans and DNA, which contribute to promote inflammatory responses in the host [54]. This interaction can take place distant from the OMV-producing bacterial cell. PAMPs on the vesicles interact with the cells of the innate immunity, including epithelial cells, macrophages and neutrophils, through surface receptors, mostly toll-like receptors, but also by fusing in lipid rafts and delivering its contents inside the cell [63]. Among other PRRs, TLR 2, 4 and 9 are activated by OMV. These interactions will in turn stimulate the release of inflammatory cytokines such as IL-6, IL-8 and IL-12, as well as antimicrobial peptides [64].

Circulating *E. coli* OMV cause sepsis in mice and intranasal administration of OMV from *P. aeruginosa* cause TLR 2 and TLR 4 dependent pneumonia in mice [115,116]. Triggering TLR 4 by LPS causes an instant activation of the innate immunity, but also polymyxin B-treated OMV lacking LPS trigger inflammation, although substantially dampened [117]. Even OMV-containing dust may induce pulmonary inflammation [118]. In summary, bacterial OMV interact with human cells and cause symptoms mimicking real bacterial infections by stimulation of surface receptors and by delivering their cargo inside the cell.

Bacterial defence against the cellular response

To counteract the pathogen-responsive cells and persist in the respiratory tract, *P. aeruginosa* has an extensive repertoire of secreted toxins and toxins that are injected directly into eukaryotic cells. Exotoxin A (ToxA, PA1148) is a secreted toxin that is highly deleterious to eukaryotic cells. It is an AB-toxin, *i.e.* it is composed of two parts, an effector unit ("A") and a carrier unit ("B"). The A-unit inhibits protein elongation by acting on elongation factor-2 (EF-2), similarly to the AB-toxin produced by *Corynebacterium diphtheriae* that causes the typical pseudomembrane of dead epithelial cells during diphteria. Exotoxin A causes rapid apoptosis in human cells and has been evaluated for its therapeutic potential against tumour growth when used in conjunction with antibodies targeting the malignant cells [119,120].

When *P. aeruginosa* is in close contact with other cells, it can use surface structures for direct cell-to-cell communication and delivery of signal proteins, toxins and genetic elements. The type III secretion system (T3SS) is a needle-like protein complex spanning the inner and the outer membranes of many Gram-negative bacteria, including *Pseudomonads*. This system is used to inject effector proteins into eukaryotic cells and is structurally similar to the flagella apparatus [121]. For *P. aeruginosa*, four effector proteins are known: ExoS (PA3841), ExoT (PA0044), ExoU (PA14_51530) and ExoY (PA2191) [122]. ExoS has been found to harm epithelial cells and leukocytes during pneumonia [123].

Additionally, the type VI secretion system (T6SS) is also a contact dependent needle-like molecular machine that injects toxins directly into eukaryotic cells, but also into other bacteria [124]. It contains bacteriophage-like proteins forming a tube that can penetrate the entire cell wall of adjacent cells and cause epithelial cell remodelling that promotes bacterial invasion [125]. Through the T6SS, *P. aeruginosa* has also been found to inject a toxin (Tse2, PA2702) into adjacent bacteria that can arrest growth in other Gram-negative bacteria [126].

As a side note, the type IV secretion system (T4SS) is a pilus-based system primarily for intercellular signalling among prokaryotes [127]. It allows for transport of proteins but also DNA across membranes, thus playing a crucial role in the horizontal gene transfer through conjugation. The transferred DNA may consist of single genes, plasmids or large, multiple open reading frames that contain genomic islands, including antibiotic-resistance genes [128].

Secreted effector molecules of the innate immunity

The pathogen-responsive cells secret molecules into the respiratory lumens to prevent further colonization. These include surfactant proteins, antimicrobial polypeptides (AMPs), the proteins of the complement, metal-ion binding proteins and degrading lysozymes to opsonize, kill or inhibit the growth of microbes. Secretory IgA may also be added to this group.

Secretory IgA is produced in the mucosal tissue, whereas IgG and IgM mainly enter the airway through transudation from plasma during inflammation. All antibodies are strictly part of the adaptive immunity, but with multiple links to the innate immunity. For instance, IgA binds and blocks bacterial adhesion to the epithelial cells which causes them to be trapped in the mucus and expelled by the mucociliary escalator [129]. IgG and IgM, but not IgA, activate the complement cascade and opsonized bacteria are cleared by the abundant alveolar macrophages. In response to protect itself, the bacteria secrete elastase and protease IV, that cleave IgA and IgG to avoid opsonophagocytosis [99,130].

A similar bacterial response is seen against surfactant proteins A and D. These proteins are produced by the respiratory epithelial cells and belong to the collectins,

proteins with a collagen-like N-terminal and a lectin-domain at the C-terminal that binds bacterial polysaccharides. The surfactant proteins opsonize bacteria which enables clearing by macrophages. Both elastase and protease IV hydrolyze surfactants to reduce opsonization and subsequent phagocytosis [131].

The AMPs function as secreted antibiotics. Some AMPs are constitutively expressed, others are induced upon stimulation [132]. They are small cationic peptides which provide defence against microorganisms. Many AMPs have a hydrophobic side which make them amphipathic and enable them to get in close contact with the bacterial membranes. For a yet to be explained reason, these compounds have retained antibacterial effects although bacteria have coevolved with them, while our clinical antibacterial drugs are rapidly rendered useless by the emerging bacterial antibiotic resistance [133].

The most studied AMPs are defensins and cathelicidins. The human beta-defensins are widely expressed by epithelial cells, including in the respiratory tract. LL-37 is a human cathelcidin that is also expressed by respiratory epithelium [134]. The AMPs act on the bacterial membranes by penetrating and disrupting the integrity. The biological importance of the AMP in the lung is not established as the concentrations are relatively low. Knock-out mice lacking human beta-defensin 1 cleared *H. influenzae* slower than the wild-type counterparts, but did not evolve more severe symptoms of disease. However, this is merely one of many AMPs that together may have an important role in the innate immunity [134]. A suggested mechanism of action behind the inability of patients with CF to clear *P. aeruginosa* is that the increased salt concentration in the lung mucus may inactivate human beta-defensin 1 [135].

The complement system

Jules Bordet was awarded the Nobel Prize in Physiology or Medicine in 1919 for identifying the complement system, a bactericidal component of serum (Figure 11). The first mention was made by Buchner a few years earlier when he identified "alexins", a heat labile factor in blood that could kill bacteria [136]. It is now known that the complement system consists of several proteins that function through a cascade of enzymatic reactions. The complement detects and kills bacteria and also promotes bacterial killing by phagocytes. The major end products are (I) chemotactic factors that attract neutrophils and macrophages, (II) anaphylotoxins promoting inflammation, (III) opsonization of bacterial cells which leads to phagocytosis and (IV) formation of the membrane attack complex (MAC) that is inserted in the cell wall to lyse bacteria [76]. Also the adaptive immunity is activated through co-

stimulation: when bacteria are recognized by the PRRs of the complement, B-cells are stimulated to produce antibodies [137].

Figure 11: Jules Bordet
Stamp portraying Jules Bordet who was awarded the Nobel Prize in Physiology or Medicine in 1919 for his discovery of the complement system. Artwork by Lennart Forsberg and Arne Wallhorn, issued by Sweden Post in 1979 and published with permission from PostNord Frimärken.

The outcome of host-pathogen interactions with the complement determines if bacterial colonization will be successful or if the microbes will be cleared. The complement can be activated by three pathways: the classical, lectin and alternative pathway, which are outlined in Figure 12. The pathways converge when C3 is cleaved, C3b is covalently attached to the cell surface (opsonization) and C3a is secreted to induce inflammation and attract neutrophils and macrophages. Deposition of C3b on the cellular surface leads to the assembly of C5 convertases that initiate the formation of the MAC. C3b is bound to the surface of *P. aeruginosa* by the abundant OprF, and possibly other proteins [138].

The classical pathway is activated by antibody-antigen complexes as C1q recognizes the Fc-region of IgG or IgM, that is bound to the bacterial surface. The lectin pathway is activated by lectin-binding (by mannose binding protein; MBL) directly to bacterial surface carbohydrates, without the need of antibodies. Finally, the alternative pathway is also antibody independent and independent of C1, C2 and C4. Instead it is initiated by spontaneously hydrolyzed C3 to C3a and C3b, the latter of which bind to the bacterial surface and catalyze the downstream reactions.

The final product of the cascade is a lytic pore, the MAC, C5b-9, which is inserted to the cellular surface causing lysis. However, the thick peptidoglycan layer outside the cell wall of Gram-positive bacteria protect from insertion of MAC. Hence, mainly Gram-negative bacteria are lysed by MAC [76].

To avoid damage to human cells, the cascade reaction is tightly controlled by regulators of complement activation (RCA). These can be membrane-bound or soluble and circulate in plasma (*e.g.* membrane bound: complement receptor 1 [CR1,

CD35] and decay accelerating factor [DAF, CD55], soluble: factor H, C4b binding protein [C4BP] and vitronectin). Presence of the RCA on self-surfaces inhibits the complement cascade and prevents damage to human cells. The lack of RCA on foreign cells or debris activates complement to ensure that invading bacteria or debris is phagocytosed. The soluble RCAs act at distinct steps of the complement cascade. For example, C4BP regulates the classical and lectin pathway convertases, factor H regulates the alternative pathway, whereas vitronectin regulates the terminal pathway to prevent assembly of the MAC. These factors are essential regulatory proteins that protect bystander host cells and tissue from damage by complement activation [76,139].

Although complement is well-characterized in serum, complement activity has also been detected in the human lung. Among others, complement factors C1q, C2-6, factor H and vitronectin (but not C4BP) have been detected in bronchoalveolar lavage fluid (BALF) [140,141]. The complement factors can follow plasma exudate into the airway, but some factors are also synthesized by cells in the respiratory tract in response to PAMP stimuli [140,142].

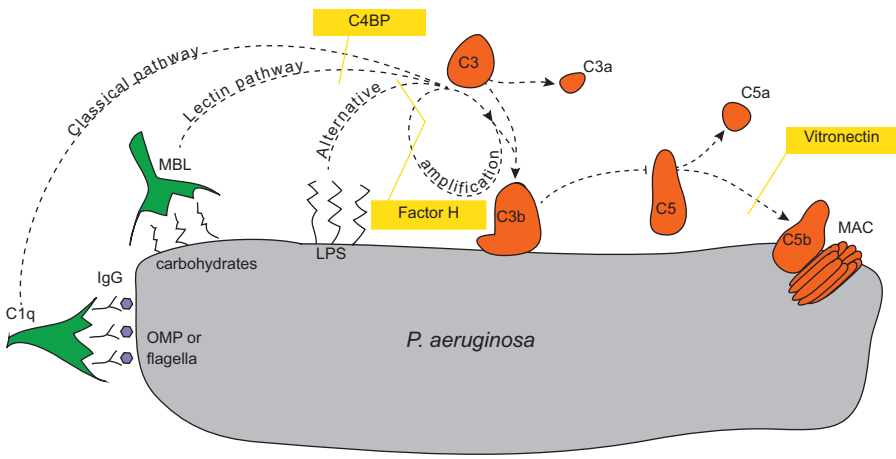

Figure 12: Complement cascade and its inhibitors
A simplified cartoon showing the three complement pathways as activated by the bacterial surface. The regulators of complement activation C4BP, factor H and vitronectin and their corresponding regulated steps are shown in yellow. Modified from [76].

Bacterial complement evasion and serum resistance

The complement is important for clearance of *P. aeruginosa* from the lung. This was experimentally demonstrated in mouse models in which hypo-complementaemic

animals were not able to clear the bacteria [143]. By escaping complement-mediated killing, bacteria can persist in the body. Several pathogens have developed multiple and abundant counterstrategies to evade complement. Bacteria can evade the complement attack by binding regulators of complement activity to the microbial surface, by antigenic masking, or by degrading the complement proteins [77].

Surface binding of complement regulators

Invading bacteria are normally quickly opsonized by the complement upon invasion, followed by phagocytosis or lysis. However, both Gram-positive and Gram-negative pathogens take advantage of soluble RCA from the host by binding these proteins to their surface and in that way regulate the complement activity directly on the bacterial cell [144]. The RCA bind to specific receptors on the bacterial surface. These receptors are usually membrane proteins with other known functions. Since many RCA share common structural features called "short consensus repeat domains" or "complement control protein" (CCP), a single bacterial receptor can sometimes bind to several RCA, which enables inhibition of the complement cascade at multiple pathways and levels [76]. Three RCAs that have been extensively studied in bacterial pathogenesis are vitronectin, C4BP, and factor H.

Vitronectin

Vitronectin is a multifunctional glycoprotein that is found in plasma, in the ECM, and in the respiratory tract. Increased vitronectin concentrations in the bronchial lumen of patients with sarcoidosis, interstitial lung disease, hypersensitivity pneumonitis, fibrotic lung disease and chronic obstructive pulmonary disease (COPD) indicate that vitronectin is associated with inflammatory processes [141,145-147]. It is a 75 kDa glycoprotein that is primarily expressed and secreted by hepatocytes, although it is also expressed by other cell types, including respiratory epithelial cells and alveolar macrophages [148-150]. It has multiple domains and motifs reflecting its many roles in the extracellular matrix (Figure 13).

Figure 13: Schematic presentation of vitronectin
Vitronectin has a multidomain structure and selected domains are presented. Near the N-terminal is a Arg-Gly-Asp (RGD) motif that allow for interaction with integrins (yellow). Three heparin binding domains (HBP) are known (orange). Multiple pathogens can bind to HBD 3. Finally, there are four hemopexin domains with unknown function that span a large portion of the protein (blue) [151].

Vitronectin it inhibits the terminal pathway of the complement cascade. With surface-bound vitronectin, assembly and insertion of the MAC are inhibited and the bacteria avoid killing by lysis. Several respiratory pathogens can bind and use vitronectin to their advantage, including NTHi and *M. catarrhalis* [152].

Other known biological functions of vitronectin include involvement in cellular attachment and migration, in tissue and wound healing and in regulation of apoptosis [153,154]. It is a component of the ECM and invading pathogens can use it to adhere to ECM but also to cells. Surface bound vitronectin can form a bridge between the bacteria and the epithelial cell as the tripeptide Arg-Gly-Asp (RGD motif) on the N-terminal attaches to αvβ5-integrins on the epithelial cell and the heparin binding domain (HBD) 3 to the bacterial surface (Figure 14) [155]. This anchoring virulence mechanism has been shown for both Gram-negative and Gram-positive bacteria and is thought to be important for subsequent invasion [156,157].

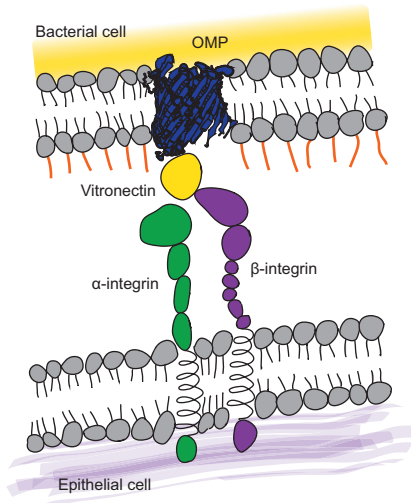

Figure 14: Bacterial adherence using vitronectin as a bridge
The proposed mechanism for vitronectin mediated bacterial adherence to epithelial cells. Vitronectin (yellow) binds to a bacterial adhesin at HBD3 and to αvβ5-integrins (green, purple) on the epithelial cell at its RGD-motif [158].

C4BP

The large (570 kDa), spiderlike regulator C4BP inhibits the classical and the lectin pathways. It is comprised of 7 identical chains with repetitive domains of CCP and can be captured onto the cellular surface by several bacterial pathogens. For instance, *M. catarrhalis* inhibits the classical pathway by binding to CCP2 and CCP7 via the surface proteins A1 and A2 (UspA1, UspA2), just like the M-proteins of *S. pyogenes* [159-161]. Although C4BP is an acute phase protein that can be upregulated four-fold during inflammation, it has not yet been identified in the human respiratory

tract and the relevance for host-pathogen interaction in the lung is thus not established [144].

Factor H

The fluid phase RCA factor H has been found in the respiratory tract and is upregulated by LPS-induced inflammation [140]. Factor H is a 150 kDa plasma glycoprotein that regulates the alternative pathway by binding C3b. It is composed of 20 CCP domains [162]. Domain 20 is a common microbial binding site used by several respiratory tract pathogens [163]. However, some pathogens interact at domain 6, for instance the factor H-binding protein in *N. meningitides* [164]. *Pseudomonas aeruginosa* binds factor H to its surface using moonlighting function of Elongation factor (TufB, PA4277) and Dihydrolipoamide Dehydrogenase (Lpd, PA1587). The cell-bound factor H retains its complement regulatory activity by mediating C3b degradation, and protects the bacteria from killing [42,43].

Shielding by cell wall structures

As it has been reported that the length and type of LPS O-antigens are of importance for the sensitivity to complement in *P. aeruginosa,* it has been suggested that the polysaccharides surrounding bacteria obstruct insertion of the MAC into the outer membrane [165-167]. *Moraxella* spp., *Neisseria* spp., and *Haemophilus* spp. are particularly susceptible to lysis by MAC as these species are enclosed in shorter lipooligosaccharides (LOS) and not LPS, and are less protected against MAC insertion, unless the LOS are sialylated [168].

Several studies support this mechanism. In a burn-wound mouse model, more than 1000-fold higher dose of a mutated *P. aeruginosa* lacking O-antigen was needed to kill 50% of mice (LD_{50}) as compared to the wild type strain. The mutants were more sensitive to killing by serum although no changes could be seen in outer membrane protein expression [169]. Likewise, persisting CF colonizing isolates were more serum sensitive than other clinical isolates [170]. The adaptation in persisting isolates usually involves changes in the O-antigen composition and sometimes loss of O-antigens. The O-antigens are potent antigens and elicit strong antibody responses and subsequent opsonophagocytic killing. This suggests that while O-antigens are involved in bacterial virulence during colonization and the acute infection, they are not crucial for long-term persistence and it is likely an evolutionary advantage in the chronic setting to drop the O-antigen even though the cells become more sensitive to the complement [44].

Similarly, Gram-negative bacteria are shielded by other encapsulating polysaccharides. The mucoid alginate and the Psl polysaccharides surrounding *P. aeruginosa* decrease complement activation by shielding and protect against neutrophilic phagocytosis [165]. As both alginate and Psl are upregulated in strains from patients with CF, and

these strains have been found to be more sensitive to the complement-mediated killing, the influence of the LPS composition may be greater than the influence of other polysaccharides [171,172].

The production of all the four cell-surface glycans (alginate, Psl, type B and type A LPS) are partly interlinked as all require *algC* (PA5322), found in the locus responsible for alginate biosynthesis. Hence, mutated strains lacking *algC* do not have any protective capsule of polysaccharides. As expected, experimental studies report that if mice were challenged with Δ*algC* mutants in the respiratory tract, the bacteria were readily cleared and both bacteraemia and mortality rates were significantly lower than if wildtype strains were used [47].

Proteolytic degradation of cell bound complement

Degradation of complement proteins into non-functional fragments stops the cascade reaction. *Pseudomonas aeruginosa* alkaline protease degrades C2, and alkaline protease and elastase degrade C1q, C3, C5, C8 and C9 [173-175]. An indirect mechanism to remove deposited IgG and C3b from the bacterial surface is to bind and activate plasmin from plasminogen. Plasmin efficiently degrades complement factors, including C3b and C5 [176]. *Pseudomonas aeruginosa* is one of the bacterial species where this mechanism has been described [42]. Several bacteria produce plasminogen activators, for instance *Yersinia pestis* (Pla), *Streptococcus pyogenes* (streptokinase) and *S. aureus* (staphylokinase). More recently discovered is nattokinase, a plasminogen activator that is expressed by some strains of *P. aeruginosa*. The clinical significance, if any, of this enzyme is yet unknown [177].

Clinical infections caused by *Pseudomonas aeruginosa*

Nosocomial infections

Pseudomonas aeruginosa rarely causes disease among the young and healthy. Instead, most infections can in some way be associated with a compromised host defence. For instance, patients with CF are infected with *Pseudomonas* in the respiratory tract, patients with indwelling catheter in the urinary tract, and burn trauma victims get invasive infections originating from the wounds.

The infection may be endogenous or exogenous. Rationally, a prerequisite for any bacterial infection is the presence of the causing organism. *Pseudomonas aeruginosa* may be a resident in the gut in healthy people. Eight per cent of healthy individuals carried *P. aeruginosa* in stool samples in a recent Spanish study [178]. In the respiratory tract, it is a less common resident (<1% of healthy people), but the

prevalence increase sharply after hospitalization or antibiotic treatment [179,180]. Assumingly, this increase is catalyzed by alterations in the composition of the commensal flora after antibiotic treatment. Competition between commensal bacteria form protection against infection by pathogenic species and disturbances in the flora may facilitate infection [181]. This was illustrated by Santiago Ewig *et al.* who compared bacterial colonization at admission and at the end of intensive care treatment. They found a significant increase in *P. aeruginosa* colonization during this time and a corresponding decrease in the most common etiologic causes of pneumonia: *S. pneumoniae*, *S. aureus* and *H. influenzae* [182]

There are abundant amounts of *P. aeruginosa* in the environment as an exogenous source of bacteria, both in nature and in hospitals. For instance, the species was detected in 24% of analyzed environmental soil samples, it has been found to contaminate the floors, bed rails, showers, flower vases and sinks of hospitals, and has been cultured from the hands of health care workers and from diagnostic endoscopes [183,184]. Hence, it is ever-present and as an opportunist ready to take advantage of temporary or permanent immunosuppression to colonize and cause infections in any part of the human body, as will be exemplified in this chapter.

Cases of severe *P. aeruginosa* infections have been described since the introduction of antibacterial agents and it is strongly associated with hospital stay [185]. In cases of nosocomial infection, cross-transmission from other patients have been found in 29.5-52.6% based on molecular subtyping, whereas only a small fraction was genotypically related to that of the hands of health care workers [186]. Most community-acquired infections are caused by environmental isolates and, in agreement with this, isolates typed in epidemiological studies are mainly non-clonal, although endemic strains exist [187,188]. Similarly, young children with CF normally acquire *P. aeruginosa* strains from environmental reservoirs unless they are siblings or otherwise linked to an on-going epidemic [189]. An exception was seen in a CF clinic in Denmark where a colistin-resistant strain spread among the patients. Only after extensive infection control measures were initiated, the outbreak could be stopped [190].

There are various typing techniques to identify clonal populations. In an outbreak situation, molecular typing can provide epidemiological evidence to identify paths of transmission. Serotyping and pulsed-field gel electrophoresis were replaced by multi-locus sequence typing (MLST) as it provided highly discriminatory data that was portable between laboratories and allowed for world-wide comparisons [187]. By typing with MLST, several studies have reported spread of successful clones carrying multi-drug resistance (MDR), whereas susceptible isolates were more diverse [191,192]. One example of such epidemic are the related MDR isolates belonging to clonal complex 111 that has repeatedly been reported in Skåne county [193]. As whole genome sequencing has become widely available, genotyping has been used to,

for instance, trace the bacterial source of hospital outbreaks and is likely to be the typing method we will use during the forthcoming years [194].

Respiratory tract infections

Respiratory tract infections caused by *P. aeruginosa* can broadly be divided into two distinct syndromes:

1. Acute infection causing respiratory distress, increased sputa, fever, and radiologic manifestations. This is seen in community-acquired pneumonia (CAP) in patients with CF, COPD, or malignant diseases. It is also seen in hospital-acquired pneumonia (HAP), often in the intensive care unit presenting as ventilator-associated pneumonia (VAP).
2. Chronic infection in patients with CF and COPD leading to frequent exacerbations and long-term damage of the lung.

The pathogenesis is different between the two syndromes. During acute infections, traditional virulence traits promoting mobility, adherence, and destruction of host defences by virulence traits including exotoxins, proteases, flagella, and LPS are important [184]. During chronic infection, *P. aeruginosa* gradually adapts to the environment and down-regulate or lose many of these virulence traits by negative selection to become less immunologically reactive. This allows bacteria to persist in the respiratory tract as biofilms for extended periods of time, sometimes decades [195,196].

Acute respiratory infections

Pseudomonas aeruginosa is a common cause of hospital-acquired pneumonia (HAP) but not a common cause of community acquired pneumonia (CAP). The species causes up to every fourth HAP, regardless of if it is ventilator-associated or not. This makes it the most commonly isolated bacteria causing HAP, in some reports second after *S. aureus* [186,196-198]. In contrast, *S. pneumoniae* is the most common pathogen in CAP, followed by *H. influenzae* and various atypical pathogens. *Pseudomonas aeruginosa* has been reported as the causing organism for 0.5-17.1% of CAP [199,200].

It should be noted that some studies did not differentiate between colonization and pneumonia and hence the true incidence rate of *P. aeruginosa* pneumonia is likely to be somewhat lower. Bacterial colonization by *P. aeruginosa* in the respiratory tract of hospitalized patients is relatively common and clinical signs of infection (for instance: fever, increased sputa, and respiratory distress) and radiologic manifestations should be assessed before antimicrobial treatment is initiated. VAP caused by *P. aeruginosa* is associated with a very high mortality, in some reports as high as 87% [186]. Hence, it

is important to initiate effective antipseudomonal treatment when indicated. To aid in this decision, various algorithms have been proposed to signal when to initiate treatment. These include variables as (i) abrupt increase in quantity and purulence of respiratory secretions, (ii) intracellular bacteria in centrifuged BALF, and (iii) microbiological quantitative criteria (bacterial counts) [186]. However, the differentiation between colonization and infection remains challenging and may cause unnecessary overtreatment at intensive care units.

A special case of HAP is caused by bacterial transmission from medical equipment. In an infamous outbreak in Baltimore, USA, in 2001, the incidence of *P. aeruginosa* isolated from BALF samples increased from 10.4 to 31.0 % during the outbreak. The source was a contaminated bronchoscope and in which a loose biopsy-port cap sheltered organisms and thus rendered disinfection procedures ineffective. This malfunction caused multiple incidents of severe infections and contributed to the death of three patients [201].

Chronic respiratory infections

The pathogenesis behind chronic infections in patients with CF or COPD differs from the pathogenesis behind acute infections. CF is an hereditary autosomal recessive disorder resulting from mutation of the cyclicAMP-regulated chloride ion channel protein known as the cystic fibrosis transmembrane conductance regulator (CFTR) [202]. It is the most common life threatening hereditary disease in the European Union, affecting about 1/2000-1/5000 new-borns [203]. The mutation results in dysfunction of CFTR, which causes production of mucus with altered properties. In the respiratory tract, the thickened secretion reduces the efficiency of the mucociliary escalator and the airways become obstructed [184]. Although this is a multisystem disease, the primary cause of death in CF is usually respiratory failure resulting from chronic pulmonary infection [204].

It is not known why CF patients are specifically infected with *P. aeruginosa*. One theory revolves around CFTR being a receptor for *P. aeruginosa*. The mutated form ΔF508, which is seen in 70% of CF patients, bind *P. aeruginosa* less efficiently than wild-type CFTR. Without the receptor, less bacteria are internalized into the epithelial cells. It is believed that lower internalization of bacteria followed by less apoptosis, detachment and expulsion by the mucociliary escalator result in abnormally high bacterial load and establishment of infection. However, more studies are required to confirm this hypothesis [184].

The seemingly idle, chronic infection with *P. aeruginosa* account for most of the morbidity of the disease leading to lung transplantation or death [184]. Once *P. aeruginosa* infection is established, the bacteria start to adapt to the new environment in the respiratory tract and frequently colonize the paranasal sinuses. Through a series of mutational steps, the colonizing *Pseudomonad* undergo phenotypic switch to a

mucoid phenotype (overproducing alginate) with high antibiotic resistance, reduction in growth rate and loss of O-antigen, T3SS and quorum sensing [205,206]. This mucoid phenotype is rare among clinical isolates from other anatomical regions [207]. In the lung, the adaptation enables the bacteria to persist in the body embedded in mucus or in biofilms to minimise immune activation and the risk of clearing.

While it is well established that most CF patients are colonized by *P. aeruginosa* during childhood, less focus has been on the role of *Pseudomonas* in the pathogenesis of COPD. The inflammatory airway disease COPD is immensely more common than CF and is ranked as the fifth most common cause of death worldwide [208].

According to both older studies based on traditional culturing methods and more recent microbiota studies based on DNA-sequencing, the respiratory tract of COPD patients are commonly colonized and infected by *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and *P. aeruginosa*, in descending order of frequency. Up to every fifth patient with severe COPD is chronically colonized by *P. aeruginosa*, but just like in the majority of patients with COPD, it seems that these patients have a diverse polymicrobial flora in their respiratory tract [209,210]. There are also several studies in which *P. aeruginosa* is the most common bacterial species isolated during exacerbation of severe COPD [211,212]. The course of COPD is characterized by intermittent exacerbations and the presence of *P. aeruginosa* in the lower airways is associated with increased symptoms and exacerbations. Unlike in CF patients, there is a frequent turnover of clones, but also persistent isolates that behave similarly to the CF-associated mucoid isolates [213,214].

Bacteraemia

It is rare that patients with CF develop bacteraemia (blood stream infection; BSI) [215]. Bacteraemia is more often seen among patients with haematological or other malignancies, high age, multiple comorbidities, organ transplants and HIV infection. As with other types of *P. aeruginosa* infections, bacteraemia is associated with frequent contact with the health care system. As many as 78.4% of the cases have been reported as health care associated [216-219]. It is the third most common Gram-negative BSI, topped only by *Escherichia coli* and *Klebsiella* spp, and cause approximately 7% of Gram-negative BSI [220]. It is, however, associated with higher mortality than other Gram-negative BSI, ranging from 21 to 60% [217,218,220,221]. This high mortality likely reflects the predilection of the bacteria for immunocompromized individuals, the elderly or patients heavily burdened by other comorbidities, but can also be an effect of the inherent virulence of *P. aeruginosa*. One example of a bacterial factor is a specific exoU genotype that has been reported as an independent marker of early mortality [123].

Burn victims are especially prone to disseminated *P. aeruginosa* infection, probably because the skin is destroyed by thermal injury and the skin is the outermost barrier of the innate immunity. As *P. aeruginosa* is common in the hospital environment and it usually takes time before the sometimes extensive wounds heal, it is very likely that the victim will be colonized. Unlike in the persistent isolates found in CF patients, strains with disrupted *lasR* regulatory gene, that lack flagella or lack pili are less prone to cause invasive infections in burn victims than motile bacteria [184,222].

There are several case-reports of bacteraemia involving vascular grafts and the heart valves, although not very common. In a recent study, *P. aeruginosa* was the most commonly isolated organism from infected peripheral prosthetic vascular grafts (31%) causing considerable morbidity and mortality in affected patients [223]. About 3% of endocarditis cases are caused by *P. aeruginosa*, importantly among intravenous drug abusers injecting contaminated water, but also among patients with a history of instrumentation [224,225]. Secondary seeding can cause bone and joint infections. Osteomyelitis caused by *Pseudomonas* was especially common among American soldiers during operations in Afghanistan and Iraq [226].

Urinary tract infections

Although the urinary tract is the most common source of BSI, urinary tract infections (UTI) caused by *Pseudomonas* are usually mild. *Pseudomonas* UTIs are more common among males and are associated with contact with the health care system in general, specifically with treatment at an intensive care unit or with carriage of an indwelling urinary catheter. As with Gram-negative BSI, *P. aeruginosa* is the third most common infecting agent in the urinary tract in the nosocomial setting, following *E. coli* and *Klebsiella* spp [227]. In the intensive care setting, 9% of *P. aeruginosa* UTI were followed by pneumonia caused by *P. aeruginosa* (median delay after the UTI, 9 days) and 3% by *P. aeruginosa* bacteraemia (median delay after the UTI, 4 days) [228].

Other infections of clinical significance

Other manifestations of *P. aeruginosa* include “swimmers ear”: an acute diffuse external otitis and “hot tub folliculitis”: multiple painful papules commonly following bathing in a whirl bath with contaminated water. Ulcerative keratitis is seen among contact lens carriers and, finally, the rapidly progressing endophtalmitis after penetration of the eye [106,229]. Keratitis has been used as a model infection to study many of the known bacterial virulence traits involved in adhesion and invasion.

Antimicrobial treatment and drug resistance

General principles of antibacterial treatment of *Pseudomonas aeruginosa* and *Moraxella catarrhalis*

Few bacteria are as difficult to treat as *P. aeruginosa*. It is intrinsically resistant to antimicrobial drugs due to the low permeability of the outer membrane that obstructs entry into the cell. Drugs with a mechanism of action inside the cell need to enter through the tightly controlled porin-like outer membrane proteins, which are frequently mutated. Once inside the cell, the binding sites can be altered and the cells have efflux pumps to expel drugs to the extracellular space. Moreover, a wide variety of beta-lactamases are produced by clinical strains of *P. aeruginosa*, including enzymes capable of hydrolyzing carbapenems (carbapenemases). Generally, resistance to antimicrobial drugs can be acquired as a random mutation in the chromosome, or through the spread of a transposon or a plasmid with the resistance gene. Increased antimicrobial resistance in general is a major threat. To put this threat in historical perspective, the average life expectancy increased by 10 years when antibiotic treatment was first introduced, [230].

Commonly used antipseudomonal drug classes include beta-lactamase stable penicillins, some cephalosporins, carbapenems, monobactams, aminoglycosides, fluoroquinolons and, as a last resort, polymyxins. Resistance mechanisms against these drugs can coexist, thereby conferring MDR (Table 1).

Clinical isolates of *M. catarrhalis* are currently to 90% producing beta-lactamase [231]. The first report of beta-lactamase in this species was published in 1977, but after the initial discovery of resistant isolates, a rapid worldwide spread of resistance was seen. Within four years of the beta-lactamase enzymes were found in 1977, up to 75 percent of *M. catarrhalis* in the United States were resistant [232].

Table 1
Commonly used antipseudomonal drugs. Abbreviations: iv=intravenous, inh.=inhalation.

Antimicrobial drug	Class	Adm. route	Target structure	Bacterial resistance	Ref.
Piperacillin-tazobactam	penicillin, beta-lactamase inhibitor	iv	Peptidoglycan synthesis (PBP)	AmpC, ESBL, MexAB	[233-235]
Cefepime	cephalosporin	iv		AmpC, ESBL, MexAB, MexXY	[235,236]
Ceftazidime					
Ceftazidime-avibactam	cephalosporin, beta-lactamase inhibitor			Efflux	[237]
Ceftolozan-tazobactam					
Meropenem	carbapenem	iv		OprD, MexXY, carbapenemase	[234,238,239]
Imipenem					
Doripenem					
Aztreonam	monobactam	iv, inh.		MexAB	[240]
Gentamicin	aminoglycoside	iv	30S ribosome	Aminoglycoside modifying enzymes	[241]
Tobramycin		iv, inh.			
Ciprofloxacin	fluoroquinolone	iv, oral	DNA gyrase, topoisomerase	MexAB, gyrase	[242,243]
Colistin	polymyxin	iv, inh.	LPS	Lipid A modification	[244]

Beta-lactam antibiotic drugs

Most antipseudomonal treatment regimens include a beta-lactam antibiotic, alone or in combination with another antimicrobial drug. Common to all beta-lactam antibiotics is the beta-lactam ring, but variations in the side chains cause differences in, for instance, beta-lactamase resistance and how the drugs translocate over the outer membrane [245]. Beta-lactam antibiotics are bactericidal drugs that cannot diffuse through the outer membrane and must instead enter the periplasm through OMPs [238]. Once across the bacterial outer membrane, the mechanism of action is by binding to penicillin binding proteins (PBP) in the periplasm, and inhibit the cross-linking between peptidoglycans [246]. This binding inhibits further peptidoglycan synthesis, but degradation of peptidoglycans resumes. Eventually the peptidoglycan matrix is weakened to the point when the cell burst due to osmotic pressure.

As the total duration of antibiotic concentration above the minimal inhibitory concentration (MIC) determines the antibacterial efficiency for beta-lactam

antibiotics, treatment strategies using continuous or prolonged infusion have been evaluated in several studies for both piperacillin-tazobactam, cephalosporins and carbapenems. Although constant infusion was found to be superior from a pharmacodynamic perspective, superior antimicrobial efficacy against *Pseudomonas* pneumonia has not been established [247].

Penicillins

The only penicillin that is frequently used against *P. aeruginosa* is piperacillin-tazobactam. The beta-lactamase inhibitor tazobactam prevents hydrolysis by many beta-lactamases, but notably not by the inducible chromosomal beta-lactamase AmpC, and only by some of the transferrable extended spectrum beta-lactamases (ESBL) [233,235,247,248]. Piperacillin-tazobactam is however one of the most efficient antipseudomonal drugs in clinical use. In a recent Cochrane report based on a meta-analysis of 40 reports, it was the recommended antibiotic for use in febrile neutropenia caused by *P. aeruginosa*, as mortality was low and few cases of diarrhoea caused by *Clostridium difficile* were seen [249].

Cephalosporins

Although the commonly used cephalosporin antibiotic cefotaxime has partial stability against beta-lactamase, it is not stable when exposed *Pseudomonas* AmpC or ESBL. Antipseudomonal cephalosporins include cefepime, ceftolozan and ceftazidime. The latter two have recently been combined with beta-lactamase inhibitors to extend the use to previously resistant strains, including carbapenem resistant isolates [250]. However, at least cefepime and ceftazidime are vulnerable to ejection from the bacterial cells by efflux pumps. As illustrated in Figure 4, the efflux pumps are fusion peptides of an OMP, often OprM or OprJ, a linker through the periplasm (MexA, MexC or MexX) and a pump in the cytoplasmic membrane (MexB, MexD or MexY). Ligands (including drugs) can enter the pump at any side of the cytoplasmic membrane and are transported out of cell. Overexpression of efflux pumps are caused by mutations in transcriptional regulatory proteins and confers resistance to cephalosporins, but also carbapenems, fluoroquinolones and aminoglycosides [236,238]. Alterations in efflux pumps have commonly been observed in clinical isolates [242].

Carbapenems

The mechanism of action of carbapenems is similar to other beta-lactam antibiotics, but they differ by their ability to bind multiple PBPs. They are also less susceptible to hydrolysis by AmpC and ESBL. Unfortunately, in many geographic regions, carbapenem hydrolysing beta-lactamases (carbapenemases) are endemic. The most common carbapenemase in *P. aeruginosa* is VIM-2, a zinc dependent metalloenzyme

[247]. In addition, several other carbapenemases have been found in clinical isolates, including KPC that was first reported about 10 years ago [234,239].

The main carbapenem resistance mechanism in *P. aeruginosa* is however mutation or loss of Porin D (OprD, PA0958) [238]. Entry of imipenem into the cell is entirely dependent on OprD. Other carbapenems are only partly dependent on OprD for entry and other beta-lactam antibiotics are not at all associated with OprD activity [247]. Imipenem was the first carbapenem in clinical use. It is a derivative from thienamycin, the archetype carbapenem and a natural product from *Streptomyces cattleya* [251]. As variations in *oprD* existed in environmental isolates before the introduction of imipenem, and as both *P. aeruginosa* and *S. cattleya* are common bacteria in soil, it is possible that these mutations were beneficial for *Pseudomonas* also in the environment. In analogy with this, a survey of the microbiome in the restricted Lechuguilla Cave in New Mexico, USA reported resistance mechanisms against 14 commercially available antimicrobial drugs even though the cave had been isolated for 4 million years [252].

Other carbapenems include meropenem, ertapenem and doripenem. Ertapenem has been reported to have lower activity against *P. aeruginosa*, whereas the newer doripenem was reported as the most efficient antipseudomonal carbapenem to date *in vitro*, although superior clinical efficacy is yet to be shown [253].

Monobactams

Finally, the monobactam aztreonam is mainly used in nebulized form against *P. aeruginosa* in the respiratory tract of patients with CF [254]. The monobactams differ from other beta-lactam antibiotics in that they only have a single beta-lactam ring. The first known monobactam was produced by *Chromobacterium violaceum*, another bacteria growing in soil and water in tropical regions. In analogy with carbapenem-resistant environmental isolates, *P. aeruginosa* from soil in Brazil were reported as highly resistant to aztreonam [255]. Aztreonam resistance may be associated with modification of efflux pumps or alterations of PBPs [240]. Aztreonam can be an alternative drug when the bacterial isolate is resistant to more commonly used drugs.

Antimicrobial combination treatment

Severe *P. aeruginosa* infections are commonly treated with a combination of two or more antimicrobial drugs, usually including one beta-lactam drug. Combination treatment can broaden the antimicrobial spectrum to include other species or resistant isolates, provide synergistic interactions between the drugs, reduce the risk of emergence of antimicrobial resistance and minimize superinfections [247]. *In vitro*, synergy is mainly seen between aminoglycosides and beta-lactams. *In vivo*, however, most observational studies on VAP, bacteraemia and febrile neutropenia have not

been able to show any benefit of combination therapy compared with correct monotherapy, although the opposite has also been reported [247,256,257].

Antimicrobial drugs not targeting the peptidoglycan synthesis

Aminoglycosides

The most commonly added drugs to a beta-lactam treatment regimen are aminoglycosides or fluoroquinolones. The first member of the aminoglycosides was streptamycin, but presently the most commonly used drugs of this class are tobramycin and gentamicin. The nomenclature –mycin and –micin depend on the natural origin of the molecule, from *Streptomyces* spp. or from *Micromonospora* spp., respectively.

These molecules consist of aminated sugars and the mechanism of action is on the bacterial ribosome [258]. The ribosomes synthesize proteins as they translate mRNA into polypeptides. The bacterial ribosomes consist of two subunits, 30S and 50S, together forming a 70S unit and differ from the eukaryotic counterpart in size. The aminoglycosides bind to the centre of the small subunit where they impair the proofreading process and decrease the translational accuracy, causing aberrant proteins and ultimately bacterial death [258-260]. Hence, aminoglycosides are only effective on metabolically active bacteria, and not on, for instance, dormant bacteria in the centre of biofilms. Aminoglycosides are concentration-dependent, and can at higher concentrations have an additional bactericidal effect outside the cell as they can cause rapid killing even when bound to albumin [261]. Aminoglycoside resistance is mediated through aminoglycoside-modifying enzymes, which according to some reports are inducible [241,247]. Patients with CF are sometimes treated with inhaled tobramycin powder in an attempt to eradicate *P. aeruginosa* [254].

Fluoroquinolones

The other commonly added drug in an antipseudomonal combination treatment regimen is ciprofloxacin, a fluoroquinolone. When introduced in 1983, it was considered to be one of the most potent antimicrobial drugs known [262]. The quinolones act on DNA gyrase and DNA topoisomerase IV, which interfere with DNA replication. DNA fragments and reactive oxygen species accumulate until the bacterial cell dies [263]. Quinolone-resistance in *P. aeruginosa* is mainly attributed to mutations in DNA gyrase, but quinolones are also effluxed through membrane pumps [242,243,247].

Polymyxins

For clinical Gram-negative bacterial isolates with MDR, *Pseudomonas* and others, a last resort antimicrobial is colistin. As strains with MDR have become increasingly

common, the interest in colistin has increased markedly. Colistin is a cationic peptide that belongs to the polymyxins, all originating from *Paenibacillus polymyxa* [253]. Polymyxins damage the Gram-negative bacterial cell by binding to the LPS, where it has a detergent-like activity that disrupts the outer membrane integrity resulting in leakage of intracellular components [253].

The colistin efficacy is undisturbed by efflux pumps as the mechanism of action is on the surface and outside the cell. Variations of the lipid A part of LPS induce resistance to polymyxins [244]. This is still rare and most commonly seen in patients with CF who have received colistin inhalation treatment [247]. However, recently, a plasmid-mediated colistin resistance gene, *mcr-1*, was reported in China [264]. This transferrable element has been found in *E. coli*, *K. pneumoniae*, *Shigella sonnei* and *Salmonella enterica*. Since its first report in China in November 2015, *mcr-1* has been detected in isolates from five continents, both from colonized and infected individuals, from animals and from environmental strains [265]. As *P. aeruginosa* can be transformed *in vitro* to include and carry the plasmid, and since such transformant mutants have increased colistin MIC, it may be expected that we will encounter more pan-resistant *Pseudomonads* in the future.

The Present investigation

Aims

The overall aim of this thesis is to explore the pathogenesis of Gram-negative respiratory tract bacteria, focusing on *P. aeruginosa,* and to study the relevant bacterial outer membrane proteins and outer membrane vesicles at a molecular level.

The specific aims are:

- To investigate if bacterial proteins enclosed in outer membrane vesicles (OMV) are protected against neutralization by host antibodies.
- To explore the interaction between vitronectin and clinical *P. aeruginosa* isolates.
- To identify *P. aeruginosa* outer membrane proteins involved in surface binding of vitronectin.
- To examine the dynamics of the complement regulator vitronectin in respiratory tract infections *in vivo* and *in vitro* during OMV-induced inflammation.
- To establish if vitronectin from clinical airway samples are available for acquisition by bacteria and investigate the effect of such binding on the sensitivity to complement.
- To identify host and treatment related factors that influence the outcome of invasive *P. aeruginosa* infections.

Methods, Results and Discussion

Paper I: Outer membrane vesicles shield *Moraxella catarrhalis* beta-lactamase from neutralization by serum IgG

Introduction and aims

In year 2000, our colleagues at the University of Copenhagen discovered that OMV from *P. aeruginosa* contained beta-lactamase [71]. At the time, it was already known that high levels of IgG antibodies directed against beta-lactamases were found in chronically colonized patients with CF [266]. In parallel, earlier studies from our group had revealed that also the Gram-negative respiratory pathogen *M. catarrhalis* secreted OMV containing Bro-1 beta-lactamase and that these OMV protected other respiratory pathogens from amoxicillin [72].

This study aimed to further investigate the clinical significance of the enclosure of bacterial enzymes inside the OMV and specifically if such enclosure protected the cargo against host IgG.

Methods and results

To establish the clinical significance of OMV secretion in the respiratory tract and to determine if beta-lactamase was present inside OMV *in vivo*, we used TEM to depict *M. catarrhalis* and their derived vesicles from nasal discharge. The beta-lactamase was detected inside vesicles using gold-labelled polyclonal antibodies (pAbs), confirming that this mechanism was not only seen in the laboratory setting.

Anti-beta-lactamase pAbs were produced through immunising rabbits with recombinantly expressed protein. The pAbs were subsequently purified on CnBr-sepharose columns containing beta-lactamase. The proteins were recombinantly expressed from transformed *E. coli* BL21(DE3) containing the beta-lactamase *bro* gene in the plasmid vector pET26b. Expressed hexa-histidine tagged proteins were purified by metal ion affinity chromatography. OMV were produced and purified according to Figure 15. Vesicle-associated beta-lactamase was found in *in vitro* analysis of OMV from a known beta-lactamase producing strain, KR526, when analyzed by flow cytometry.

To ensure the presence of IgG *in vivo*, we analysed IgG against *Moraxella* beta-lactamase in healthy adults. Sera were collected from 40 healthy blood donors and reactivity against beta-lactamase was measured with enzyme-linked immunosorbent assay (ELISA). For reference, titers against the surface exposed *Moraxella* proteins MID, UspA1 and UspA2 were determined. Six of forty cases had titers against beta-lactamase.

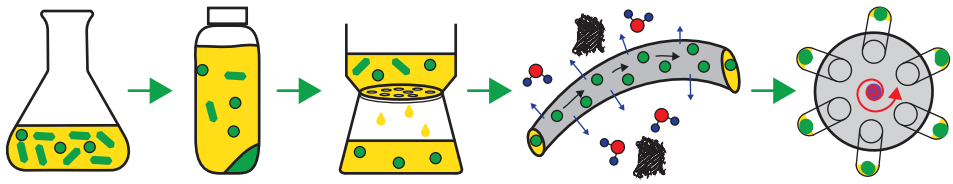

Figure 15: Preparation of OMV in the lab
Stepwise presentation of OMV purification. From the left: Bacteria are cultured in growth medium, then removed from the medium by pelleting and filtration. OMV are concentrated by removing salts, proteins and water in a semipermeable tube and finally further concentrated by ultracentrifugation.

To investigate if the neutralizing effect of IgG on beta-lactamase was affected by the enclosure of the enzyme within OMV, OMV were incubated with anti-beta-lactamase pAbs followed by co-incubation with nitrocefin. Nitrocefin is a chromogenic beta-lactam that is hydrolyzed by beta-lactamases. When analysing intact OMV, the enzymatic activity was decreased by 61%. In contrast, if the membranes of the vesicles were permeabilized using saponin, the activity decreased by 95%.

In summary, we could show that OMV were produced *in vivo* in the respiratory tract and that these vesicles contained beta-lactamase. We were also able to show that a proportion of healthy adults carried antibodies against *M. catarrhalis* beta-lactamase and that these IgG neutralise beta-lactamase less efficiently if the enzyme was contained inside a vesicle.

Discussion and future perspectives

This study used *M. catarrhalis* as a model bacterium. As all Gram-negative bacteria (including *P. aeruginosa*) shed OMV during growth, the findings from the present study can likely be extrapolated to include most beta-lactamase producing Gram-negative bacteria. The vesicles are containers that store and protect their interior, but also deliver their cargo to specific sites, as surface exposed molecules have specific receptors on the cells of the innate immunity. This enables complex reactions involving delivering several proteins at sites distant from the producing bacteria.

Hydrolysis of beta-lactams by beta-lactamases requires that the molecules are at the same place. Beta-lactams normally cross the outer membrane through porins [267]. As the OMV originate from the outer membrane and contain outer membrane proteins, including porins, it is possible that beta-lactam antibiotics diffuse into the OMV in the same way as they cross the outer membrane in the bacterial cell. Hence, the nitrocefin hydrolysis observed in this study may have taken place inside the OMV.

Complete eradication of *P. aeruginosa* among patients with CF is often very difficult. *Pseudomonas aeruginosa* resides in the mucus layer above the cilia, out of reach of direct cell-to-cell interactions. If these bacteria are cleared, subpopulations persist in the paranasal sinuses and the chronic infection causes a low-grade inflammation, slowly deteriorating the tissues in the lung. As the OMV are stable products capable of withstanding IgG, it would be of interest to investigate the presence and importance of bacterial vesicles in the lower respiratory tract of patients that are colonized only in the sinuses.

Paper II: Identification of outer membrane Porin D as a vitronectin-binding factor in cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*.

Introduction and aims

From previous reports from our group we knew that the Gram-negative respiratory tract pathogens *H. influenzae* and *M. catarrhalis* could evade killing by the complement system through binding of complement regulators to OMPs on their surface [268]. Moreover, a correlation between the severity of disease and the ability to evade lysis by serum had been found [269]. Vitronectin was identified as the main mediator of complement inhibition, which is logical as these bacteria are prone to insertion and lysis of the MAC, and vitronectin is a regulator of the terminal pathway of the complement cascade. Therefore, it was reasonable to hypothesise that *P. aeruginosa* also may regulate complement using OMPs, although reports from the 1980s suggested that the LPS composition determined serum resistance by inhibiting MAC insertion [166]. Intriguingly, other studies reported that vitronectin bound to bacteria also promoted adhesion to epithelial cells, an important step in the early respiratory tract colonization [155,156]. Hence, at least two different vitronectin-related mechanisms of potential significance for bacterial virulence were known.

This study aimed to investigate to which extent clinical *P. aeruginosa* isolates had the ability to capture vitronectin to their surface and to identify proteins in the outer membrane responsible for this interaction.

Methods and results

As no previous studies had been performed on vitronectin interactions and *P. aeruginosa*, the first part of this study was conducted to establish if the species were able to bind vitronectin to the cell. We collected clinical isolates cultured from the respiratory tract and from blood samples. Vitronectin was labelled with radioactive ^{125}I and the iodinated protein was incubated with each clinical isolate to determine the total vitronectin-binding capacity. The experiments revealed that isolates from the respiratory tract bound more vitronectin.

To identify the responsible OMP, we isolated all outer membrane proteins from five strains of *P. aeruginosa*. These were subjected to gel separation in two dimensions. First, proteins were separated according to mass using electrophoresis followed by separation according to isoelectric point after application of a perpendicular pH gradient. The proteins were transferred to a hydrophobic membrane and incubated with vitronectin. Finally, vitronectin-binding proteins were revealed using anti-vitronectin pAbs. In PAO1 and all clinical isolates, one spot was consistently found to bind vitronectin. This protein was extracted from the gel and identified by mass spectrometry as OprD (Figure 16).

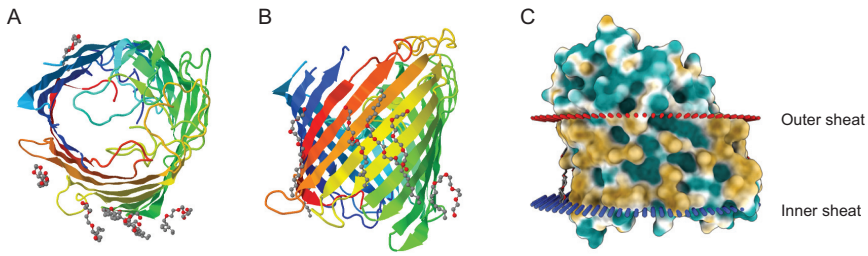

Figure 16: Barrel-shaped outer membrane protein
The *P. aeruginosa* OMP OprD (Porin D) has a typical beta-barrel shape enclosing a central opening (A: top view and B: front view). The electron density model (C) illustrates its integration in the outer membrane, with hydrophilic epitopes (blue) extending both into the periplasm and outwards into the extracellular space. All models were generated at the Protein Data Bank (available at http://www.rcsb.org) and the structure based on PDB ID: 2ODJ.

OprD was recombinantly expressed using the same technique as in Paper I. The used expression plasmid is illustrated in Figure 17. The protein was used to immunize rabbits to obtain antibodies and protein-protein interactions between OprD and vitronectin were explored using two separate experimental methods, ELISA and biolayer interferometry (Octet RED; Pall ForteBio, Fremont, CA, USA). Biolayer interferometry is an antibody independent method where vitronectin was coated on light emitting sensors. As the analyte, OprD, bound to the vitronectin, the thickness of the layer increased, resulting in a wavelength shift of the reflected light [270]. Through the use of truncated vitronectin fragments and heparin inhibition, the site of interaction was found to be in the heparin-binding domain, which is the site for most bacterial interactions of vitronectin [152].

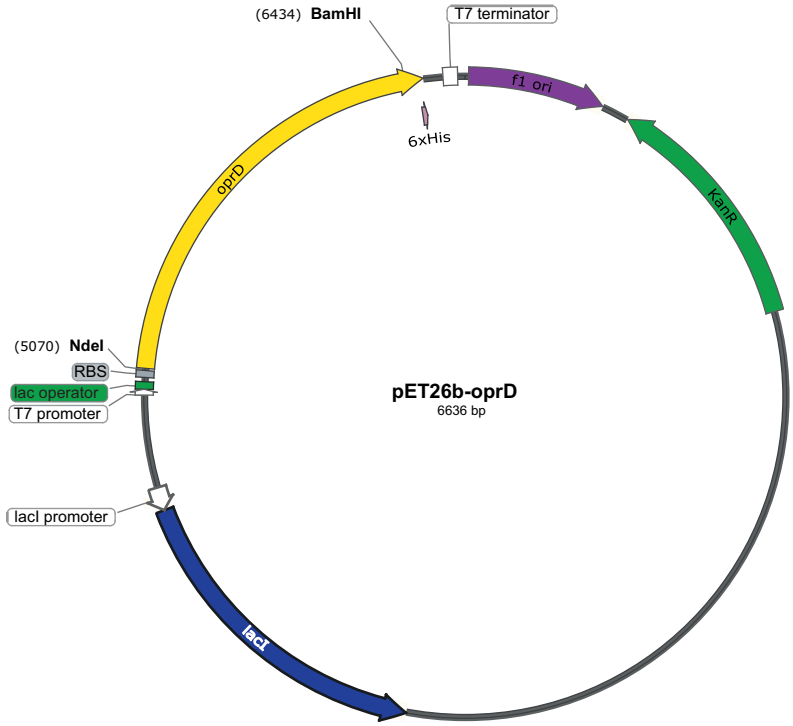

Figure 17: Plasmid map of pET26b-*oprD*
Map representing the DNA sequence of the expression plasmid pET26b, with a Porin D insert. This plasmid attaches a His-tag to the protein that is used for purification after expression in the *E. coli* host. Plasmid map created in Snapgene (GSL Biotech, Chicago, IL, USA)

As one of the main OMP of *P. aeruginosa*, OprD was found in all tested clinical strains. An *oprD*-deficient mutated strain was obtained from "Seattle *P. aeruginosa* PAO1 transposon mutant library". This strain had a transposable genetic element inserted in the PA0958 locus, which effectively disabled the gene. Consequently, the vitronectin binding ability was significantly decreased, as was the ability to adhere to immobilized vitronectin. Conversely, when transformed with a plasmid for surface expression (pET16b) containing the *oprD* gene, *E. coli* gained the ability to bind vitronectin to its surface as measured by flow cytometry.

Discussion and future perspectives

In this study, we were able to show that isolates responsible for chronic infections of the respiratory tract had higher vitronectin-binding capacity than invasive isolates causing bacteraemia. This was an unexpected finding as it has been known for 40 years that CF isolates are more sensitive to killing by complement than other clinical isolates and were expected to be less apt vitronectin-binders [172]. However, this result also highlights the importance of vitronectin binding for persistence in the

airway. It can be speculated upon what benefit the bacteria adapted for chronic infection may have from this, but as vitronectin is a part of the extracellular matrix and the OprD-expressing strains adhered better to immobilized proteins, it may be related to the adhesion to epithelial cells using vitronectin as a bridging molecule.

OprD was one of the first identified proteins in the *Pseudomonas* outer membrane and originally named protein D2 [36]. It has given name to a whole family of OMPs, the OprD-family, comprised of semi-specialized uptake channels with different substrate specificity [271]. The main member, OprD, drew attention to the research community when it in 1987 was clear that this porin-like OMP was the previously uncharacterized 46 kDa protein responsible for imipenem resistance, and not a new beta-lactamase [272,273]. The natural substrate for OprD in *P. aeruginosa* is, however, not likely to be the synthetic imipenem. Just like all the members of the OprD-family, the channel transports specific amino acids and dipeptides into the periplasm. In the case of OprD, arginine and lysine is transported [271].

It is common that bacterial outer membrane proteins have several functions, in this case a nutrient channel and a potential adhesion molecule. The extracellular loops protrude to the extracellular space and allow for interaction with other molecules or cells (Figure 16) [274]. OprQ (PA2760), another OprD-family member, has been suggested to be involved in adherence to fibronectin. Fibronectin is a part of the ECM and this interaction may be important in the initial colonization of the host epithelial tissue [275].

Shortly after this manuscript was published, another group in collaboration with us reported that the moonlighting protein Lpd also bound vitronectin to the surface of *Pseudomonas* [276]. Moreover, they reported that bacterial survival was higher when challenged with complete human serum, than with vitronectin-depleted serum. This corroborated the role of vitronectin as complement regulator also for *Pseudomonas*.

We did not quantify the OprD expression, more than semi-quantitively in western blot and hence it was not possible to correlate levels of OprD expression to vitronectin binding capacity. Neither did we characterize the LPS in this study, and hence it is still unknown if the polysaccharides surrounding the cells, OprD expression or Lpd is the most important determinant for *Pseudomonas* sensitivity to complement.

In retrospect, the use of the NTHi protein UHP_03526 as negative control was a poor choice, as this protein cross-reacted with the anti-vitronectin pAbs in ELISA. The choice was based on that this protein was a known non-binder to vitronectin from other experiments, and had been expressed in the same system as OprD.

In a study by Skurnik *et al*. in 2013, a transposon mutant library was used to screen for virulent phenotypes, thousands of *P. aeruginosa* mutants with different gene deletions were inoculated in the gut of mice. For unknown reason, *oprD*-deficient

mutants were more invasive and disseminated more readily to the spleen than any other mutated strain. A similar change in fitness has not been replicated in *in vitro* experiments, but this report gave rise to an editorial about resistance mechanisms and fitness. The remarkable part about this finding is that OprD mutants are imipenem resistant, as well as seemingly more invasive [277,278].

By identifying molecules on the surface of the bacterial cell that are of importance for pathogenesis, we unravel possible epitopes for a future vaccine. Similarly to the factor H binding protein in *N. meningitides* used in the serogroup B vaccine, Porin D is a potential target as it is surface exposed, expressed by most clinical cells and is important for nutrient uptake as well as interaction with the innate immunity. However, as mutations in OprD is a common cause of imipenem resistance among clinical strains, it may be a less feasible target to include in a future vaccine.

Paper III: Vitronectin is produced in the lung upon infection by respiratory pathogens, and is utilized to conquer the innate immunity

Introduction and aims

The final manuscript in this thesis explores the interplay between the innate immunity and components of the outer membrane of Gram-negative bacteria. In Paper II, we found that *P. aeruginosa* isolates from chronic respiratory tract infections were apt vitronectin binders, which suggested that this property was important for bacterial persistence in the respiratory tract. However, vitronectin is primarily produced in the liver and circulated in plasma [279]. Vitronectin levels in the respiratory tract have been reported to be elevated during chronic pulmonary diseases, but little was known about the relation to infection and pneumonia [141].

Two proposed effects of bacterial vitronectin binding are evasion of complement-mediated killing and adhesion to the epithelial cells [152]. It has not been investigated whether concentrations in the respiratory tract were sufficient for significant binding to the bacterial surface and if other molecules in the respiratory tract would block the binding by competitive inhibition.

The aim of this study was to measure vitronectin concentrations in the lung during pneumonia, to explore if concentrations were increased *in vivo* and *in vitro* following stimuli from bacterial surface structures (LPS and OMV) and to investigate if the proposed bacterial use of vitronectin was possible using vitronectin from BALF.

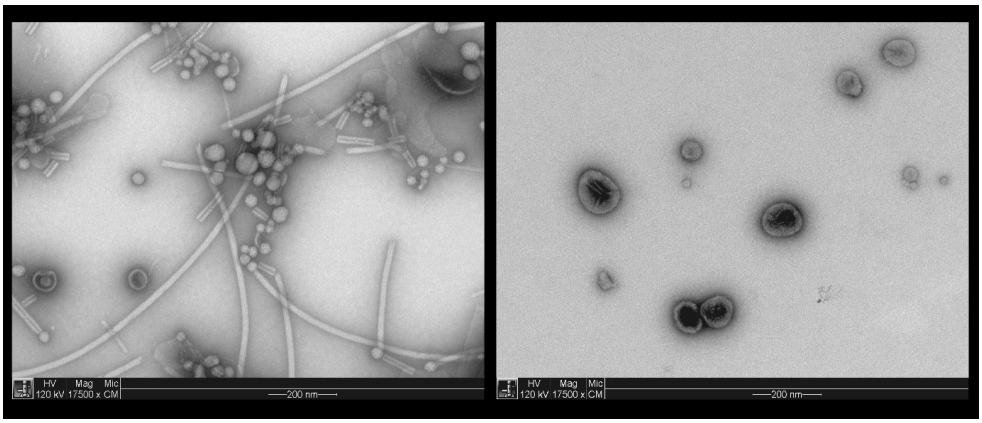

Figure 18: OMV purification method affects the purity of the preparation
TEM pictures of two batches of OMV purified from the same isolate, *P. aeruginosa* PAO1. The preparation to the left also contain other cellular components such as flagella and pili. While preparing OMV for Paper III, these contaminants were removed in a nonionic density gradient medium. Photo by Linda Sandblad, Umeå Core Facility for Electron Microscopy.

Methods and results

To determine vitronectin levels in the human lung during pneumonia, we recruited 8 patients with suspected pneumonia that were undergoing diagnostic bronchoscopy at the Department of Infectious diseases at the Skåne University Hospital in Malmö. Healthy volunteers were recruited to provide control samples and for investigations on the effect of LPS instillation on vitronectin levels in the lung. All BALF samples were collected using the same protocol (washing with 3x50ml phosphate-buffered saline) to allow for adequate comparisons between the groups.

Vitronectin concentrations were measured from centrifuged, cell free, BALF by sandwich ELISA. Concentrations were interpolated from a curve generated from known standards included in the experiment. In this case, as the BALF were scarce samples were analyzed in duplicates and not in triplicates, which is the standard procedure. We found higher concentrations of vitronectin in the samples from patients with pneumonia and from endotoxin challenged lungs than in the control material.

This host-pathogen interaction was further investigated *in vitro* by coincubating OMV from *P. aeruginosa* and NTHi with A549 epithelial cells *in vitro*. This cell line originates from a 58-year old male that suffered from lung cancer and are since 1972 routinely used for laboratory studies on respiratory epithelial cells [280]. Through measuring the vitronectin mRNA after conversion to cDNA with reverse transcription polymerase chain reaction (RT-PCR), we found that stimulated cells increased their vitronectin translation as a response to OMV from *P. aeruginosa* or

NTHi. In accordance, we found increased amounts of vitronectin on the epithelial cells surface as measured by flow cytometry.

During OMV purification, an additional step was used in this study compared to Paper I. The OMV were purified in a density gradient medium to avoid interference by other bacterial structures that potentially could cause immune responses irrelevant to the present analysis. As seen in Figure 18, this procedure efficiently isolated OMV from flagella and pili.

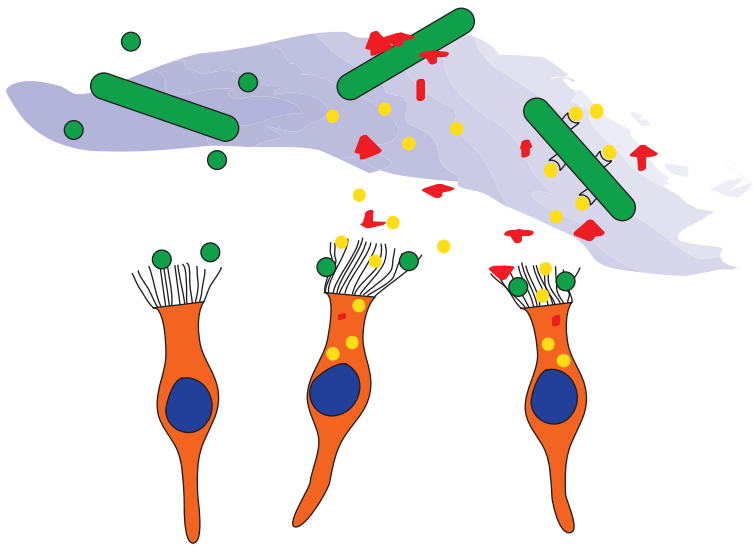

Figure 19: The interplay between bacteria and cells cause persisting infections
OMV from distant bacteria (green) stimulate respiratory epithelial cells to produce antibacterial peptides, complement (red) and regulators of complement activity (yellow). Vitronectin is a regulator of complement activity that is captured onto the bacterial surface. The cell-bound vitronectin protect the bacteria against the terminal stage of the complement cascade, the formation of a lytic membrane attack complex (MAC) and the bacteria persist in the respiratory tract.

Finally, bacteria were incubated in the clinical BALF samples from patients with pneumonia. Using flow cytometry, we found that both *P. aeruginosa* and *H. influenzae* captured vitronectin to the bacterial surface. The bacteria were challenged with human serum to determine sensitivity to complement-mediated killing. By pre-incubating bacteria in BALF or BALF that had been depleted of vitronectin, we could show that the vitronectin in BALF was biologically active and protected the bacterial cells against complement-mediated killing.

Discussion and future perspectives

This study shows that vitronectin is an acute phase protein in the lung during pneumonia and that epithelial cells are an inducible local source of vitronectin. Vitronectin concentrations were elevated in patients with pneumonia of various

etiology and this could be reproduced by instilling endotoxin in healthy lungs as well as by stimulating cell cultures with OMV. The produced vitronectin was subsequently exploited by the pathogen to evade clearing by complement (Figure 19).

The number of clinical samples used in this study is relatively few (n=8) and the infectious agents were diverse, including fungi. These are not a representative sample reflecting the most common agents causing CAP or HAP. Inclusion of more patients would be preferred, but an obstacle is that relatively few patients with pneumonia are routinely examined by bronchoscopy and hence the patient recruitment is slow.

Different bacterial products were used in the challenge of human lungs and cell cultures. The endotoxin originated from a large pool of standardized endotoxins that has been well characterized. It is however not pure LPS. Although mainly LPS, it also contains OMPs, lipoproteins, DNA and peptidoglycans and hence well reflect the composition of OMV [284].

Epithelial cells are not the sole source of vitronectin in the lung. Although epithelial cells are the most common cells in the lower respiratory tract, alveolar macrophages are also common and another potential source of vitronectin. Isolation of these cells would allow for *in vitro* analysis of their relative importance. Another potential source of vitronectin in the airway is plasma, as plasma is exudated to the respiratory tract as a response to inflammation. Both of these potential sources could be further explored in future studies.

The results from this study implies that the interaction between bacterial surface structures and epithelial cells result in increased vitronectin levels and that this protein is subsequently used by the pathogens for increased virulence. The results also imply that this is a clinically and biologically significant interaction and not merely a phenomenon seen *in vitro*.

A possible future application of the described mechanism is to block the bacterial binding of vitronectin with an inhibiting molecule. This would likely render the bacteria more sensitive to the effects of the complement system and less likely to cause long term persistent infections.

Paper IV: Antimicrobial combination treatment including ciprofloxacin decreased the mortality rate of *Pseudomonas aeruginosa* bacteraemia: a retrospective cohort study.

Introduction and aims

The fourth paper in this thesis is an observational epidemiological study based on medical record review. It was well known that bacteraemic *Pseudomonas* infections primarily affect individuals with multiple comorbidities, of high age or with some

specific diseases such as CF. Some uncertainty remains concerning the optimal treatment of invasive disease. Conflicting reports were available on the importance of the adequate empirical treatment at admission and, although commonly practised in the clinical setting, a meta-analysis had introduced doubt about using a combination of more than one antimicrobial drug [221,256,281]. The primary objective of this study was to report the effect of antimicrobial treatment choices on the outcome of *P. aeruginosa* bacteraemia, unadjusted as well as adjusted for potential confounders.

Methods and results

We identified all incidents (*n*=292) of *P. aeruginosa* growing in clinical blood cultures during an 8-year period in a database search at the local clinical microbiological laboratory. Detailed population data allowed for calculation of incidence rates that could be stratified according to gender and age. The overall annual incidence rate was 8 / 100,000 inhabitants, but for males aged over 80 years, the incidence rate exceeded 50 / 100,000 inhabitants.

Medical records were available for 235 patients, which enabled collecting of baseline demographics and comorbidities. Complete treatment medication charts were available in 219 patients. The Charlson Comorbidity Index was calculated from comorbidities and age. This index was originally developed to predict one-year mortality based on selected underlying conditions [282]. The variables were correlated to 30-day mortality with and without correction for other variables in a multivariate regression analysis model to calculate the odds ratio (OR) and *p*-values. As in the other papers of this thesis, 95% confidence intervals (CI) of the OR not including 1 or *p*-values at or below 0.05 were considered significant.

As expected, high age, multiple comorbidities, advanced malignancies with metastasis and haematological disease correlated to 30-day mortality. The same was seen for polymicrobial infections and an infectious origin in the lung. These observations confirm previous findings.

On initial presentation at the emergency ward, it is very difficult to predict if a patient suffers from *P. aeruginosa* bacteraemia and start antipseudomonal treatment. In most cases, the relevant clinical treatment question is what antimicrobial therapy to use when cultures indicate growth of *P. aeruginosa.* Hence, empirical treatment and definitive treatment were analyzed separately.

If an adequate empirical antipseudomonal drug was introduced when the cultures were sampled, the mortality was significantly lower (OR 0.37, 95% CI 0.16–0.89). Hence, although very difficult, the clinician should at least attempt to evaluate risk markers for *P. aeruginosa* such as multiple comorbidities, previous *Pseudomonal* growth, previous antibiotic therapy, and recent hospitalization.

Intuitively, administration of adequate definitive antipseudomonal therapy when cultures indicated *P. aeruginosa* bacteraemia correlated to lower mortality. Likewise, patients that received a combination therapy including ciprofloxacin had lower mortality compared with other patients (OR 0.16, 95% CI 0.05–0.55). As ciprofloxacin is the only available oral antipseudomonal drug, we suspected that there were uncorrected confounding factors affecting the results. To account for covariates that predicted receiving ciprofloxacin, a propensity model was created. We introduced all relevant covariates into the multivariate analysis model, and the group receiving ciprofloxacin was compared to those not receiving the drug. However, no difference after adjusting for the propensity to be treated with ciprofloxacin could be found.

Discussion and future perspectives

In this study, we were able to show that both the empirical treatment and the definitive treatment against *P. aeruginosa* were of importance. The two most widely used additions in a combination regimen were an aminoglycoside or a fluoroquinolone (usually ciprofloxacin). Most previous studies have compared monotherapy to combination therapy in general and not found any benefit of combination. Definitive combination treatment including ciprofloxacin was associated with lower mortality. No support for the addition of aminoglycosides was found in the present study. Although not entirely in line with previous reports, these results are not contradictory. For instance, the first choice treatment recommendation from the Infectious Diseases Society of America and the American Thoracic Society against *P. aeruginosa* pneumonia is a antipseudomonal beta-lactam plus a fluoroquinolone [283].

Lost or unavailable medical records caused a noteworthy loss of data. By comparing age and sex for missing and non-missing files, we could not find any bias in the missing files and they were treated as missing at random. Future observational studies will be less prone to such loss as paper-based medical records are presently rare.

A secondary objective of this study was to establish a dataset with corresponding bacterial isolates. Through bacterial genomic and transcriptomic analysis, *in silico* analysis of virulence genes can be performed and correlated to disease severity and mortality. This approach could potentially identify important virulence genes that subsequently can be explored using molecular microbiological techniques.

Ethical considerations

Before any of the studies included in this thesis were initiated, the ethical implications were considered. In all cases were any personal data were collected, the data was made anonymous before analysis and publication.

Papers I and II did not include any personal data, but since rabbits were immunized for antibody production, permit was applied for and given by the Swedish Board of Agriculture.

Paper III required extensive considerations and three separate ethical applications were filed, to the regional committees in Lund, Gothenburg, and Stockholm. Moreover, as the collected samples were stored, a biobank permit was applied for. Volunteers for LPS instillation were recruited through advertising and informed consent was obtained at a separate visit to the clinic. The volunteers were informed about possible side effects caused by pulmonary endotoxin instillations, although several previous studies had successfully used this preparation of a highly standardized endotoxin batch from *E. coli*.

To obtain clinical patient samples as well as to collect other personal data from the hospital records, each patient was required to be informed and give permission to be included in the study. As some of the included patients were expected to be under general anaesthesia or be unable to give informed consent because of the medical condition, these patients had to be informed and give consent as soon as their condition had improved.

The retrospective collection of personal data for Paper IV required ethical permission from the regional ethics committee. After the application, the committee reviewed the aim of the study and the potential harm for study individuals. The potential harm was judged to be limited and permission from each study subject was not required. Instead, the general public was notified about the study through a notice that was published in local newspapers.

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Outer membrane vesicles shield *Moraxella catarrhalis* β-lactamase from neutralization by serum IgG

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Objectives: The aim of this study was to detect the presence of IgG against *Moraxella catarrhalis* β-lactamase in healthy adults, and to determine whether outer membrane vesicles (OMVs) could protect the enzyme from inhibition by anti-β-lactamase IgG.

Methods: Transmission electron microscopy was used to detect the presence of β-lactamase in OMVs. Sera were examined by ELISA for specific IgG directed against recombinant *M. catarrhalis* β-lactamase in addition to the outer membrane adhesins MID/Hag, UspA1 and A2. Binding of anti-β-lactamase IgG from serum to OMVs was analysed by flow cytometry. The chromogenic substrate nitrocefin was used to quantify β-lactamase enzyme activity.

Results: The presence of β-lactamase was determined in OMVs from a 9-year-old child suffering from *M. catarrhalis* sinusitis. Furthermore, anti-β-lactamase IgG was detected in sera obtained from healthy adults. Out of 40 adult blood donors (aged 18–65 years) tested, 6 (15.0%) carried anti-β-lactamase IgG. No correlation between IgG titres against β-lactamase and the adhesins was found. Flow cytometry analyses revealed that anti-β-lactamase IgG from serum bound to β-lactamase-positive OMVs. By comparing the β-lactamase activity of intact OMV with OMV that were permeabilized with saponin we found that OMVs shielded active β-lactamase from the anti-β-lactamase IgG.

Conclusions: *Moraxella catarrhalis* β-lactamase is found in, or associated with, OMVs, providing clinical relevance for the vesicles in the spread of antibiotic resistance. Furthermore, OMVs protect β-lactamase from specific IgG.

Keywords: amoxicillin, antibiotic resistance, sinusitis

Introduction

Moraxella catarrhalis is a Gram-negative human pathogen that causes respiratory tract infections. The species is found as a commensal in pre-school children and causes ~15%–20% of acute otitis media (AOM) cases. *M. catarrhalis* is also the third most common cause of exacerbations in patients with chronic obstructive pulmonary disease (COPD) after *Streptococcus pneumoniae* and *Haemophilus influenzae*.[1] *M. catarrhalis* adheres to and infects pulmonary epithelial cells, and has been found to hide in the palatine tonsils.[2,3] Since the 1980s >97% of *M. catarrhalis* strains have been β-lactamase positive.[4]

In parallel with most other Gram-negative species, *M. catarrhalis* releases outer membrane vesicles (OMVs), which are small spheres released from the outer membrane as the membrane bulges out and pinches off. The OMVs thus reflect the composition of the outer membrane, carrying mainly lipids and outer membrane proteins.[5–8] As the release of OMVs is an energy-demanding process, it has been suggested that this is an essential virulence mechanism for Gram-negative bacteria. Evidence from various studies supports this notion, as OMVs not only have a role in pathogenesis, but also in biofilm formation, nutrient acquisition and horizontal gene transfer.[7,9–11] OMVs interact both with host cells and with other bacteria residing in a mutual niche. Moreover, OMVs act as vehicles for secretion whereby vesicle components are protected from destruction and delivered to target cells at a distance.[5,8,12]

We recently showed that OMVs from β-lactamase-positive *M. catarrhalis* also contain the enzyme and consequently can absorb and hydrolyse amoxicillin.[13] β-Lactamase is known as a

periplasmic enzyme, and therefore its storage in OMVs and subsequent secretion would potentially be an important virulence mechanism.[14] Since *M. catarrhalis* is often found with other bacterial species,[15] we hypothesized that conferring antibiotic resistance on susceptible bacteria might make co-infection a highly advantageous mechanism also for other species. Intriguingly, we found that β-lactamase-positive *M. catarrhalis* OMVs confer resistance on amoxicillin-susceptible *H. influenzae* and *S. pneumoniae* in addition to the now rare β-lactamase-negative *M. catarrhalis*.[13]

The antibody levels against major *Moraxella* outer membrane proteins, such as *Moraxella* IgD-binding protein (MID)/haemagglutinin (Hag) and ubiquitous surface proteins (Usp) A1 and UspA2, have previously been studied in both adults and children.[16–18] IgG levels are generally lower in children compared with adults, leading to a higher incidence of infections in children. However, the human antibody response to *M. catarrhalis* β-lactamase is currently unknown. In this study, we determined IgG levels against *Moraxella* β-lactamase in healthy adults using a recombinant protein. A group of individuals that had significant anti-β-lactamase IgG titres was identified, and this polyclonal antibody (pAb) recognized β-lactamase-positive OMVs. Interestingly, we discovered that the hydrolysing β-lactamase activity was partially protected within the OMV. This suggests that *Moraxella* OMVs not only play a role in polymicrobial infections but also act as protective reservoirs for β-lactamase,[13] avoiding neutralization by the host adaptive immune system.

Materials and methods

Bacterial strains and growth conditions

M. catarrhalis clinical and reference strains KR526 and Bc5, respectively, were cultured on chocolate agar plates. Bacteria were grown at 37°C in 5% CO_2. To determine amoxicillin MICs, both Etests (Biodisk, Solna, Sweden) and colony counting after growth in liquid media with varying antibiotic concentrations were used.

Production of recombinant β-lactamase, UspA1, A2 and MID

The manufacture of full-length recombinant β-lactamase$^{26-318}$ from *M. catarrhalis* strain RH4 was done as described previously.[13] Briefly, the β-lactamase *bro* gene was cloned into the vector pET26b(+) and, after selection in *Escherichia coli* DH5α, the protein was produced in *E. coli* BL21(DE3) by induction with IPTG. Bacteria were sonicated and proteins were purified using affinity chromatography. Recombinant full-length UspA1$^{50-770}$ and UspA2$^{30-539}$ in addition to the truncated protein MID$^{962-1200}$, which was selected due to a size approximately similar to that of the β-lactamase$^{26-318}$, were all from *M. catarrhalis* Bc5. Recombinant proteins were produced as previously described.[17]

Isolation of M. catarrhalis OMVs

OMVs were isolated according to the method described by Rosen *et al.*[19] Briefly, bacteria were grown in brain heart infusion broth overnight at 37°C with shaking, and after centrifugation the resulting supernatant was filtered through 0.2 μm pore filters (Sartorius, Goettingen, Germany) to obtain a cell-free solution. The flow-through was filtered with 100 kDa Vivaspin centrifugal concentrators (Vivascience, Hannover, Germany). The remaining concentrate was further concentrated by ultracentrifugation at 100000 **g** and washed with PBS followed by centrifugation. The protein concentration was measured using NanoDrop (NanoDrop Technologies, Wilmington, DE), and plated on chocolate agar plates in order to confirm that the preparations were free from bacteria.

Transmission electron microscopy (TEM)

A fresh nasal discharge from a 9-year-old child with *M. catarrhalis* sinusitis (pure growth of *M. catarrhalis* from a nasal aspirate) was examined. The sample was prepared by suspending a drop of the purulent nasal discharge in 1 mL of PBS with 4% paraformaldehyde. The cellular fraction was obtained by centrifuging the specimen at 14000 rpm. Following fixation of samples, ultrathin sections of specimens were mounted on gold grids and subjected to antigen retrieval with metaperiodate. The grids were floated on drops of immune reagents displayed on Parafilm, and 50 mM glycine was used to block free aldehyde groups. Grids were subsequently blocked with 5% (vol/vol) goat serum diluted in incubation buffer (0.2% BSA-C™ in PBS, pH 7.6) (Aurion, Wageningen, Netherlands) for 15 min. OMVs were incubated with primary antibodies (dilution 1:50 and 1:100) overnight at 4°C. The grids were washed in incubation buffer and floated on drops of gold conjugate reagents of sizes 10 and 5 nm, diluted 1:10 to 1:20 in incubation buffer, for 1 h at room temperature. After washes in incubation buffer, the sections were fixed in 2% glutaraldehyde. The sections were then washed in distilled water and post-stained with uranyl acetate and lead citrate. Sections were examined with an election microscope (JEM 1230; Jeol, Tokyo, Japan) operated at a 60 kV accelerating voltage. The images were recorded with a Gatan Multiscan 791 charge-coupled device camera (Gatan, Pleasanton, CA).

ELISA

To analyse antibody concentrations in serum, ELISA was done as described previously.[17] Briefly, 96-well plates (Nunc, Roskilde, Denmark) were coated with 1 μg recombinant protein (UspA1/A2, MID or β-lactamase) per well in Tris–HCl buffer (pH 9.0) overnight at 4°C. After washing and blocking steps, human sera from healthy blood donors ($n=40$; aged 18–65 years) were added in duplicate for 1 h at room temperature. Horseradish peroxidase (HRP)-labelled anti-human IgG polyclonal antibody (pAb) (1:6000) (Dako, Glostrup, Denmark) was added as a secondary layer to plates for 20 min, and after subsequent washing steps the plates were developed and measured at OD_{450}. Each sample was tested in duplicate.

Purification of human anti-β-lactamase IgG

Human sera were purified against a recombinant β-lactamase from *M. catarrhalis* strain RH4 on a CnBr–Sepharose coupled column (VWR International, Leicestershire, UK) as previously described.[13] Sera were diluted 1:5 in PBS. The β-lactamase binding fraction was eluted with 0.1 M glycine (pH 2.4) and immediately mixed with 3 M Tris–HCl (pH 8.8) and 5 M NaCl. The flow-through after purification was used as a negative control serum devoid of specific anti-β-lactamase IgG. Both fractions were absorbed against β-lactamase-negative *M. catarrhalis* strain Bc5 for 1 h at room temperature in order to remove non-specific antibodies.

Flow cytometry analysis

To analyse the recognition by IgG of β-lactamase-positive OMVs from *M. catarrhalis*, OMVs (2 μg) were fixed with 3.5% formaldehyde for 15 min at room temperature. After a subsequent wash (100000 **g** for 30 min), the OMVs were incubated with purified anti-β-lactamase IgG. After another washing step, FITC-labelled rabbit anti-human pAb

(Dako) was added as a secondary step in PBS/BSA (1%) for 20 min at room temperature. Samples were analysed in an EPICS XL-MCL flow cytometer (Beckman Coulter, Hialeah, FL) and a gate was set to exclude signals ≤2.0%.

SDS-PAGE and western blotting

To check the binding of purified anti-β-lactamase IgG to the recombinant RH4 β-lactamase, SDS–PAGE (12%) and western blotting were performed. Proteins were transferred from gels to Immobilon-P membranes (Millipore, Bedford, MA) at 20 V overnight, and following transfer the membranes were blocked with 5% milk in PBS containing 0.1% Tween (PBS-Tween). After subsequent washing with PBS-Tween, the membranes were incubated with anti-β-lactamase pAb purified from normal human or rabbit serum for 1 h at room temperature as described previously.[17] After several washing steps, membranes were incubated for 1 h with HRP-conjugated secondary rabbit anti-human or swine anti-rabbit pAb (Dako) respectively, which were diluted 1:1000. Membranes were washed and developed using enhanced chemiluminescence western blot detection reagents (Amersham Pharmacia Biotech, Uppsala, Sweden).

Determination of the inhibitory effect of serum anti-β-lactamase antibodies blocking β-lactamase activity in *M. catarrhalis* OMV

The enzyme activity in OMVs was quantified with a nitrocefin assay as previously described.[13] The chromogenic cephalosporin nitrocefin (Oxoid, Thermo Scientific, Cambridge, UK) was used. Briefly, OMVs (0.3 μg/mL) were pre-incubated with saponin (0.2%) for 5 min at room temperature to lyse them,[20] and incubated with purified anti-β-lactamase pAb isolated from normal human serum or the flow-through control serum (dilution 1:10) for 1 h at room temperature. OMVs were incubated with nitrocefin (500 μg/mL) for 30 min at 37°C in the dark, followed by centrifugation at 13 000 **g** for 3 min. After chromogenic hydrolysis the subsequent colour change was determined using NanoDrop at OD_{485}. The enzyme activity of the OMV preparations was estimated using a standardized curve from a recombinant β-lactamase (VWR International, Leicestershire, UK). The activity was quantified as the number of moles of nitrocefin hydrolysed per minute per milligram of protein.

Statistical analysis

The statistical analyses were performed with GraphPad Prism 5 software (San Diego, CA). The unpaired Student's *t*-test was used to determine the statistical differences between control and treated samples. All data are expressed as the mean ± SEM, and *n* represents the number of experiments performed.

Results

Outer membrane vesicles from *M. catarrhalis* carry β-lactamase in vivo

We have recently shown that *Moraxella* releases OMVs that are loaded with β-lactamase.[13] To determine whether β-lactamase is also associated with OMVs *in vivo*, a specimen obtained via a nasopharyngeal swab from a 9-year-old child with *Moraxella* sinusitis was analysed by TEM. As can be seen in Figure 1(a), *M. catarrhalis* readily released OMVs in the nasopharynx.

To manufacture specific detection antibodies for *M. catarrhalis* β-lactamase, we produced a full-length recombinant β-lactamase$^{26-318}$ in *E. coli* followed by immunization of rabbits. The resulting anti-β-lactamase pAb was conjugated with gold granules and used with our clinical samples for detection in TEM (Figure 1a). The presence of β-lactamase was clearly seen in OMVs or in the close vicinity as seen in several sections (Figure 1b–e).

Sera obtained from healthy adults contain IgG directed against *M. catarrhalis* β-lactamase

In order to determine whether adults carry IgG against β-lactamase, sera were collected from 40 healthy individuals (aged 18–65 years). To quantify the IgG concentration, recombinant β-lactamase$^{26-318}$ was immobilized on microtitre plates followed by ELISA (Figure 2a). In addition, the well-defined adhesins MID$^{962-1200}$, UspA1$^{50-770}$ and UspA2$^{30-539}$ were included for comparison. Both UspA1$^{50-770}$ and UspA2$^{30-539}$ are full-length recombinant proteins, whereas MID$^{962-1200}$ is a 238 amino acid truncated fragment of the native MID protein (mol. wt ∼200 kDa). The IgG antibody titres against β-lactamase$^{26-318}$ showed large variation between individuals, and titres were found to be significantly lower than the average anti-UspA1/A2 IgG titres, but higher than the mean anti-MID$^{962-1200}$ IgG titres. The high antibody titres for UspA1 and A2 were in agreement with results previously obtained in our laboratory.[17] Finally, sera from the healthy donors in general contained higher mean antibody levels against UspA1 compared with UspA2 (1.5-fold difference).

To confirm the specificity of anti-β-lactamase IgG, binding of antibodies against our recombinant β-lactamase$^{26-318}$ was analysed by ELISA. An example can be seen in Figure 2(b), where we show a serum that bound to β-lactamase$^{26-318}$ in a dose-dependent manner when compared with a non-binding serum. Intriguingly, out of sera from 40 patients, only 6 (15.0%) were detected positive for anti-β-lactamase IgG as verified by the binding specificity curve of the antibiotics. When the human sera were compared in detail, we found that sera with either high or low titres of antibodies against β-lactamase ($n=3$ of each) had equal levels of IgG against MID and UspA1/A2 in both groups (Figure 2c). In conclusion, no correlation was found between anti-β-lactamase and anti-UspA1/A2 IgG antibody titres.

OMVs protect β-lactamase from specific anti-β-lactamase IgG

Since we have previously found that β-lactamase is associated with OMVs,[13] we wanted to investigate whether human anti-β-lactamase IgG could inhibit the enzyme activity. Specific antibodies were affinity purified on a Sepharose column containing recombinant β-lactamase$^{26-318}$. The resulting anti-β-lactamase$^{26-318}$ IgG efficiently detected recombinant β-lactamase and was comparable to the rabbit anti-β-lactamase pAb, as shown by western blotting (Figure 3a).

To analyse whether the purified human IgG has the capacity to recognize β-lactamase-loaded OMVs, we included flow cytometry in our analysis. Anti-β-lactamase IgG significantly

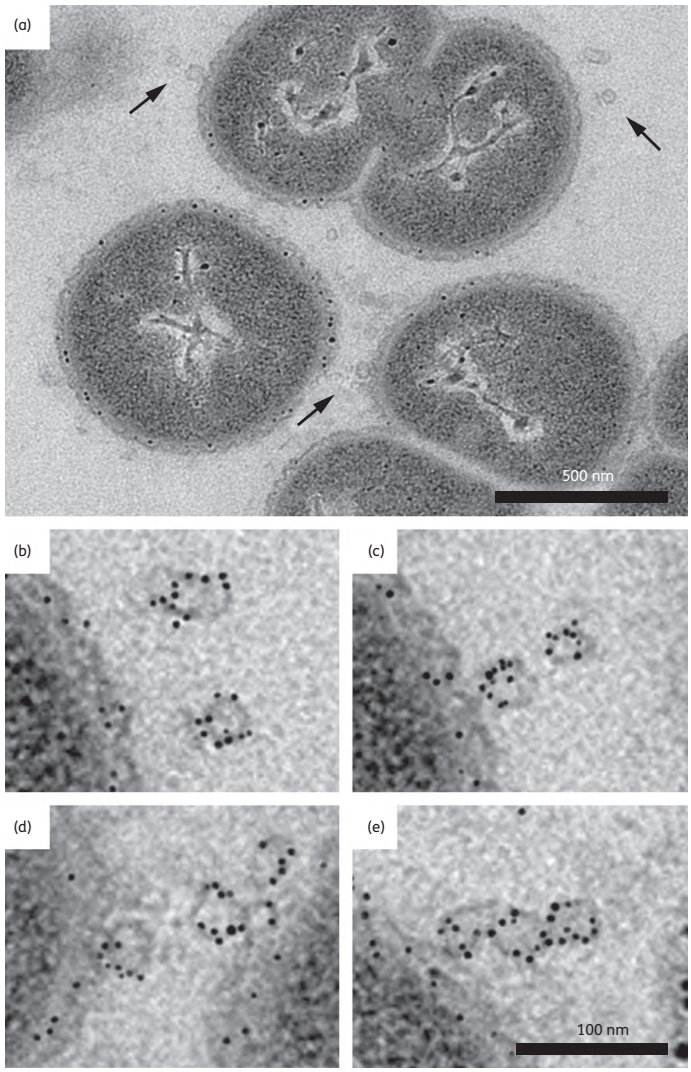

Figure 1. OMVs from *M. catarrhalis* contain β-lactamase *in vivo*. (a) Arrows show vesicles secreted from β-lactamase-positive *Moraxella* in a 9-year-old patient. A sample taken with a nasopharyngeal swab was analysed by TEM. The *M. catarrhalis* produced β-lactamase as confirmed by gold-labelled pAb in TEM. (b–e) The presence of β-lactamase inside or close to OMVs could clearly be seen in several TEM sections.

detected OMVs isolated from the β-lactamase-positive *M. catarrhalis* KR526 (Figure 3b). A 5.6-fold increase in mean fluorescence intensity was observed with strain KR526 OMVs compared with the control with the FITC-conjugated secondary detection antibody only (Figure 3c). In contrast, no binding was seen with the β-lactamase-negative strain Bc5.

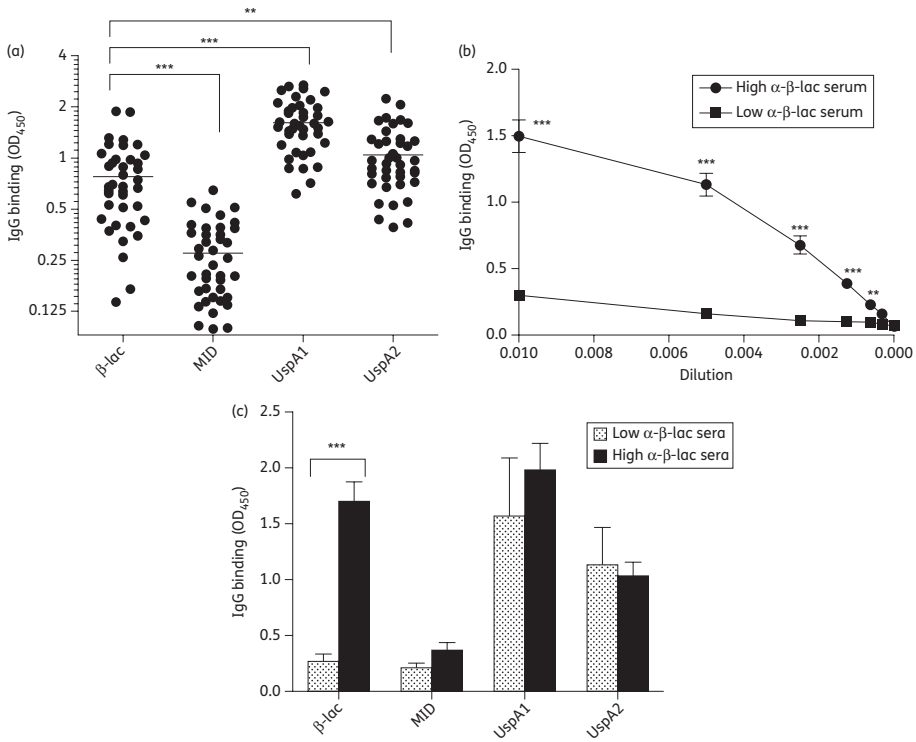

Figure 2. A small proportion of healthy adults have IgG antibodies directed against *M. catarrhalis* β-lactamase and MID compared with UspA1/A2. (a) Concentrations of IgG against β-lactamase$^{26-318}$, UspA1$^{50-770}$, UspA2$^{30-539}$ and MID$^{962-1200}$ in healthy adults were quantified by ELISA. (b) The specificity of a β-lactamase$^{26-318}$ binding serum (High α-β-lac) was compared with that of a non-binding (Low α-β-lac) serum in ELISA. (c) The anti-β-lactamase$^{26-318}$, anti-UspA1$^{50-770}$, anti-UspA2$^{30-539}$ and anti-MID$^{962-1200}$ IgGs in the weakest β-lactamase-binding sera ($n=3$) were compared with those in the highest β-lactamase binding sera ($n=3$). Human sera were diluted 1:200. Binding was measured as a function of absorbance at 450 nm. Horizontal bars in (a) represent mean values. In (b) mean values and SEM are shown. All results represent triplicate values from two separate experiments. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

To investigate whether the anti-β-lactamase antibodies inhibit the enzymatic activity of β-lactamase, OMVs were incubated with purified anti-β-lactamase IgG for 1 h to allow binding. Thereafter, β-lactamase activity was measured using the chromogenic substrate nitrocefin. When OMVs were incubated with anti-β-lactamase IgG, the enzyme activity decreased by 60.8%±19.6%, confirming that antibodies partially inhibited β-lactamase activity in the OMVs (Figure 3d). However, since there was still significant enzymatic activity in the OMV preparation, vesicles were treated with saponin in order to lyse them. Interestingly, this opening of the vesicles increased the enzymatic activity of β-lactamase-positive OMVs by 32.8%±16.8%, albeit not statistically significant, it suggests the presence of β-lactamase inside OMVs. The inhibitory effect by the anti-β-lactamase IgG was more prominent, resulting in a decrease of enzymatic activity by 94.6%±3.0%. Taken together, our results suggest that β-lactamase was located both inside and on the surface of the OMVs.

Discussion

OMVs were first observed in the 1970s,[21] and since then increasing evidence has emerged showing that these small spheres play an important role in both bacterial survival and pathogenesis.[6] In this study we show that OMVs not only act as protective vesicles, whereby proteins can be delivered in complex with other cellular material, but are also important factors in the interplay between bacteria and the host's humoral immunity.

We have previously demonstrated the presence of β-lactamase in *M. catarrhalis* OMVs, and showed that these

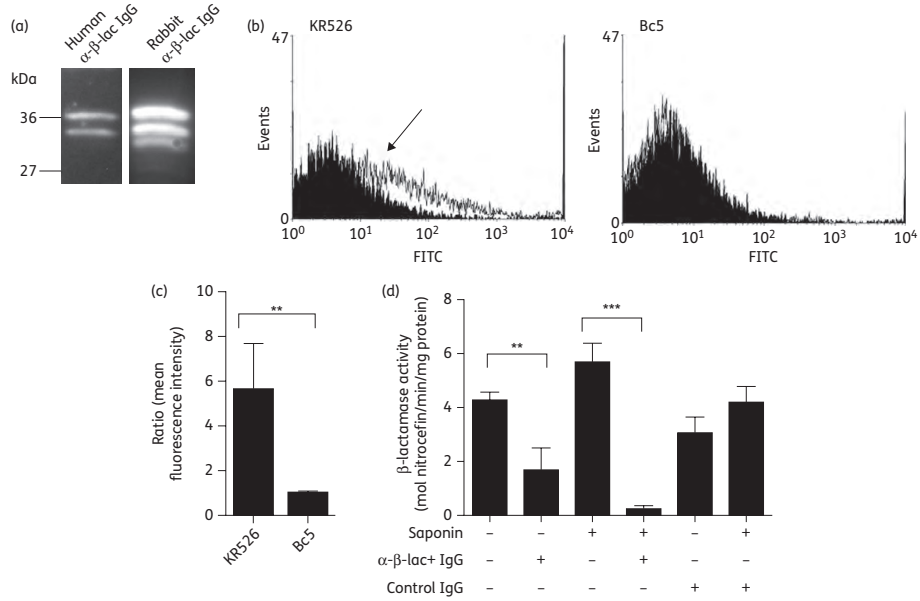

Figure 3. Purified anti-β-lactamase IgG from human serum does not inhibit β-lactamase enzymatic activity inside OMVs. (a) Western blotting showed that purified human anti-β-lactamase IgG detected recombinant β-lactamase (35 kDa). Recombinant RH4 β-lactamase$^{26-318}$ (1 μg) was run in each lane. Rabbit anti-β-lactamase IgG was used as a positive control. (b) Flow cytometry analysis confirmed that purified anti-β-lactamase bound OMVs from the β-lactamase-positive *M. catarrhalis* KR526, illustrated by a positive shift (arrow), but not the β-lactamase-negative strain Bc5. OMVs (2 μg) without IgG were compared with OMVs incubated with purified anti-β-lactamase IgG (arrow). (c) Bar graph representing the ratio of mean fluorescence intensity between control and IgG-treated KR526 and Bc5 OMVs. Flow cytometry results represent three independent experiments. (d) A nitrocefin assay determined that β-lactamase enzyme activity of OMVs from KR526 was inhibited when OMVs were lysed with saponin (0.2%) and incubated with anti-β-lactamase IgG purified from human serum. In (d), 250 μg/mL OMVs was used and serum was diluted 1:100. β-Lactamase activity was quantified as a function of the change in absorbance from OD_{380} to OD_{485} as determined by spectrophotometry. Data in (d) are mean and standard error of the mean (SEM) of at least three independent experiments. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

OMVs confer antibiotic resistance on amoxicillin-susceptible *M. catarrhalis*, *S. pneumoniae* and *H. influenzae*. The last two species are important pathogens causing AOM and exacerbations in COPD patients. *M. catarrhalis* is often isolated as a co-pathogen in infections with *S. pneumoniae* and *H. influenzae*. We suggest that *Moraxella* has an important role in protecting its co-pathogens in the upper respiratory tract, by helping them resist antibiotic treatment. Interestingly, electron microscopy analysis of a nasopharyngeal sample from a child with sinusitis caused by *M. catarrhalis* revealed the enzyme β-lactamase. This further proves that β-lactamase is also found in or associated with OMVs *in vivo*, providing a clinical relevance for the vesicles in the spread of antibiotic resistance.

We also show that there is an immunological response against β-lactamase, as revealed by serum analysis. When IgG levels in sera obtained from healthy adults were compared, it was found that 15% carried anti-β-lactamase IgG. We analysed specific anti-β-lactamase IgG levels using recombinant β-lactamase produced in *E. coli*, and observed higher antibody titres against β-lactamase compared with titres against MID. In contrast, significantly lower antibody titres existed against β-lactamase when compared with anti-UspA1/A2 IgG titres. This suggests that UspA1, in contrast to MID, contains immunogenic epitope(s), generating a strong serological response. It has to be kept in mind, however, that the truncated MID$^{962-1200}$ is considerably shorter than the UspAs and represents only a small portion of the large MID molecule. Nevertheless, it seems that β-lactamase is significantly less immunogenic compared with UspA1/A2. To our knowledge, this is the first report on antibodies against *M. catarrhalis* β-lactamase in serum. Taking into consideration that *M. catarrhalis* β-lactamase is a unique enzyme that is highly conserved within the species,[22] we assume that these antibodies were specific for *Moraxella* β-lactamase.

In a paper by Giwercman *et al.*,[23] the levels of β-lactamase in cystic fibrosis (CF) patients with *Pseudomonas aeruginosa* infections were analysed. These authors found that the β-lactamase activity in sputum was high in CF patients and that levels significantly increased in patients treated with certain antibiotics. In a more recent paper the presence of IgG against chromosomal β-lactamase in serum and sputum samples of CF patients was demonstrated.[24] In healthy controls, no anti-β-lactamase IgG was detected. The authors speculated that antibodies could potentially increase the efficiency of treatment with β-lactam antibiotics by inhibiting the β-lactamase present in serum, and thereby contributing some degree of protection against infection.

In our study, anti-β-lactamase IgG from sera obtained from healthy adults was found to bind to OMVs carrying β-lactamase. However, significant enzyme activity still remained in vesicles after incubation with antibodies, indicating that OMVs protected the enzyme from inhibition. In the light of the observations made by Giwercman *et al.*,[23] this could give the bacterium an advantage against removal of the serum anti-β-lactamase IgG. Since we have recently shown that vesicles confer antibiotic resistance in cultures with other bacterial species, it is interesting to speculate that protection from antibody neutralization of β-lactamase inside OMV might to some level also play a role in the interplay with other bacteria. However, considering that ∼97% of *M. catarrhalis* strains are β-lactamase positive, it is worth noting that only 15% of healthy adults carry β-lactamase antibodies. Since OMVs function as a storage pool for β-lactamase, it is suggested that proteins located inside the OMV perhaps are not properly taken care of by the adaptive immune system. Although speculative, this perhaps may result in less efficient antibody production against β-lactamase compared with the adhesins UspA1 and A2.

M. catarrhalis was considered a harmless commensal for a long time. Although invasive disease may occur, evidence shows that *Moraxella* is mainly involved in AOM as well as in exacerbations in COPD patients, rendering it a genuine respiratory pathogen.[25,26] With the introduction of a pneumococcal conjugate vaccine the relative frequency of isolating *Moraxella* as a pathogen has also increased, suggesting a shift in the bacterial niche.[1,18] We suggest that another important role for *Moraxella* is, by means of OMVs, to provide an advantageous environment for other more severe disease-causing bacteria. Our results clearly show that specific IgG exists against β-lactamase in healthy donors, but also that OMVs protect the periplasmic β-lactamase residing inside OMVs from inhibition by antibodies.

Bacterial resistance against antimicrobial agents is an emerging problem, and it is of the greatest importance to take into account all aspects of the field. The present study may be an impetus for further studies on the role of OMVs in infections caused by antibiotic-resistant bacteria. It is highly relevant to examine in detail these mechanisms considering the long-term goal of finding new approaches to antimicrobial therapy.

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Transparency declarations

None to declare.

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Paper II

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Original Article

Identification of outer membrane Porin D as a vitronectin-binding factor in cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*

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Abstract

Background: Pseudomonas aeruginosa is a pathogen that frequently colonizes patients with cystic fibrosis (CF) or chronic obstructive pulmonary disease (COPD). Several pathogens are known to bind vitronectin to increase their virulence. Vitronectin has been shown to enhance *P. aeruginosa* adhesion to host epithelial cells.
Methods: We screened clinical isolates from the airways of CF patients and from the bloodstream of patients with bacteremia for binding of vitronectin. Two-dimensional SDS-PAGE and a proteomic approach were used to identify vitronectin-receptors in *P. aeruginosa*.
Results: P. aeruginosa from the airways of CF patients ($n = 27$) bound more vitronectin than bacteremic isolates ($n = 15$, $p = 0.025$). Porin D (OprD) was identified as a vitronectin-binding protein. A *P. aeruginosa oprD* transposon insertion mutant had a decreased binding to soluble and immobilized vitronectin ($p \leq 0.001$).
Conclusions: P. aeruginosa isolates obtained from CF patients significantly bound vitronectin. Porin D was defined as a novel *P. aeruginosa* vitronectin-receptor, and we postulate that the Porin D-dependent interaction with vitronectin may be important for colonization.

Keywords: Adhesion molecules; Extracellular matrix; Porin D; *Pseudomonas aeruginosa*; Vitronectin

1. Introduction

The Gram-negative opportunistic pathogen *Pseudomonas aeruginosa* is responsible for a substantial burden of disease and mortality, particularly for patients with other underlying diseases. *P. aeruginosa* causes various infections in humans, notably pulmonary infections, keratitis, wound infections and septicemia [1]. It has been shown to be the main pathogen causing increased morbidity and mortality in patients with cystic fibrosis (CF) [2]. *P. aeruginosa* is also associated with a high morbidity in patients with advanced stage of chronic obstructive pulmonary disease (COPD) [3], and is reported as the most common bacterial species isolated during exacerbations [4]. In parallel, *P. aeruginosa* is often found as an infectious agent in patients suffering from ventilator associated pneumonia (VAP) [5].

Though *P. aeruginosa* primarily exists in mucus plugs and sputum plaques during long term colonization of CF patients, it is known to adhere to epithelial cells, exposed basement membrane or proteins of the extracellular matrix (ECM) [6]. For both

Abbreviations: aa, amino acid; BSA, bovine serum albumin; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; CPM, counts per minute; DBA, direct binding assay; ECM, extracellular matrix; FCS, fetal calf serum; HBD, heparin binding domain; IPTG, isopropyl-1-thio-β-D-galactoside; LB, lysogeny broth; mAb, monoclonal antibodies; NHS, normal human serum; OMP, outer membrane protein; pAb, polyclonal antibodies; PBS, phosphate buffered saline; PBST, PBS with Tween; SEM, standard error of the mean; VAP, ventilator associated pneumonia; WT, wild type bacterium.

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P. aeruginosa and *P. fluorescence* it has been suggested that the ECM protein vitronectin is important for bacterial adherence to respiratory epithelial [7,8].

Vitronectin is a plasma protein that exists naturally as a 75 kDa intact protein and a truncated 65 kDa form. It was first discovered for its ability to stick to glass surfaces where subsequently human primary cells could grow [9]. It has later been found to be associated with cell to cell adhesion, wound repair and regulation of the complement system. Furthermore, vitronectin promotes coating on polymer surfaces and it may aid to build up biofilms, as exemplified by coagulase-negative staphylococci on cerebrospinal shunts [10]. During wound healing, vitronectin facilitates mammalian cell adhesion and forms an ECM with other glycoproteins that is attached to mammalian cells through $\alpha_v\beta_5$ integrins, which are upregulated during inflammation [11]. Although vitronectin is mainly produced by hepatocytes, it is also released by respiratory epithelial cells and present in the healthy lung [12]. The vitronectin mRNA expression is upregulated in neutrophils derived from patients with CF and the concentration of vitronectin in the airway lumen is increased in patients suffering from sarcoidosis and interstitial lung disease, which may reflect the level of inflammation [13,14].

The goal of this study was to investigate whether *P. aeruginosa* has vitronectin-binding surface receptors, and to evaluate the ubiquity of this interaction by screening clinical isolates. Moreover, we also aimed to identify vitronectin-binding proteins at the surface of *P. aeruginosa*, and to characterize the nature of this interaction in detail. We identified Porin D as the main vitronectin-binding receptor, which is a previously unknown function attributed to this outer membrane protein (OMP).

2. Material and methods

2.1. Bacterial strains and culture conditions

Forty-two clinical *P. aeruginosa* isolates and the reference strain PAO1 were supplied by the Clinical Microbiology Laboratory (Malmö, Sweden) and the Department of Clinical Microbiology at Rigshopitalet (Copenhagen, Denmark) [15]. All isolates were verified as *P. aeruginosa* by using MALDI-TOF. A transposon insertion mutant was obtained from the *P. aeruginosa* two-allele library (Washington university, Seattle, WA): PW2742 oprD-E12::IS*pho*A7/hah [16]. *P. aeruginosa* was grown on blood agar plates or in liquid Lysogeny broth (LB). *Escherichia coli* DH5α and *E. coli* BL21 (DE3) were also cultured in LB medium supplemented with appropriate antibiotics (Table 1).

2.2. Vitronectin direct binding assay (DBA)

Vitronectin was labeled with [^{125}I] using the Chloramine-T method [17]. One loop of *P. aeruginosa* was taken from a glycerol stock kept at −80 °C and grown overnight on blood agar plates. Bacteria were scraped from the plates and resuspended to OD_{600} = 1.0 in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA). After washing, 15 ng of [^{125}I]-vitronectin in 100 μl PBS was added to the bacterial suspension and incubated for 1 h at 37 °C. The unbound [^{125}I]-vitronectin was removed by 2 washes with PBS. Bacterial surface-bound radioactivity was measured in a Tri-Carb liquid scintillation counter (Perkin Elmer, Waltham, MA).

Table 1
Clinical *Pseudomonas aeruginosa* isolates and laboratory strains used in the present study.

Name	Description/genotype	Reference
Clinical isolates		
PA KR794	Urine isolate	This study
PA KR796	Airway isolate from patient with CF	This study
PA KR799	Blood isolate	This study
PA KR801	Airway isolate from patient with CF	This study
Laboratory strains		
PAO1	*P. aeruginosa* reference strain	[15]
E. coli BL21(DE3)	*E. coli* laboratory strain	[19]
E. coli DH5α	*E. coli* laboratory strain	
E. coli—OprD	*E. coli* BL21(DE3) pET16b-*oprD*	This study
MPAO1	*P. aeruginosa* reference strain. Clone of PAO1 used in two allele transposon library.	[16]
PW2742 oprD-E12::IS*pho*A7/hah	MPAO1 mutant with transposon insert in *oprD*	[16]

2.3. Outer membrane protein identification with two-dimensional (2D) gel electrophoresis

Bacterial OMP fractions were prepared from overnight cultures based upon the method of Alteri and Moble and analyzed by 2D-SDS-PAGE [18].

2.4. Expression and purification of recombinant proteins

The full-length gene encoding for Porin D (*oprD*) were amplified from genomic DNA of *P. aeruginosa* PAO1 using primers 5′-CTGAGGATCCGGACGCATTCGTCAGCGATCAGGCC-3′ and 5′-CTGACAAGCTTCAGGATCGACAGCGGATAGTCGACGATCAG-3′. The amplified gene products were cloned into the expression vector pET26b (Novagen, Darmstadt, Germany), and used for protein expression and purification [19]. The *oprD* gene was also cloned into pET16b (Novagen) for expression of proteins at the surface of *E. coli* using primers 5′-TATACGCATATGAAAGTGATGAAGTGGAGCGCCATTGCA-3′ and 5′-TCAATTGGATCCTTACAGGATCGACAGCGGATAGTCGA-3′. For expression and purification, *E. coli* DE3 (Novagen) with the appropriate vector was used. Vitronectin fragments were expressed in HEK 293T cells and purified by Ni-NTA resin [18].

2.5. Antibody production

Two rabbits were immunized with 200 μg of recombinant protein emulsified in complete Freund's adjuvant (CFA; Difco and BD Biosciences, Franklin Lakes, NJ). Booster doses were injected on days 18 and 36 with the same dose of protein in incomplete FA. Blood was drawn three weeks later. Antibodies

were purified by CN-bromide agarose conjugated with OprD [20].

2.6. Enzyme-linked immunosorbent assay (ELISA)

Initial protein–protein interactions were analyzed with ELISA. Proteins (50 nM) were coated in 96-well PolySorp® plates (Thermo Fisher Scientific, Waltham, MA) in coating buffer (100 nM Tris–HCl, pH 9.0) and stored overnight at 4 °C. Thereafter a standard protocol was followed [18]. *Haemophilus influenzae* hypothetical protein UHP_03526 (GI:144986114) was included as a negative control. It is an OMP derived from non-typable *H. influenzae* 3655 and does not bind Vn (unpublished data). UHP_03526 was expressed and purified using the same method as described for Porin D and was used to exclude the possibility of unspecific binding derived from vector sequences including the Histidine-tag (6xHis), or from trace amounts of co-purified *E. coli* contaminants.

2.7. Porin D-vitronectin affinity measurements

Kinetic analysis was performed by Biolayer interferometry using a forteBio OctetRed96 platform (Pall, Menlo Park, CA). Vitronectin aa 80–396 was immobilized on AR2G sensors (Pall) by amino coupling. The analyte (Porin D) was serially diluted in running buffer (PBS) ranging from 0.016 μM to 1 μM. The experiments were conducted at 30 °C. Data analysis was performed using the Fortebio Data Analysis software 8.1 (Pall). Curves were fitted with 1:1 binding kinetics and the K_{ass}, K_{diss}, and affinity (K_D) was calculated.

2.8. Western blot

OMPs were prepared by resuspending bacteria in 50 nM Tris-phosphate buffer containing 3% Empigen (Calbiochem, Merck Millipore, Darmstadt, Germany) and a protease inhibitor (Complete; Roche, Basel, Switzerland). This suspension was incubated at 37 °C with glass beads and end-to-end rotation. Proteins were separated on a NuPAGE 4–12% or 10% Bis–Tris gels (Life technologies, Carlsbad, CA) and blotted as described [18].

2.9. Flow cytometry

E. coli BL21 (DE3) containing pET16b *oprD* or pET16 without insert (negative control vector) were induced overnight with isopropyl-β-D-1-thiogalactopyranoside (IPTG). Bacterial pellets were washed and resuspended to OD_{600} = 1.0 in PBS and incubated with recombinant vitronectin aa 80–396 in PBS-2% BSA followed by addition of an anti-vitronectin monoclonal antibody (mAb) VN58-1 (Abcam, Cambridge, UK). After washes, FITC-conjugated rabbit anti-mouse polyclonal Abs (Dako, Glostrup, Denmark) was added. Finally, samples were analyzed in a flow cytometer EPICS-XL (Beckman Coulter, Pasadena, CA). Gates were set to include 2% of the background and any reading above this was considered positive. Controls were prepared in the presence of primary and secondary Abs, but in the absence of vitronectin.

2.10. Binding of P. aeruginosa to immobilized vitronectin

Glass slides were coated with 0.5 μg human plasma vitronectin and air-dried at 37 °C. Bacteria were grown in LB medium, washed and resuspended in PBS (OD_{600} = 0.5). The slide was submerged in the bacterial suspension and incubated at 37 °C for 1 h. After washing and drying, the sample was Gram-stained and the main investigator and one independent researcher counted adherent bacteria in six randomly selected fields.

2.11. Statistical analysis

Comparisons of means were evaluated with unpaired Student's *t*-test. *P*-values ≤ 0.05 and were considered as statistically significant. Statistical analyses were performed using Graph-Pad Prism® version 6.0 (GraphPad Software, La Jolla, CA).

2.12. Ethics statement

Permit (M193-11) was obtained from Malmö/Lund District Court (Djurförsöksetiska nämnden, Lund, Sweden) for immunization of rabbits.

3. Results

3.1. P. aeruginosa isolated from the respiratory tract of patients with cystic fibrosis have increased vitronectin-binding capacity

To analyze the vitronectin-binding capacity of clinical *P. aeruginosa* isolates, we performed a DBA with [^{125}I]-labeled vitronectin. Isolates from the airway of patients with CF (n = 27) and blood isolates from bacteremic patients (n = 15) were selected. Binding of vitronectin was normalized to the reference strain *P. aeruginosa* PAO1 that was set to 1.0. Intriguingly, we found that airway isolates from CF patients bound significantly (p = 0.025) more vitronectin in comparison to blood isolates (Fig. 1).

3.2. Proteomics reveals Porin D as a vitronectin receptor of P. aeruginosa

To identify vitronectin-binding proteins in the outer membrane of *P. aeruginosa*, OMPs from PAO1 and four selected clinical strains were separated by 2D-SDS-PAGE. A typical gel with PAO1 is exemplified in Fig. 2A. Vitronectin was used as a probe and the vitronectin-binding proteins were identified by a far Western blot immunoassay (Fig. 2*B*). We observed a unique spot corresponding to a putative vitronectin-binding protein with molecular mass of 50 kDa in all strains. This spot was excised from the 2D-gel, analyzed by MALDI-TOF and subsequently identified as Porin D (GI: 15596155, PA0958 according to the *Pseudomonas* Genome Project [21]). This indicated that vitronectin acquisition at the surface of *P. aeruginosa* involved

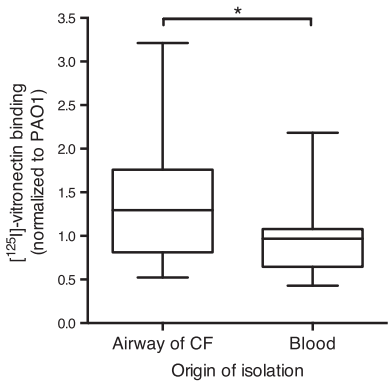

Fig. 1. *P. aeruginosa* binds vitronectin. Clinical isolates bind vitronectin as determined by an [^{125}I]-vitronectin DBA. *P. aeruginosa* from the lower airway of patients with cystic fibrosis (n = 27) and isolates cultured from blood from patients with bacteraemia (n = 15) were included. Binding (cpm) of replicate values were normalized to PAO1. Mean value of airway isolates: 1.37 ± SEM 0.12, blood isolates: 0.98 ± SEM 0.12, p = 0.025. Error bars indicate the minimum and maximal values. Each experiment was repeated three times with triplicates.

Porin D. The protein was thereafter recombinantly expressed in *E. coli* (Fig. 2*C*), and used for immunization of rabbits. Resulting pAbs recognized the recombinant protein (Fig. 2*D*).

3.3. Recombinant Porin D from P. aeruginosa has a high affinity for vitronectin

To confirm that the vitronectin-binding property of the putative vitronectin receptor was not an artifact, protein–protein interactions between vitronectin and recombinant Porin D were analyzed by ELISA. Porin D was coated on microtiter plates and increasing concentrations of vitronectin were added. A dose-dependent binding of vitronectin to Porin D was observed, that is, significantly more vitronectin was bound by Porin D in comparison to the negative control. (Fig. 3*A*). To further evaluate the kinetics of this interaction, we performed a Biolayer interferometry assay with vitronectin immobilized to the sensors. The interaction was analyzed with Porin D in serial dilutions starting with 1 μM. Under these experimental conditions and a vitronectin concentration of 125 nM, the dissociation constant (K_D) was calculated to 3.6 ±0.11 nM (Fig. 3*B*).

Vitronectin contains three heparin-binding domains (HBD) (Fig. 3*C*). We have previously shown that *Haemophilus* surface fibrils (Hsf) from *H. influenzae* bind to the HBD 3 of vitronectin (amino acids 352–374) [22]. In the present study, we used truncated vitronectin fragments to in detail test the interaction between Porin D and vitronectin (Fig. 3*C*). Our results implied that Porin D bound to vitronectin between amino acid (aa) sequence 352 to 374, which corresponds to HBD 3 (Fig. 3*D*). Moreover, we found that heparin completely blocked the binding of vitronectin to Porin D (Fig. 3*E*), which confirmed involvement of HBDs in the Porin D-dependent vitronectin binding.

3.4. Porin D is functional and binds vitronectin at the surface of E. coli

The specific function of Porin D was further investigated using *E. coli* as a heterologous host. After transformation, porin D-expressing *E. coli* was analyzed by flow cytometry using anti-Porin D pAbs (Fig. 4*A*). Porin D was readily expressed at the surface of the *E. coli*-OprD transformant as opposed to the control with an empty expression vector. Vitronectin binding was confirmed by flow cytometry after incubation with increasing concentrations of vitronectin (0–128 nM). A significantly higher vitronectin-binding capacity was observed with *E. coli*-OprD as compared to the negative control (Fig. 4*B*). Taken together, these results confirmed that Porin D is a surface-exposed protein that significantly attracts vitronectin at the bacterial surface.

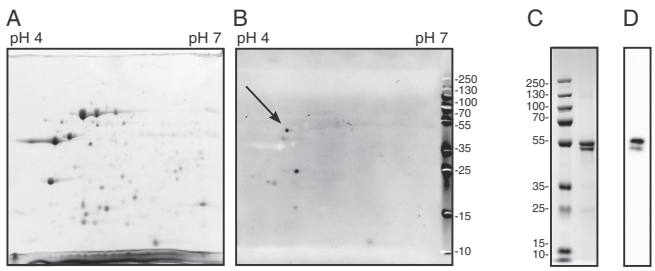

Fig. 2. Porin D is identified as a vitronectin-binding surface protein of *P. aeruginosa*. (A) The outer membrane proteome of PAO1 was separated by 2D-SDS-PAGE (pH 4–7) and stained with Coomassie-blue (left panel). (B) Another gel was in parallel blotted to a PVDF membrane and vitronectin binding was determined by Far-Western blotting using vitronectin as bate (right panel). The arrow point at a spot corresponding to a 50 kDa vitronectin-binding protein, which was subsequently identified by MALDI-TOF as Porin D. (C) Recombinant Porin D (5 μg) was separated on a SDS-PAGE and stained with Coomassie-blue. (D) An identical gel as in (C) was blotted to a PVDF membrane that was incubated with anti-Porin D pAbs followed by incubation with HRP-conjugated secondary pAbs.

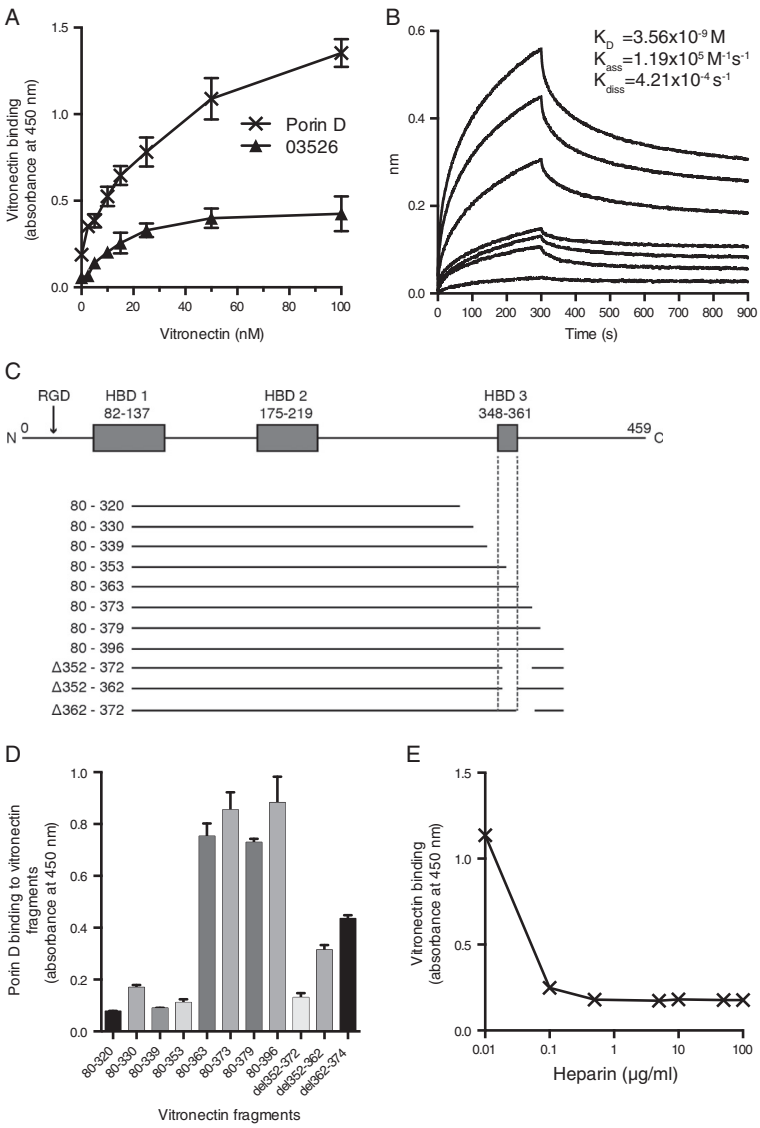

Fig. 3. Porin D interacts with vitronectin at the C-terminal HBD 3. (A) Vitronectin bound to Porin D as shown by ELISA. *H. influenzae* UHP_03526 was used as a negative control. Increasing concentrations of recombinant vitronectin aa 80–396 was added and bound vitronectin was detected by an anti-vitronectin mAb. (B) Kinetic analysis was performed with Biolayer interferometry (Octet Red96). (C) Truncated vitronectin fragments were recombinantly expressed and purified by Ni-NTA affinity chromatography. The integrin binding domain (RGD) domain and heparin-binding domains (HBD) 1–3 are denoted in the figure. (D) ELISA showing binding of truncated vitronectin fragments (50 nM) to Porin D. (E) The interaction between Porin D and vitronectin was inhibited by heparin. Vitronectin at 50 nM was added together with increasing concentrations of heparin. In (A) and (D), mean values and SEM of three independent experiments are presented.

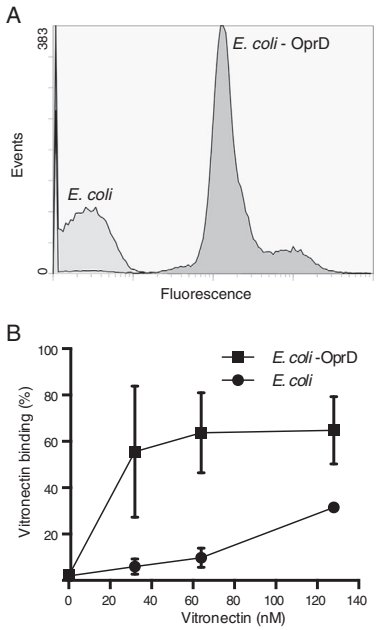

Fig. 4. Porin D expressed at the surface of *E. coli* binds vitronectin. (A) *E. coli* transformed with pET16-*oprD* expressed Porin D at the surface. *E. coli*-OprD was analyzed by flow cytometry after labeling with anti-Porin D pAbs. *E. coli* with an empty vector (pET16b) was used as a negative control. (B) Porin D was functionally active and bound more vitronectin at the bacterial surface than the control ($p = 0.037$ at vitronectin 64 nM). Mean values and SEM of three independent experiments are shown.

3.5. Porin D is expressed in clinical P. aeruginosa isolates, and a Porin D-transposon insertion mutant has a decreased vitronectin-binding capacity

To confirm expression of Porin D in 12 randomly selected clinical isolates, OMPs were isolated followed by Western blot using anti-Porin D pAbs. Four of those are presented in Fig. 5*A*. As seen in the Coomassie stained SDS-PAGE, approximately equal amounts of proteins were loaded in the gel (Fig. 5*A*, *left panel*). Porin D was expressed in the clinical isolates at similar levels as the reference strains tested (Fig. 5*A*, *right panel*), except in one strain in which no Porin D was detected (data *n.s.*). To further elucidate the role of Porin D as a vitronectin-binding protein, we used an *oprD*-deficient transposon mutant. Porin D expression was completely abolished in the *P. aeruginosa oprD* mutant when compared to the wild type (WT) *P. aeruginosa* MPAO1 in Western blot (Fig. 5*A*, *right panel*). Importantly, the growth rate of the *oprD* mutant was similar to that of the wild type *P. aeruginosa* MPAO1 (Fig. 5*B*).

To further verify that Porin D in *P. aeruginosa* is important for vitronectin binding, we analyzed bacteria in a DBA using [^{125}I]-vitronectin. The *oprD* mutant bound significantly less [^{125}I]-vitronectin than the Porin D-expressing wild type counterpart ($p \leq 0.001$) (Fig. 5*C*). Moreover, the importance of Porin D-dependent vitronectin binding was further demonstrated when *P. aeruginosa* was added to vitronectin-coated glass slides. By counting the adherent bacteria after rinsing and Gram-staining, we found that the *P. aeruginosa oprD* transposon insertion mutant adhered significantly less to the vitronectin-coated glass slides in comparison to the wild type strain ($p \leq 0.001$) (Fig. 5*D–F*). Thus, Porin D mediates *P. aeruginosa* adhesion to soluble and to immobilized vitronectin.

4. Conclusions

The opportunist pathogen *P. aeruginosa* causes a great burden of disease, particularly to patients with CF. The host–pathogen interactions leading to the first stage of infection have not yet been fully elucidated. In the present study, we have shown that *P. aeruginosa* isolates bind the human ECM protein vitronectin and that isolates from the airway of CF patients have stronger vitronectin-binding phenotypes than isolates obtained from the blood stream. Moreover, we identified Porin D as the first known vitronectin-binding protein on the surface of *P. aeruginosa*.

Vitronectin is available in the lower respiratory tract as it is produced by respiratory epithelial cells. This production is upregulated in the airway of CF patients [13]. Despite adhesion may promote the host response and clearance, our observations suggest that vitronectin-dependent adhesion may be a virulence strategy promoting colonization of the airway of CF patients. Similar mechanisms are used by e.g. *Streptococcus pneumoniae* [24]. For *P. aeruginosa*, we speculate that adhesion is of importance at least during initial colonization and exacerbations.

To identify vitronectin-binding proteins in the outer membrane of *P. aeruginosa*, we used a proteomic approach with 2D-SDS PAGE using vitronectin as a probe. Following this, we confirmed the specific interaction between recombinant Porin D and vitronectin by ELISA and calculated the dissociation constant to 3.6 nM, which indicates a strong binding affinity. Surface expression of Porin D was confirmed in the outer membrane of several clinical *P. aeruginosa* isolates by Western blotting. Moreover, the vitronectin-binding function of Porin D was verified by heterologous expression on the surface of *E. coli*. Importantly, the vitronectin binding capacity was significantly reduced in the Porin D-deficient mutant, for which the interaction with vitronectin was decreased both with soluble vitronectin and immobilized vitronectin. Our current data thus clearly indicate that Porin D is a strong vitronectin-binding protein of *P. aeruginosa*. Vitronectin-binding is, however, likely to be multifactorial. We did not observe a complete reduction of the vitronectin binding capability in the transposon mutant and hence we would expect *P. aeruginosa* to possess other vitronectin-binding proteins. Consequently, it is likely that the clinical strains express multiple ECM binding proteins.

Though *P. aeruginosa* often exists in sputum plaques, adhesion to the epithelial surface is likely an important step for successful colonization of the airway. It has previously been shown that vitronectin bridges the *P. aeruginosa* surface and

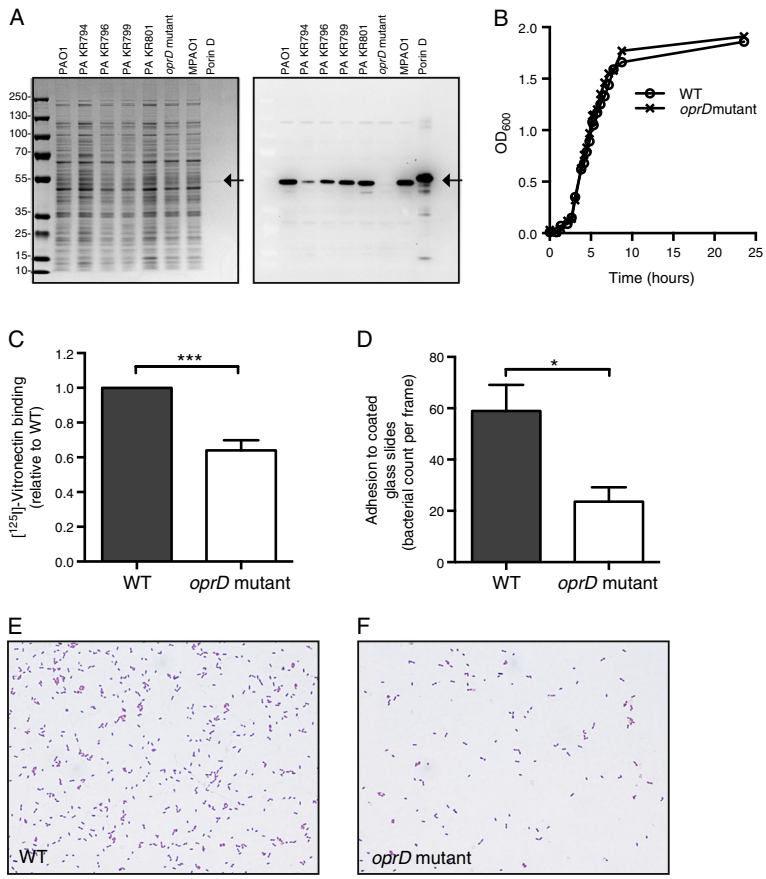

Fig. 5. Porin D is important for vitronectin binding in *P. aeruginosa*. (A) Analysis of Porin D expression in *P. aeruginosa* PAO1, four clinical *P. aeruginosa* isolates and confirmation of the *oprD* transposon insertion mutant. The *oprD* mutant is compared to the wild type (WT) counterpart *P. aeruginosa* MPAO1. OMP were loaded on NuPAGE 4–12% Bis–Tris gels that were Coomassie stained (*left*) and analyzed by Western blot (*right*) using anti-Porin D pAbs. The localization of Porin D is marked with an arrow. One representative experiment out of three performed is shown here. (B) Growth curve of *P. aeruginosa* WT (MPAO1) and the *oprD* insertion mutant cultured in LB medium. (C) The vitronectin-binding capacity of the *P. aeruginosa oprD* insertion mutant and corresponding WT as determined by DBA using [^{125}I]-vitronectin. CPM values were normalized to *P. aeruginosa* WT and presented as mean ± SEM of three separate experiments. (D) Adhesion of the *P. aeruginosa* WT and *oprD* mutant to immobilized vitronectin (0.5 μg) on glass slides. The bar diagram represents mean and the error bars SEM of three independent experiments. (E–F) Raw data for the results shown in panel D. Adherent bacteria were Gram-stained and representative pictures were taken at 100× amplification.

$\alpha_v\beta_5$-integrins of host lung epithelial cells, an interaction that ultimately contributes to the adherence and internalization of bacteria [7]. Supporting this observation, we were able to show that *P. aeruginosa* interacts with vitronectin at the C-terminal (HBD 3), leaving the RGD motif at the N-terminal free to bind integrins. Recruitment of vitronectin by Porin D on the surface of *P. aeruginosa* may thus contribute to adherence to not only the ECM but also indirectly to the epithelial cell surface by using vitronectin as a bridging molecule.

Porin D is an OMP of approximately 50 kDa that is also known as OprD, PA0958, occD1 or Outer membrane protein D2. The crystal structure of Porin D has recently been solved [26]. Following the introduction of imipenem in clinical practice in the late 1980s, Porin D was identified as a channel for basic amino acids and imipenem [27]. Mutations in Porin D of *P. aeruginosa* have been shown to increase the bacterial resistance to imipenem, and worldwide it is one of the most commonly observed imipenem resistance mechanisms [28].

Porin D consists of 9 loops of which loops 2 and 3 have been most extensively studied. Deletions of loops cause conformational changes that lower the sensitivity to imipenem. It has recently been reported that *oprD* deficient strains had an increase in *in vivo* fitness based on gastrointestinal tract colonization and systemic dissemination [29]. The mechanisms behind systemic dissemination from the gastrointestinal tract are likely to be different to the mechanisms promoting local colonization of the respiratory tract, which is why our results are not conflicting. We hypothesize that OprD binding to Vn is of biological importance for local colonization of the respiratory tract.

In conclusion, we found that clinical isolates from the airways of CF patients bind more vitronectin in comparison to isolates cultured from blood. Furthermore, we identified Porin D as the first known surface protein of *P. aeruginosa* that binds vitronectin. Our study sheds light upon the interaction between *P. aeruginosa* and the human ECM protein vitronectin. The findings also pave the way for further studies that aim to analyze the importance and function of this interaction in bacterial pathogenesis.

Acknowledgments

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Paper III

Vitronectin is produced in the lung upon infection by respiratory pathogens, and is utilized to conquer the innate immunity

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Running title: Bacterial utilization of vitronectin in the respiratory tract

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ABSTRACT

Bacterial extracellular vesicles (EVs) are shed during growth by the common respiratory pathogens *Pseudomonas aeruginosa* and *Haemophilus influenzae* and are intricately involved in host-pathogen interactions. These EVs contain bacterial membrane structures that activate the bacteriolytic complement cascade, which is regulated partly by vitronectin (VTN). We aimed to show that VTN is an acute phase protein that was elevated in bronchoalveolar lavage fluid (BALF) during pneumonia, produced by respiratory epithelial cells upon stimulation by bacterial EVs and utilized by the respiratory bacteria for increased virulence.

We collected BALF from patients with pneumonia and from healthy volunteers after subsegmental endotoxin (LPS) instillation and analyzed VTN concentrations. Higher VTN concentrations were found in BALF from patients with pneumonia than from controls (p=0.0063) and in endotoxin-challenged lung segments than control segments after 12h (p=0.031) or 48h (p=0.016). Bacteria that were incubated in BALF captured VTN and were less killed compared to bacteria devoid of VTN (*P. aeruginosa* p=0.016, NTHi p=0.011). Respiratory epithelial cells were stimulated with bacterial EVs to mimic bacterial invasion *in vitro*. *VTN* mRNA transcription rose during the first hour (p=0.022) and surface bound VTN increased for two days (p=0.0003 and p=0.010).

In conclusion, elevated VTN concentrations are found in BALF from patients with pneumonia. Endotoxin or EVs derived from *P. aeruginosa* and *H. influenzae* caused increased VTN levels *in vivo* and *in vitro*. The same bacterial species captured VTN on their surface to boost the virulence by evading lysis by complement. Hence, VTN is an acute phase protein, produced by respiratory epithelial cells upon bacterial infection and utilized by pathogens to persist in the respiratory tract.

INTRODUCTION

The initial encounter between the human host and pathogens involves the interplay between microbial virulence factors and the innate immunity that protects the human lung from pathogens. The innate immune system consists of physical barriers as the mucociliary movement and the epithelial cell lining, but also of antimicrobial antimicrobial peptides, complement proteins and pathogen responsive cells [1-3]. The host responds to pathogen-associated molecular patterns (PAMPs), for example, bacterial cell wall components and lipopolysaccharide (LPS), to initiate inflammation, cell recruitment and clearance of bacteria through killing by lysis or phagocytosis [4]. As counterattack, pathogens have evolved sophisticated strategies to conquer the host defense including firm adhesion to the respiratory epithelium to prevent removal by the mucociliary escalator, and to evade killing by complement proteins [5,6].

Invading as well as mucosal pathogens escape complement-mediated killing by recruiting complement regulators to the bacterial surface. Several pathogens have evolved the ability to bind and use the complement regulator vitronectin (VTN) to their advantage. VTN is found in human plasma and in the extracellular matrix. This 75 kDa glycoprotein is primarily expressed and secreted by hepatocytes, although it is also produced by other cell types including respiratory epithelial cells [7,8]. VTN protects human cells and tissues from self-damage induced by complement through inhibition of the membrane attack complex (MAC) [9]. Other known biological functions of VTN include involvement in cellular attachment and migration, in tissue healing and in regulation of apoptosis [10,11]. Non-typeable *Haemophilus influenzae* (NTHi) Protein E, *Pseudomonas aeruginosa* Porin D, and *Streptococcus pneumoniae* PspC are examples of VTN-binding outer membrane proteins (OMP) on common respiratory pathogens [6,12-14]. Microbes inhibit the formation of MAC and gain resistance against complement by recruiting VTN [2,6,13]. Moreover, surface-bound VTN can form a bridge between bacteria and the epithelial cell, and hence is used for increased adherence to the epithelium [15,16]. The importance of VTN binding for *Pseudomonas*-dependent virulence in the respiratory tract is illustrated by the greater VTN-binding capacity of airway isolates compared to counterparts isolated from blood [12].

All Gram-negative bacteria shed outer membrane vesicles (extracellular vesicles; EVs) during growth [17,18]. Bacterial EVs are nanosized spherical structures composed of a lipid membrane with proteins and LPS on the surface, and carry periplasmic proteins, RNA and DNA [19]. Purified LPS trigger an immunological response in respiratory epithelium via TLR4, whereas the components of EVs are recognized by several pattern recognition receptors (PRR) on mammalian cells [17,20]. Endotoxin instilled into a lung segment triggers a local inflammation in humans and EVs from *P. aeruginosa* instilled in mice induce pulmonary inflammation, partly through TLR2 and TLR4 but also via other receptors yet to be identified [21]. Increased VTN concentrations in the bronchial lumen of patients with chronic lung diseases indicate that VTN is an effector molecule associated with inflammatory processes [22-25]. The effects of bacterial PAMPs on VTN levels and concentrations during pneumonia are, however, unknown.

The aim of this study was to investigate whether respiratory epithelial cells produce VTN as a result of infection. We hypothesized that VTN in the human airway is an acute phase protein released as a response to bacterial PAMPs. Bronchoalveolar lavage fluid (BALF) was analyzed from a cohort of patients with suspected pneumonia. In addition, *Escherichia coli* endotoxin (lipopolysaccharide; LPS) was instilled into the bronchial lumen of healthy volunteers, followed by collection of bronchoalveolar lavage fluid (BALF) and analysis of VTN concentrations. We found that both pneumonia and endotoxin challenge *in vivo* resulted in increased VTN concentrations in the lung. *H. influenzae* or *P. aeruginosa* were incubated with BALF

and, intriguingly, bacteria captured VTN derived from BALF resulting in increased resistance against complement-mediated killing. To verify our clinical findings *in vitro*, we also incubated type II alveolar respiratory epithelial cells with EVs derived from the respiratory pathogens *P. aeruginosa* and *H. influenzae*, and found that cell-bound VTN and *VTN* mRNA increased in stimulated cells compared to controls. Taken together, these result show that VTN is an acute phase protein in the respiratory tract that is locally produced. This production is stimulated by respiratory pathogens and used to gain in virulence.

MATERIAL AND METHODS

Clinical study subjects

Study subjects (*n*=8) were recruited among admitted patients with suspected pneumonia by the treating clinician. All subjects were planned for bronchoscopy at the Dept. of Infectious Diseases, Skåne University Hospital (Malmö, Sweden) between September 1st, 2015 and April 30th, 2016 as part of microbial investigations. The most affected lung segments were identified and bronchoalveolar lavage (BAL) was performed with 3x50 ml PBS. In two cases, protected bronchial brush specimens were also obtained. Further patient data were collected from the hospital medical record Melior (Siemens Healthcare, Upplands Väsby, Sweden) and stored in the online database REDCap hosted at Lund University (www.project-redcap.org) [26].

Endotoxin stimulation of study subjects and controls

Healthy volunteers were recruited for bronchoscopy with endotoxin installation and repeated bronchoscopy for BAL after 12h or 48h. The procedure has previously been described in detail [27,28]. Briefly, 10 ml phosphate-buffered saline (PBS) was instilled in corresponding segmental bronchi in both lungs, on one side with the addition of 4 ng/kg *E. coli* 0113:H10 endotoxin (USP, Rockville, MD). Using this procedure we obtained subject case specific vehicle control as well as data to calculate a study specific normal range (±2SD from the mean) to compare with the clinical samples. After 12h or 48h, BAL fluid (BALF) was collected with three 50 ml aliquots of PBS in each segment. One subject was excluded because of adverse effects (vomiting). The remaining subjects (*n*=13) had mild systemic and local symptoms during the first 12 h observation after exposure as previously reported [28].

Handling of BALF and Enzyme-linked immunosorbent assay (ELISA)

BALF samples were filtered and centrifuged at 300 RCF for 10 min to obtain a cell free preparation. The protein quality and semi-quantitative concentration were evaluated by separation on SDS-PAGE followed by staining with Coomassie Blue. VTN concentrations in BALF were determined by ELISA per the manufacturer´s

instructions (Thermo Fisher, Frederick, MD). Optical density at 450 nm were measured (Tecan, Männedorf, Switzerland) and VTN sample concentrations were extrapolated from a standard curve (Prism, GraphPad Software, La Jolla, CA) using VTN standards included in the ELISA kit.

Handling of brush specimens and immunofluorescence

The protected brush specimens were vortexed and released cells were washed in PBS and subjected to slides with cytospin followed by air drying. PBS-Tween BSA 2% was used for blocking and antibody dilutions. An anti-VTN mouse monoclonal antibody (mAb) VN58-1 (Abcam, Cambridge, UK) was added, followed by FITC-conjugated rabbit anti-mouse polyclonal antibodies (pAbs) (Dako, Glostrup, Denmark). Slides were flooded with FM4-64fx to counterstain plasma membranes and cytosols (Invitrogen, Carlsbad, CA) before adding Mounting media (Dako) with 0.3 µg/ml DAPI (Sigma) for staining of nuclei. Specimens were studied in a Zeiss LSM 700 confocal microscope with Plan-Apochromat 63x/1.40 Oil and the accompanying Zen software (Zeiss, Oberkochen, Germany) was used for image processing.

Bacterial strains, culture conditions and EV isolation

Non-typeable *H. influenzae* (NTHi) 3655 was routinely cultured in 37°C and 5% CO_2 either in brain-heart infusion (BHI) liquid broth supplemented with 10 µg/ml NAD and 10 µg/ml hemin or on chocolate agar plates. *P. aeruginosa* PAO1 was grown in Luria-Bertani (LB)-agar. Extracellular vesicles were isolated according to the method described by Rosen *et al.* with an additional size separation step [29]. Briefly, bacteria were grown overnight, then removed by centrifugation and filtration through 0.45 µm pore filters. The supernatant was concentrated in Vivaflow 200, 100,000 MWCO (Sartorius, Goettingen, Germany), debris and salts further removed by ultracentrifugation at 100,000g and repeated centrifugation in density gradient medium (HistoDenz, Sigma).

Stimulation of A549 epithelial cells with EVs

Respiratory type II alveolar epithelial cells (A549; $5x10^4$) were cultured in 24-well plates in F-12 medium (Life Technologies, San Diego, CA) with 10% fetal bovine serum (FBS) and 5 µg/ml gentamicin at 37°C with 5% CO_2 for 20h. Before (16h) initiation of experiments, cells were starved in F12. EVs (1-5 µg/ml) from NTHi or *P. aeruginosa* were diluted in F12 medium. At selected timepoints, the supernatant was collected and cells were harvested using Accutase (Innovative Cell Technologies, San Diego, CA) or lysed by RTL-buffer (Qiagen, Hilden, Germany) followed by RNA purification using RNeasy Mini Kit (Qiagen) and DNase I treatment (TURBO DNA-free; Life Technologies). For flow cytometry, PBS with 2% BSA was used for blocking, washing and dilutions of anti-VTN mouse mAb VN58-1 (Abcam) and

FITC-conjugated rabbit anti-mouse pAbs (Dako). Cells were analyzed on a FACSVerse (BD Biosciences, San Jose, CA).

Analysis of mRNA expression

cDNA was prepared from RNA using GeneAmp PCR system 2400 (Perkin Elmer, Wellesley, MA) with random primers (Roche, Basel, Switzerland). Real-time PCR amplification assay was performed on a CFX-96TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using sequence-specific primers (TIB MOLBIOL Syntheslabor, Berlin, Germany) and LightCycler 480 SYBR Green I Master (Roche). Target genes were normalized to the reference gene hypoxanthine guanine phosphoribosyl transferase using CFX Manager Software (Bio-Rad). The following primers were synthesized by Eurofins (Ebersberg, Germany) and used for VTN amplification: forward 5′-GGCTGTCCTTGTTCTCCAGTG-3′ and reverse 5′-GTGCGAAGATTGACTCGGTAGT-3′.

Bacterial surface acquisition of VTN and serum bactericidal assay

Any complement proteins in BALF were inactivated (56°C for 20 min) followed by sterile filtration. Bacteria were resuspended in 66 % BALF and incubated for 1h at 37 °C. To investigate VTN-binding to bacteria, rabbit anti-VTN pAbs (ab20091; Abcam) were added followed by FITC-conjugated swine anti-rabbit pAbs (Dako) for NTHi or Alexa fluor 647 goat anti-rabbit pAbs (Invitrogen) for *P. aeruginosa*. In controls, the secondary pAbs were omitted. Surface bound VTN was detected by flow cytometry (FACSVerse; BD Biosciences). VTN complement inhibitory activity of the terminal pathway was determined by a serum bactericidal assay as previously described [30]. Briefly, NTHi was preincubated in BALF followed by challenge with normal human serum (NHS) at 5 and 10 % for NTHi and *P. aeruginosa*, respectively. Surviving bacteria were determined by counting colony-forming units (CFU).

Statistical analysis

Statistical differences were analyzed using Wilcoxon signed-rank test, Mann–Whitney test, nonlinear regression model or t-test, as appropriate (GraphPad Prism). *P*-values ≤0.05 were considered significant.

Ethics

The present study was approved by the Regional Ethical Review Boards in Gothenburg (T683-07) and Lund (2014/529), respectively. Written consent was obtained from all study subjects.

RESULTS

High VTN concentrations in BALF from clinical cases with suspected pneumonia

To enable us to measure VTN levels in the bronchial lumen of patients with pneumonia, BALF was collected from patients (n=8). In addition, we obtained protected brush specimens from two patients. All included patients were treated with antibiotics and had clinical signs of pneumonia at inclusion (shortness of breath and any of fever, purulent sputa or radiographic infiltrate) [31]. Demographic and clinical data of all included clinical study subjects are summarized in Table 1. The isolated microbial species indicate pneumonia caused by Gram-negative bacteria in three, Gram-positive bacteria in two and a fungal species in two cases. In retrospect, one patient (#4) had symptoms that were regarded as aspiration and not by infection, although *Staphylococcus aureus* was cultured from a protected brush specimen.

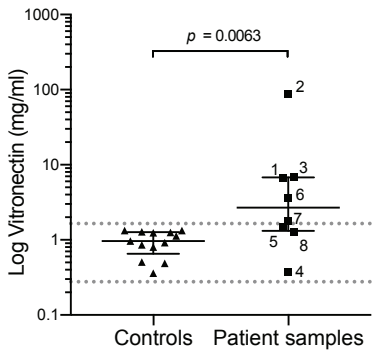

Figure 1: Higher VTN concentrations in patients with pneumonia.
BALF collected from patients with pneumonia (n=8) and healthy controls subjects (n=13). Patients had significantly higher concentrations The dotted lines represent the upper and lower ±2 SD of the mean of healthy controls and indicate a study specific reference range. All VTN concentrations were determined by ELISA.

VTN concentrations in BALF were determined by sandwich ELISA. Interestingly, patients with pneumonia had 14-fold increased concentrations of VTN in the extracellular fluid as compared to the 13 healthy control subjects (mean±SD: 13.9±30.33 vs. 0.96±0.34; p=0.0063) (Fig. 1). A study specific reference range for VTN was calculated from control samples to be 0.28-1.65 µg/ml and VTN concentrations in BALF from five of the 8 pneumonia patients exceeded this interval. The VTN concentration in BALF sample from subject #2 was strongly elevated (88.7 µg/ml). Taken together, our data suggest that VTN concentrations in BALF were significantly higher in patients with clinical signs of pneumonia as compared to healthy controls.

Table 1: Demographic and microbiological data of all included patients

Patient characteristics of all included patients with pneumonia in this study.

Patient id	1	2	3	4	5	6	7	8
Age (years)	81	69	63	59	64	82	39	82
Sex	Female	Female	Male	Female	Male	Male	Male	Male
Smoking habits	No data	No data	Non-smoker	Non-smoker	Previous smoker	Non-smoker	Non-smoker	Non-smoker
Underlying conditions								
COPD/Asthma/ Emphysema/Fibrosis	No	No	No	No	No	No	No	No
Immunodeficiency	Neutropenic, steroids	Leukemia	No	No	Steroids, Methotrexate	No	Steroids	Steroids
Inhaled steroid treatment	No	No	No	No	No	No	No	No
Comment	Acute myeloid leukemia	CLL, Lungcancer	Frequent aspirations	Frequent aspirations	Urinary bladder cancer, RA			Recent PE
Current disease								
Radiographic lung infiltrate	Yes	Yes	Yes	No	Yes	Yes	No	Yes
Purulent secretions	Not known	No	Yes	No	Not known	Yes	Yes	No
Temp >38 last 24h	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
Duration of symptoms (days)	22	14	2	18	7	13	34	7
Sepsis	Severe sepsis	No	Severe sepsis	No	Septic shock	Septic shock	Severe sepsis	Septic shock
Mechanical ventilator	No	No	No	No	Yes	Yes	Yes	Yes
FiO2 / Oxygen (L/min)	- / 1	- / 1	- / 4	- / -	40% / -	60% / -	21% / -	35% / -
Days of ventilator					2	5	7	5
Microbiological data								
Isolated microbial species	*Penicillium species*	*Nocardia farcinica*	*Klebsiella pneumoniae*	*Staphylococcus aureus*	*Pneumocystis jiroveci*	*Legionella pneumophila*	*Staphylococcus aureus*	*Serratia marcescens*
		Enterococcus faecium	*Pseudomonas aeruginosa*			Influenza A H1N1		
Ongoing antibiotic treatment	Meropenem	Sulfamethoxazole - trimethoprim	Imipenem	Cloxacillin	Cefotaxime	Levofloxacin	Sulfamethoxazole - trimethoprim	Cefotaxime
	Clindamycin	Amoxicillin-Clavulanic Acid			Erythromycin	Oseltamivir		Vancomycin
	Voriconazole							

Endotoxin challenge increases VTN concentrations in the lung of healthy individuals

Since elevated VTN concentrations were found in the airway of patients with pneumonia, we wanted to determine if endotoxin (LPS) from *E. coli* could induce VTN release in the lungs of healthy individuals. Healthy study subjects had PBS with and without endotoxins subsegmentally instilled during bronchoscopy. One side was used for the endotoxin challenge, whereas the contralateral lung segment received PBS only and hence was an endogenous case specific control. BALF was sampled from each segment after 12 h from 6 subjects and after 48 h from 7 subjects. After centrifugation, the VTN levels in cell-free BALF were determined by ELISA. Interestingly, after 12 and 48h, in all study subjects, VTN levels were significantly increased in BALF sampled from endotoxin-exposed lung segments compared to the unexposed contralateral side (Fig. 2).

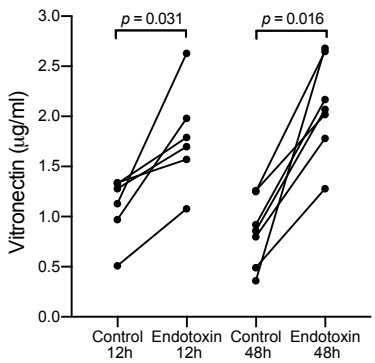

Figure 2: Endotoxin instillation caused local inflammation and increased VTN concentrations.
Healthy volunteers (*n*=13) were subsegmentally exposed to endotoxin in one lung. After 12h (*n*=6) or 48h (*n*=7), BALF were collected from both lungs and VTN concentrations determined by ELISA. All subjects had increased VTN concentrations in the exposed lung than in the contralateral control lung as indicated.

In individuals sampled after 12 h, the VTN concentrations increased from 1.09±0.32 µg/ml (mean±SD) in the control segments to 1.79±0.51 µg/ml in endotoxin-exposed segments (p=0.031). A 2.5-fold greater increase was observed in individuals sampled after 48 h, from 0.85±0.34 µg/ml (mean±SD) in the control segment to 2.09±0.49 µg/ml in the endotoxin-exposed segment (p=0.016).

Respiratory tract pathogens utilize pulmonary-derived VTN for acquiring serum resistance

We have recently shown that several bacteria including respiratory tract pathogens bind VTN at the surface when incubated with recombinant VTN or VTN purified from plasma [12,16]. We wanted to confirm that VTN in BALF was available for bacterial binding and not quenched or inhibited by other components in the pulmonary extracellular fluid. Therefore, we incubated NTHi and *P. aeruginosa* with BALF from clinical study subjects (*n*=8), and measured cell-bound VTN in flow

cytometry using anti-VTN pAbs and conjugated fluorescent secondary antibodies (Fig. 3). Both NTHi and *P. aeruginosa* bound VTN from most BALF (NTHi: 3.4-fold increase in MFI, p=0.009 and *P. aeruginosa*: 5.5-fold increase, p=0.001), and the levels of bacterial VTN binding were generally proportional to VTN concentrations measured by ELISA.

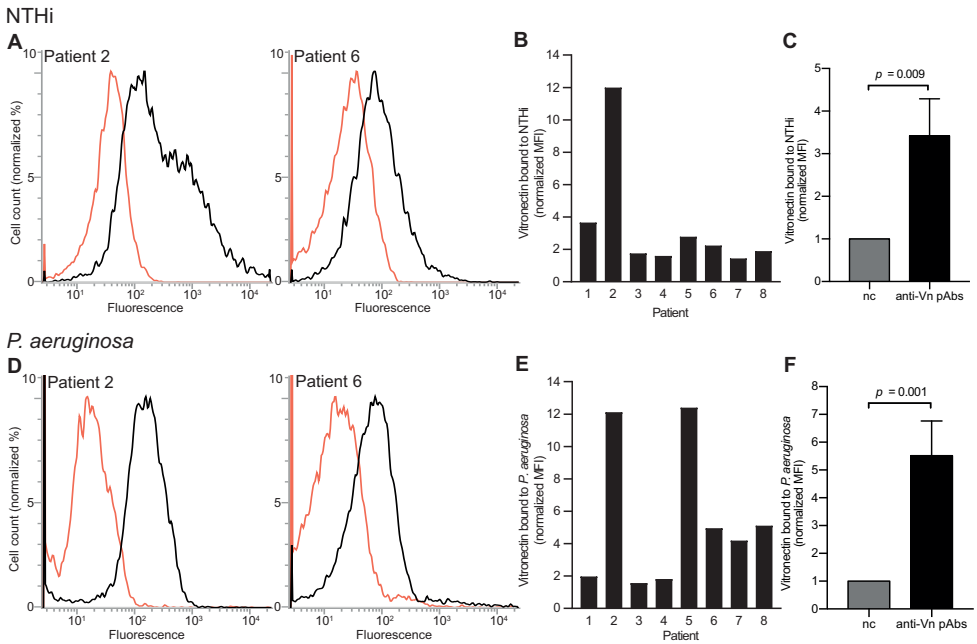

Figure 3: Bacterial respiratory pathogens capture VTN from BALF.
The respiratory pathogens *H. influenzae* and *P. aeruginosa* were incubated with BALF samples from pneumonia patients. Cell-bound VTN was revealed by flow cytometry using anti-VTN pAbs and FITC-conjugated secondary pAbs. Histograms of two representative experients per bacteria are shown (A and D). The fluorescence intensity reveals that bound VTN is proportional to the amount of VTN in each patient sample as shown in Fig. 1 (B and E). Each bar represents mean values of two independent experiments. Finally, negative controls (nc) without anti-VTN pAbs had lower fluorescence intensity, indicating low unspecific binding of the conjugated antibody (anti-Vn pAbs), and low autofluorescence at the analyzed wavelengths (C and F).

Bound VTN is used by bacterial respiratory pathogens to inhibit the complement terminal pathway and evade killing by the host innate immunity [6]. To examine whether the VTN present in BALF was in sufficient amount and in active configuration to protect bacteria from killing by normal human serum (NHS), we examined the biological activity in a serum bactericidal assay. The activity was confirmed by incubating NTHi or *P. aeruginosa* in BALF or BALF depleted of VTN. Non-incubated bacteria, or bacteria that was preincubated in VTN-depleted BALF were killed more readily by the complement components in serum (Fig. 4 A and B, $p \leq 0.001$ for both pathogens). After 30 min, 3.4- and 2.5-fold more NTHi and *P. aeruginosa*, respectively, survived the challenge if preincubated with BALF (p=0.016

resp. $p<0.001$). These experiments provided corroborative evidence that biologically active VTN from BALF was captured on the microbial surface and used to inhibit bacterial lysis by the MAC.

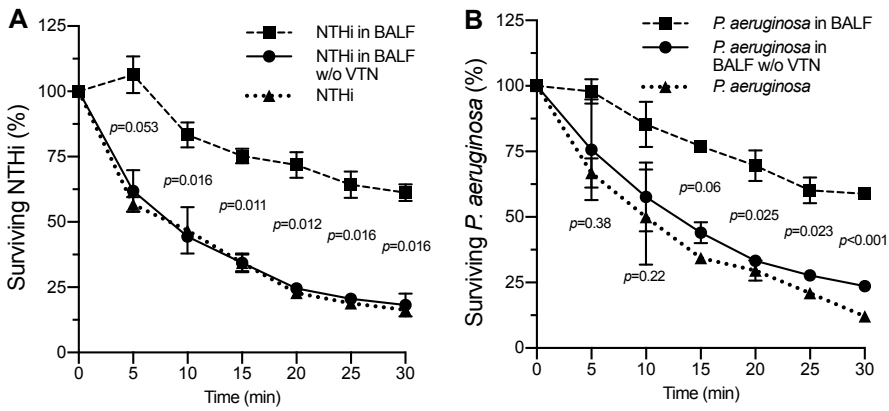

Figure 4: Respiratory pathogens use VTN from BALF to evade killing by complement.
Bacteria were challenged with human serum after preincubation in "crude" BALF from a patient with pneumonia, or VTN-depleted BALF from the same patient. Both *H. influenzae* (A) and *P. aeruginosa* (B) gained resistance to human serum by preincubation with BALF, compared to preincubation in VTN-depleted BALF or to no preincubation. Mean values and standard error of two independent experiments done in triplicates are shown. *P*-values indicate the relation between preincubation with BALF and BALF without VTN at each time point.

VTN is produced by epithelial cells during pneumonia and upon stimuli by EVs from NTHi or P. aeruginosa

Since VTN is an abundant plasma protein, we wanted to study whether epithelial cells might be an endogenous source of VTN. To visualize VTN associated with the respiratory epithelial cells from the lower respiratory tract, cytospin slides from protected brush specimens were stained with an anti-VTN mAb and secondary FITC-labelled pAbs. The cells were counterstained with FM4-64fx and DAPI to visualize cell membranes and nuclei, respectively. VTN was detected in the cytoplasm and at the surface of respiratory epithelial cells, especially on the cilia (Fig. 5).

This observation strongly indicated that respiratory epithelial cells have the capacity to produce VTN *in vivo*. To determine if VTN production was induced by stimuli of bacterial origin, we purified EVs from the respiratory pathogens *P. aeruginosa* and *H. influenzae*. Thereafter, A549 type II alveolar epithelial cells were exposed to purified EVs and harvested at different time points. In an initial set of experiments, cells were harvested and subjected to flow cytometry analyses after 24h and 48h of incubation. Importantly, we found that surface-bound VTN significantly increased at the epithelial cell surface in the presence of EVs as compared to controls (Fig. 6A).

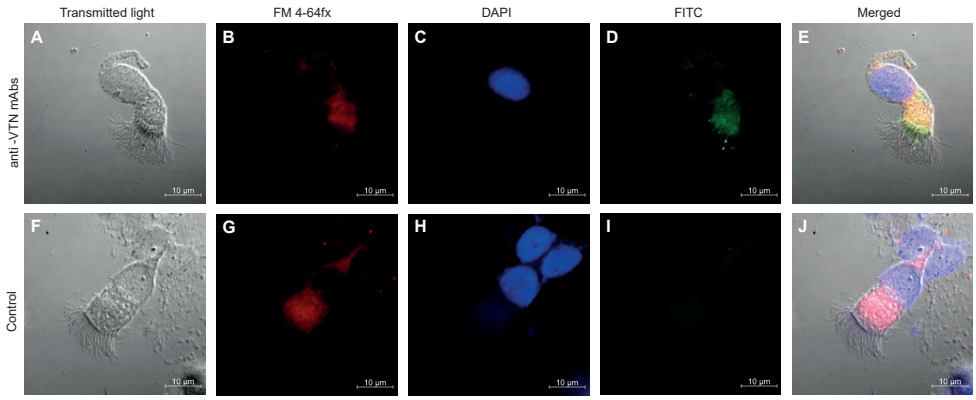

Figure 5: Respiratory epithelial cells with VTN in the cytosol and on cilia.
Ciliated epithelial cells from sterile brush specimens subjected to slides by cytospin centrifugation. Upper panel, from left to right: (A) transmitted light image, (B) membranes and vacuoles stained red by FM 4-64fx, (C) nuclei stained blue by DAPI, (D) VTN stained green by an anti-VTN mAb and secondary FITC-conjugated pAbs, and (E) a merged picture. Lower panel, corresponding control micrograph where the anti-VTN mAb was omitted (F-J). Each color channel was adjusted individually using the same parameters for the corresponding control picture.

To verify this finding in another set of experiments, RNA was isolated from A549 epithelial cells at multiple time points after incubation with EVs, followed by RT-PCR and qPCR. As can be seen in Figure 6B, increased levels of *VTN* mRNA were observed already after 1h stimulation with bacterial EVs. The increase was statistically significant in cells stimulated with EVs from *P. aeruginosa* (p=0.02), but not in cells that were stimulated with NTHi EVs (p=0.29). After an initial peak in *VTN* mRNA production at 1-3 h, the transcription levels returned to baseline. Taken together, these cellular experiments confirmed that respiratory epithelial cells constitute a local source of VTN in the respiratory tract and that the translation is triggered by PAMPs, in this case bacterial EVs derived from two common pathogens dwelling in the respiratory tract.

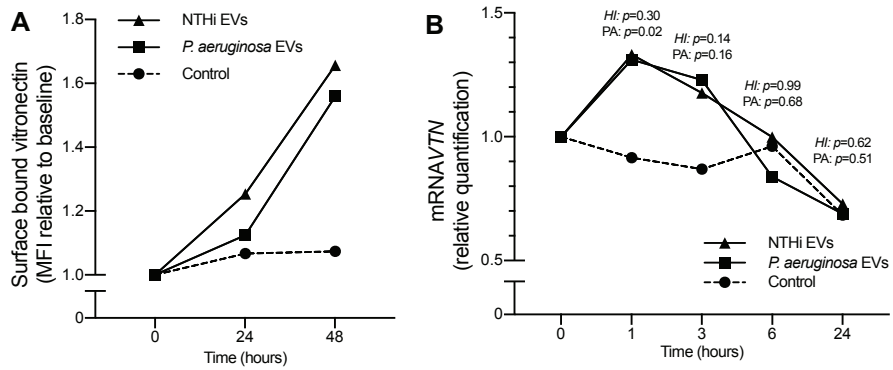

Figure 6: Type II respiratory epithelial cells produce VTN after stimulaton with EVs.
Surface bound VTN (A) was detected by flow cytometry on A549 epithelial cells using anti-VTN pAbs and FITC-conjugated secondary pAbs. Cells were grown in complete medium and starved without serum 18h prior to the experiment. EVs (5 µg/ml) from *P. aeruginosa* or *H. influenzae* were added to cells, which were harvested at the indicated time points. Significantly more VTN was found after EV stimulation than controls, as determined by a non-linear regression model (NTHi: p=0.0003, *P. aeruginosa*: p=0.010). One representative of two independent experiments is shown. A corresponding early increase in *VTN* mRNA transcription was seen 1h after stimulation with EVs (*P. aeruginosa* p=0.022, NTHi p=0.30). After 6h, the transcription had returned to baseline values. Symbols represent median value of four experiments.

DISCUSSION

In this study, we report that VTN is an acute phase protein in the human lung, and that two important respiratory pathogens that trigger a pro-inflammatory response can use VTN to its own advantage. VTN concentrations were elevated in BALF (corresponding to the extracellular fluid in the lower respiratory tract) of patients with pneumonia and was boosted to similar levels in the lung of healthy individuals after instillation of endotoxin. The increase in the bronchial lumen was detectable 12h after endotoxin exposure, and it remained elevated two days after exposure. Both *P. aeruginosa* and *H. influenzae* could bind VTN from BALF and use it to evade killing by complement. We also observed VTN inside ciliated epithelial cells collected from a patient with pneumonia and could confirm epithelial cell VTN transcription and translation *in vitro*. This translation was triggered by EVs from *P. aeruginosa* or *H. influenzae* and resulted in both increased mRNA and increased cell bound VTN.

VTN is a normal component of the extracellular fluid in the human lung and we show that the concentration is elevated during pneumonia caused by a variety of pathogens. In the present study, 5 of 8 patients with suspected pneumonia had increased levels of VTN in their lungs compared to healthy control subjects. Two outliers were identified among the included patients. The patient with the lowest concentration (#4) had no chronic lung disease and never smoked, had but frequent aspirations after a severe cerebrovascular insult. *Staphylococcus aureus* grew 18 days

earlier from a protected brush specimen and the patient was treated with cloxacillin, although the patient had no purulent sputa, radiographic infiltrates or fever. The other outlier was patient #2, whose BALF had a VTN concentration 10-200 times higher than the other patients. This patient was a previous smoker and had been treated for lung carcinoma. At inclusion, the patient was currently treated for pulmonary nocardiosis and died shortly after. It is not clear which condition that caused the remarkably high VTN concentration. As sampling and analysis conditions differ between the previous studies on lung VTN, exact values are not possible for comparison. However, the range of VTN concentrations in patients with pneumonia or endotoxin-exposed subjects in this study resemble the VTN concentrations of subjects with untreated sarcoidosis [23].

All healthy study cases that were subjected to endotoxin instillation developed increased extracellular VTN levels in BALF after 12h and this elevated level remained after 48 h. The increase was local and limited to the lung segment that was exposed to endotoxin. The experimental setup mimics the presence of Gram-negative bacteria in the respiratory tract where LPS is exposed on shed EVs and at the bacterial surface. Hence, the bacterial components cause an inflammatory response by the pathogen responsive cells of the innate immunity that in turn lead to increased VTN in the extracellular fluid of the respiratory tract. In addition to any VTN that entered the bronchial lumen through exudate, it has been reported that bronchial epithelial cells express *VTN* mRNA [32]. Since we observed VTN in the cytoplasm and on the cilia of bronchial epithelial cells collected from a patient with pneumonia, we hypothesized that epithelial cells were a local source of VTN in the lower respiratory tract. By stimulating immortalized respiratory epithelial cells *in vitro* with EVs from *H. influenzae* and *P. aeruginosa*, we could show that epithelial cells were not only capable of producing VTN, the production was increased as a response to stimuli by PAMPs on EVs. Hence, respiratory epithelial cells are a local source of VTN in the respiratory tract and this expression is altered by local bacterial stimuli.

VTN has been attributed roles in, for instance, tissue repair, chemotaxis and complement regulation, which are all related to inflammation or tissue damage. Though VTN contain antimicrobial heparin-binding domains (HBD), VTN is protective for many pathogens as surface acquisition protects from the formation of and subsequent lysis by the MAC complex [6,33]. In the current study, we report that VTN in BALF is biologically active and at a sufficient concentration for bacterial capture, even though the concentration in BALF is severely diluted and may contain potentially blocking or interfering molecules. After incubation in crude (but heat-inactivated) BALF, bacteria were protected from complement, while VTN-depleted BALF did not give any protection. As complements are ever ready and potent parts of the innate immunity, this property is imperative for the invading and colonizing pathogenic bacteria.

In this study, we report that VTN is an acute phase protein in the human lung. It is significantly increased during pneumonia, as a response of LPS instillation, and is produced by respiratory epithelial cells *in vitro* upon stimulation by LPS in the context of EVs. Finally, we could confirm an intricate host-pathogen interaction, where bacterial PAMPs trigger VTN production in respiratory epithelial cells, which the same bacteria subsequently can use to evade the innate immunity.

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Paper IV

Eur J Clin Microbiol Infect Dis
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ORIGINAL ARTICLE

Antimicrobial combination treatment including ciprofloxacin decreased the mortality rate of *Pseudomonas aeruginosa* bacteraemia: a retrospective cohort study

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Abstract Ineffective antimicrobial therapy of *Pseudomonas aeruginosa* bacteraemia increases mortality. Recent studies have proposed the use of antimicrobial combination therapy composed of a beta-lactam with either ciprofloxacin or tobramycin. To determine if combination therapy correlates to lower mortality and is superior compared to monotherapy, we investigated the effect of antimicrobial treatment regimens on 30-day mortality in a cohort with *Pseudomonas aeruginosa* bacteraemia. All cases of *P. aeruginosa* bacteraemia ($n = 292$) in southwest Skåne County, Sweden (years 2005–2010, adult population 361,112) and the whole county (2011–2012, 966,130) were identified. Available medical and microbiological records for persons aged 18 years or more were reviewed ($n = 235$). Antimicrobial therapy was defined as empiric at admission or definitive after culture results and was correlated to 30-day mortality in a multivariate regression model. The incidence and mortality rates were 8.0 per 100,000 adults and 22.9% (67/292), respectively. As expected, multiple comorbidities and high age were associated with mortality. Adequate empiric or definitive antipseudomonal treatment was associated with lower mortality than other antimicrobial alternatives (empiric $p = 0.02$, adj. $p = 0.03$; definitive $p < 0.001$, adj. $p = 0.007$). No difference in mortality was seen between empiric antipseudomonal monotherapy or empiric combination therapy. However, definitive combination therapy including ciprofloxacin correlated to lower mortality than monotherapy ($p = 0.006$, adj. $p = 0.003$), whereas combinations including tobramycin did not. Our results underline the importance of adequate antipseudomonal treatment. These data also suggest that *P. aeruginosa* bacteraemia should be treated with an antimicrobial combination including ciprofloxacin when susceptible.

Abbreviations

AIDS	Acquired immune deficiency syndrome
BSI	Bloodstream infection
CCI	Charlson comorbidity index
CF	Cystic fibrosis
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
MIC	Minimum inhibitory concentration
OR	Odds ratio
SD	Standard deviation

Introduction

Bacteraemia caused by the opportunist *Pseudomonas aeruginosa* is a serious condition that has increased in incidence [1]. It is associated with high age, multiple comorbidities and advanced or prolonged healthcare [1]. Hence, the incidence rate of *P. aeruginosa* bacteraemia is likely to continue to rise as healthcare services are advancing and life expectancy continues to increase [1, 2]. It is one of the most common Gram-negative bloodstream infections (BSI), only preceded by *Escherichia coli* and *Klebsiella* spp. However,

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the mortality of *P. aeruginosa* BSI has consistently been reported to be higher than that of *E. coli* BSI (23–36%) [1, 3–8].

Treatment of pulmonary infections caused by *P. aeruginosa* has been the topic of several reviews [9–11]. Patients with cystic fibrosis (CF) are often colonised by *P. aeruginosa*, but it is rare that CF patients suffer from bacteraemia [12]. *Pseudomonas aeruginosa* also occasionally colonises chronic wounds and the gastrointestinal and urinary tracts, particularly in hospitalised patients or patients with indwelling catheters [13]. *Pseudomonas* strains causing long-term colonisation of CF patients have adapted to become less virulent, but are extremely resistant to antimicrobials due to altered efflux pumps, porins, beta-lactamases with extended substrate specificity and biofilm formation [14]. On the other hand, invasive isolates are generally more susceptible to antimicrobials, although strains with extensive antimicrobial resistance have been reported [15]. The optimal antimicrobial treatment regimens against *P. aeruginosa* in the airway may, thus, differ from optimal treatment regimens against bacteraemia and conclusions drawn from studies on pneumonia may not be generalisable to bacteraemia.

The most adequate treatment regimen of *P. aeruginosa* bacteraemia is a matter of debate. Despite the species being often highly resistant to antimicrobials, there are normally still several antimicrobial treatment regimens to choose from, either as monotherapy or combination therapy. Ineffective empiric antimicrobial therapy has been associated with increased mortality [16, 17]. Commonly used antipseudomonal drugs include penicillins with beta-lactamase inhibitors, certain cephalosporins, carbapenems, colistin, fluoroquinolones and aminoglycosides. Combination therapy is often administered to critically ill patients and most combinations include a beta-lactam antimicrobial together with either a fluoroquinolone or an aminoglycoside. Several studies have addressed the question as to whether to use monotherapy or combination therapy and the conclusions drawn are conflicting [16–20]. The assumption that combination therapy including either fluoroquinolones or aminoglycosides would have an equivalent effect on *P. aeruginosa* bacteraemia is, however, not necessarily correct. Earlier studies are inconclusive due to the insufficient number of patients in each group or stratification into combined groups of both fluoroquinolones and aminoglycosides. Hence, the effect on bacteraemia of adding a fluoroquinolone or an aminoglycoside to a beta-lactam is unclear at present.

We investigated the effect on 30-day mortality of different antimicrobial treatment regimens against *P. aeruginosa* bacteraemia. We observed that both empiric therapy on admission and definitive therapy after culture results affected mortality. Combination therapy with a beta-lactam and ciprofloxacin was significantly associated with a lower mortality compared to monotherapy. Moreover, the design of our population-based retrospective cohort allowed us to observe unbiased incidence rates and antimicrobial susceptibility patterns.

Materials and methods

Study population and setting

The cohort comprised the adult population (aged ≥18 years) of southwest Skåne County, Sweden during a 6-year period (2005–2010; adult population 361,112) in addition to the entire county during a period of 2 years (2011–2012; adult population 966,130). The area corresponded to the catchment area of the microbiological laboratory in Malmö that was expanded in 2011 because of fusion with an adjacent laboratory. The included laboratories analysed 100% of the blood cultures sampled in the area. Hospital care was provided by Skåne University Hospital and surrounding regional hospitals.

Participants, study protocol and variables

We identified 292 unique individuals with *P. aeruginosa* bacteraemia. Recurrent cases were only included once. Incidence rates were calculated using yearly population data collected from Statistics Sweden [21]. Microbiological culture data were collected from the laboratory's records (wwLab; Autonik, Sköldinge, Sweden). Data on susceptibility and concurrent infections were collected, as were the time and date of preliminary and definitive culture results. Clinical data were collected from the hospital patient records (Melior; Siemens Healthcare Services, Upplands Väsby, Sweden). Thirty-day mortality was analysed as the outcome variable for all study cases ($n = 292$), as presented graphically in Fig. 1. Patients with available hospital medical records were included for correlations with comorbidities ($n = 235$). Sixteen patients had incomplete medication charts and were excluded from correlations with treatments ($n = 219$). Missing data were missing at random, with more missing records during the first years of the study, gradually decreasing over the study period. Randomness was controlled by comparing sex and age for missing and non-missing files. All recorded clinical variables and healthcare-related data are presented in the supplementary data, Table A1. The Charlson comorbidity index (CCI) was calculated to estimate the level of illness prior to the current bacteraemia (supplementary data, Table A2) [22]. Compound variables included pulmonary disease [chronic obstructive pulmonary disease (COPD), pulmonary fibrosis, asthma and cystic fibrosis] and heart disorder (congestive heart failure, ischaemic heart disease, cardiac arrhythmia and heart valve disease).

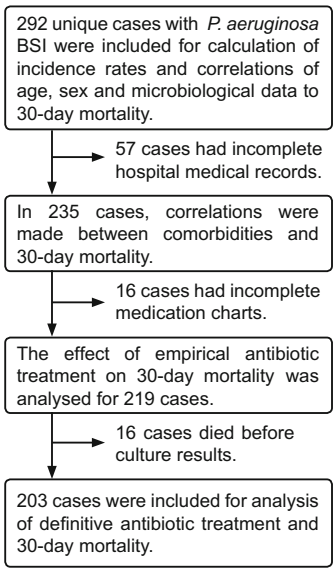

Fig. 1 Patients included in this study. Flow chart summarising the number of cases in the present study and the reason for exclusion for some analyses

Microbiological definitions and methods

Pseudomonas aeruginosa bacteraemia was defined as the isolation of the bacterial species in a blood culture collected from a patient at a hospital out-patient unit, a hospital ward or an emergency department using standard aseptic techniques. The term 'bacteraemia' was considered to be equivalent to BSI. All samples were cultured using the automated BacT/ALERT system (bioMérieux, Marcy l'Etoile, France). Isolates were identified by typical characteristics on agar plates and biochemical tests. Antimicrobial resistance was determined by disc diffusion on Mueller–Hinton agar plates, Etests (Biodisk, Solna, Sweden) or Vitek (bioMérieux) and subjected to antimicrobial susceptibility testing according to guidelines from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [23]. Physicians were notified when preliminary results on positive blood cultures were available.

Antimicrobial treatment

Empiric therapy was defined as antimicrobial therapy administered from 8 h before sampling of the initial blood culture to the time the preliminary culture result was available. Antimicrobial treatment was classified as antipseudomonal if it included any of the following: ceftazidime, imipenem, meropenem, ciprofloxacin, piperacillin, colistin or combinations including gentamicin or tobramycin. Definitive therapy was defined as antimicrobial therapy given after preliminary or final culture results. Administered antimicrobials were compared with obtained antibiograms and classified as adequate if the bacterium was susceptible to the given drug in question.

Statistical analysis

All data were entered into an Excel spreadsheet (Microsoft, Redmond, WA) and exported to SPSS Statistics version 23 (IBM, Armonk, NY) and Stata 14 (StataCorp, College Station, TX) for statistical analysis. Graphs were drawn in Prism 6 (GraphPad, La Jolla, CA). The results were expressed as median and interquartile range for continuous variables and as frequencies and percentages for categorical variables. Two-tailed p-values were calculated with Wilcoxon's and Fisher's exact test and values ≤ 0.05 were considered statistically significant. Odds ratios (ORs) were determined in the univariate analysis and presented with 95% confidence intervals (CIs). The adjusted OR for mortality was determined in a multivariate logistic regression model. We used stepwise backward selection to create the multivariable model in which all variables with p-values of ≤ 0.1 were entered, and variables with resulting p-values exceeding 0.2 were excluded. Age was stratified into categories, with 18–49 years as the index. A propensity score-adjusted analysis was performed that included the following covariates: age, sex, pulmonary, cardiovascular, renal, hepatic, neurological and malignant comorbidities, together with healthcare indicators: catheters, surgery, intensive care and coinfections. The propensity scores of receiving ciprofloxacin as combination therapy as well as monotherapy were each introduced into separate logistic regression models using ciprofloxacin treatment as a binary regression variable.

Results

The incidence of *P. aeruginosa* BSI increases with high age and male sex

The average annual incidence rate of *P. aeruginosa* bacteraemia was 8.0 [standard deviation (SD) ± 1.22] cases per 100,000 adult inhabitants. The median age of the study cases was 74 years, well above the median age of the adult population (47 years, Fig. 2a) and the calculated incidence rate was highest for the oldest age groups (age ≥80 years: 31.0/100,000 adult inhabitants). As seen in previous studies, the incidence was higher for males than females (Fig. 2b) [5]. The most common infection focus was the urinary tract (94/235, 40.7%), followed by the respiratory tract (42/235, 18.2%), wounds (38/235, 16.5%) and intravenous catheters

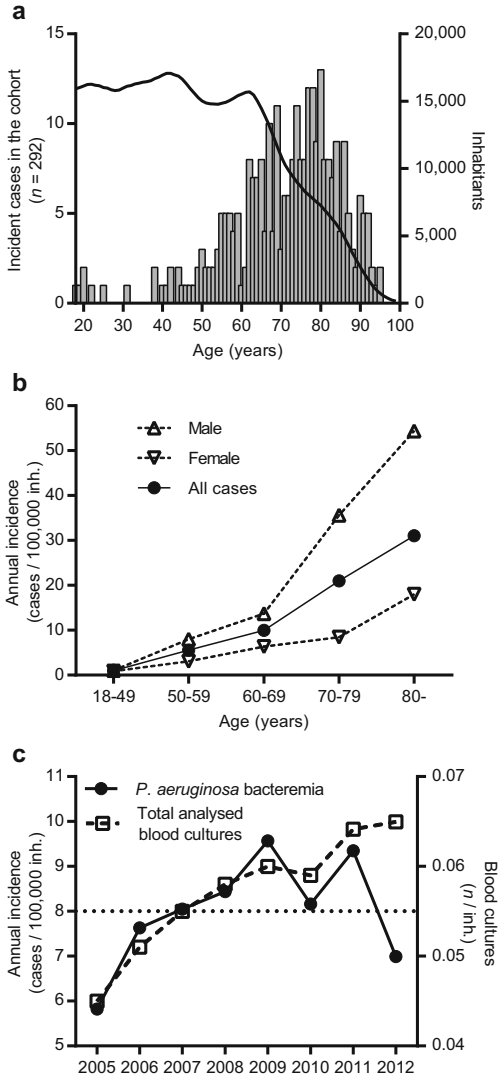

Fig. 2 *Pseudomonas aeruginosa* bacteraemia incidence increases with age. Incident cases of *P. aeruginosa* bacteraemia were older (**a**, bars, left *y*-axis) than the population in Skåne County (**a**, curve, right *y*-axis). In all age groups, the incidence of *P. aeruginosa* bacteraemia was higher among males than females and increased with higher age (**b**). No increase in incidence was seen over the entire study period (**c**, left *y*-axis), even though the number of analysed blood cultures increased (**c**, right *y*-axis)

(9/235, 3.9%). The remaining individuals (48, 20.8%) had unknown infection site. Urinary tract focus was more common among men (males 77/159, 49.3% vs. females 17/75, 22.6%, $p < 0.001$) and most men with this focus had had a urinary catheter for more than one week (51/77, 66.2% vs. other foci 18/61, 22.8%, $p < 0.001$). During the first five years of the study, a yearly increase was seen in incident cases. This trend, however, ceased in 2010 and, in total, we could not observe any significant change in incidence rate, although the number of analysed blood cultures increased throughout the study period (Fig. 2c).

Comorbidities, high age and respiratory tract focus correlate to increased mortality

The overall 30-day mortality for the study population was 22.9% (67/292). As expected, the surviving population was younger (mean age 69.8 vs. 74.4 years, $p = 0.02$). Basic demographic data, comorbidities and healthcare-related data of all included patients are presented in Table 1. To assess the effect of multiple comorbidities, we calculated the CCI from age and comorbidities. The CCI and selected variables were correlated to 30-day mortality in univariate and multivariate regression analysis (Table 2). Females had a higher mortality rate than males (adj. $p = 0.006$) and a higher CCI correlated strongly to higher 30-day mortality (survivors mean CCI 6.9 vs. non-survivors 8.5, $p = 0.002$). Pulmonary, haematological or metastatic malignant diseases were significantly associated with high 30-day mortality, whereas recent chemotherapy or diabetes mellitus correlated with lower 30-day mortality. Infection focus in the respiratory tract was associated with higher mortality and urinary tract infection or the presence of a urinary catheter with lower mortality. Coinfection with other pathogens was detected in 90 (31%) cases. The most common copathogens were *Enterobacteriaceae* but also a diverse flora of enterococci, staphylococci and anaerobes were recorded. Coinfections were associated with increased 30-day mortality ($p = 0.02$). Nosocomial infection defined as being a resident at a nursing home or being hospitalised for more than one week before blood culture was not associated with increased mortality rates.

Antimicrobial resistance against *P. aeruginosa* did not increase during the study period

Antimicrobial resistance was generally low for the studied bacterial strains compared to previous studies. Imipenem resistance was 6.8%, meropenem 6.3%, piperacillin–tazobactam 6.2%, tobramycin 0.7%, ceftazidime 5.5% and ciprofloxacin 10.0%. Except for a peak in meropenem resistance in 2008, the antimicrobial resistance did not increase during the study period (supplementary data, Table A3). In univariate correlations, only meropenem resistance was associated with increased 30-day mortality (6 fatalities from 13 cases, 46.2%, $p = 0.033$).

Table 1 Basic demographic data of cases in this study

Characteristic	Male ($n = 159$)	Female ($n = 76$)
Age	74 (63–80)	74 (63–83)
Charlson score	7 (5–9)	6 (5–9)
Pulmonary disease*	34 (21.4)	15 (19.7)
COPD	17 (10.7)	7 (9.2)
Cystic fibrosis	1 (0.6)	0 (0.0)
Heart disorder*	62 (39.0)	25 (32.9)
Peripheral vascular disease	40 (25.2)	15 (19.7)
Vascular graft	11 (6.9)	4 (5.3)
Diabetes mellitus	50 (31.4)	22 (28.9)
Renal failure	36 (22.6)	6 (7.9)
Chronic liver disease	4 (2.5)	4 (5.3)
Neurological paresis	4 (2.5)	0 (0.0)
Immunosuppression	35 (22.0)	17 (22.4)
Chemotherapy in the last 6 months	30 (18.9)	22 (28.9)
Solid malignancy	45 (28.3)	28 (36.8)
Metastasis	24 (15.1)	15 (19.7)
Haematological disease	26 (16.4)	10 (13.2)
Neutropaenia	24 (15.1)	14 (18.4)
AIDS	1 (0.6)	0 (0.0)
Burn wounds	4 (2.5)	2 (2.6)
Urinary catheter >1 week	70 (44.0)	18 (23.7)
Hospitalised >1 week	58 (36.5)	24 (31.6)
Surgery in the last month	34 (21.4)	14 (18.4)
Resident at nursing home	26 (16.4)	7 (9.2)

Age for all cases ($n = 292$) and characteristics for cases with full medical records ($n = 235$). Continuous variables are expressed as median (interquartile range) and categorical variables as observed numbers (percentage). Compound variables are marked with *

Empiric antimicrobial treatment and importance for mortality

Of the 219 patients with complete medication charts, almost all patients (93.2%) received antimicrobial treatment after cultures were drawn. In 47.5%, adequate empirical antimicrobial agents were given according to the antibiograms. The most common empiric treatment was cefotaxime (36.5%), followed by tobramycin (18.3%) and piperacillin (16.9%). The empiric use of any other antimicrobial was less than 10%. The administration of tobramycin was, in all cases, combined with another antimicrobial. Monotherapy was given to 125 patients (57.1%) and 79 patients received combination therapy (36.1%).

The choice of empiric treatment was associated with 30-day mortality in our cohort in direct correlations and when entered into a multivariable regression model (Table 3, upper section). Treatment with any effective antipseudomonal significantly decreased the 30-day mortality to 15.4% (adj. $p = 0.03$). In contrast, an increased 30-day mortality (53.3%; adj. $p = 0.01$) was observed when antimicrobials were not administered. Forty cases (18.2%) received empiric combination treatment including tobramycin, which did not decrease the mortality rate compared to monotherapy. Only five cases (2.3%) received empirical combination therapy including ciprofloxacin.

Definitive antimicrobial therapy with ciprofloxacin combinations increases survival

Sixteen patients (7.3%) died before any culture results were available. All surviving patients ($n = 203$) were administered definitive antimicrobial treatment following positive blood culture results with *P. aeruginosa*. The majority of cases (174; 85.7%) were given an effective antipseudomonal treatment. Preliminary culture results were provided for 93.1% and the mean time to preliminary culture result was 1.96 days (SD ± 1.19) and 3.68 days (SD ± 1.63) to final results. More than half were treated with ciprofloxacin (50.7%), as a part of a combination therapy (38.4%) or as monotherapy (12.3%). Piperacillin–tazobactam was given to 33.0%, carbapenem to 21.2%, tobramycin to 17.2% and, finally, ceftazidime to 10.3% of the cases. Other antimicrobial regimens were administered to less than 10% of patients.

The choice of an adequate definitive antipseudomonal treatment matching the antibiogram significantly decreased 30-day mortality (adj. $p = 0.007$) when analysed in our multivariable regression model (Table 3; lower section). Targeted monotherapy was not significantly associated with decreased 30-day mortality, whereas inadequate treatment with cefotaxime or cefuroxime as monotherapy increased mortality up to 40% (4/10).

In contrast to any individual monotherapy, definitive combination therapy with ciprofloxacin decreased the 30-day mortality (adj. $p = 0.003$), whereas combinations including tobramycin did not affect the mortality. To disclose involuntary selection bias in the group receiving ciprofloxacin, a propensity score was calculated. The effect of the addition of ciprofloxacin was independent of confounders (age, sex, comorbidities and intensive care treatment, as well as choice of antimicrobial agent). With the available covariates, we did not find any evidence of selection bias to any of the ciprofloxacin/no ciprofloxacin groups. Propensity score-adjusted analyses did not change the effect on mortality for ciprofloxacin combination therapy (propensity score-adjusted OR 0.16, 95% CI 0.05–0.53, $p = 0.003$ vs. OR 0.16, 95% CI 0.05–0.53, $p = 0.003$). For ciprofloxacin monotherapy, the association remained non-significant (propensity score-adjusted OR 0.59, 95% CI 0.04–9.9, $p = 0.72$ vs. OR 0.32, 95% CI 0.06–1.83, $p = 0.20$). The lower mortality associated with ciprofloxacin combination therapy was independent of

Table 2 Thirty-day mortality correlated to selected characteristics

Characteristic ($n = 292$)	n	Died (%)	OR (95% CI)	p-value	adj. OR (95% CI)	adj. p-value
Male sex	201	40 (19.9)	0.59 (0.33–1.04)	0.07	**0.35 (0.17–0.74)**	**0.006**
Age 18–49 years	22	2 (9.1)	1.00			
Age 50–59 years	36	6 (16.7)	2.00 (0.37–10.92)	0.42	2.72 (0.39–19.08)	0.31
Age 60–69 years	62	14 (22.6)	2.92 (0.61–14.03)	0.18	3.21 (0.51–20.03)	0.21
Age 70–79 years	83	20 (24.1)	3.17 (0.68–14.78)	0.14	3.58 (0.58–22.17)	0.17
Age ≥80 years	89	25 (28.1)	3.91 (0.85–17.95)	0.08	**6.60 (1.13–38.49)**	**0.04**
Comorbidity ($n = 235$)						
Pulmonary disease	49	18 (36.7)	2.58 (1.29–5.14)	0.01	**3.05 (1.34–6.94)**	**0.008**
COPD	24	8 (33.3)	1.89 (0.76–4.69)	0.20	0.61 (0.16–2.37)	0.47
Cystic fibrosis	1	0 (0.0)				
Heart disorder	87	22 (25.3)	1.33 (0.71–2.49)	0.42	1.50 (0.64–3.51)	0.35
Peripheral vascular disease	55	13 (23.6)	1.14 (0.56–2.34)	0.71	2.26 (0.96–5.30)	0.06
Vascular graft	15	1 (6.7)	0.24 (0.03–1.83)	0.20	0.18 (0.02–1.69)	0.13
Diabetes mellitus	63	9 (14.3)	0.49 (0.22–1.08)	0.08	**0.46 (0.22–0.96)**	**0.04**
Renal failure	42	10 (23.8)	1.12 (0.51–2.46)	0.84	1.96 (0.74–5.17)	0.18
Chronic liver disease	9	3 (33.3)	1.79 (0.43–7.40)	0.42	1.27 (0.22–7.21)	0.79
Neurological paresis	3	1 (33.3)	1.16 (0.12–11.36)	1.00	7.61 (0.45–128.36)	0.16
Immunosuppression	52	11 (21.2)	0.91 (0.43–1.93)	0.85	0.75 (0.26–2.18)	0.59
Chemotherapy in the last 6 months	52	12 (23.1)	1.05 (0.50–2.19)	1.00	**0.21 (0.07–0.66)**	**0.007**
Solid malignancy	73	20 (27.4)	1.55 (0.81–2.94)	0.23	0.99 (0.37–2.68)	0.98
Metastasis	39	14 (35.9)	2.33 (1.11–4.90)	0.03	**7.12 (2.32–21.79)**	**0.001**
Haematologic disease	42	11 (26.2)	1.63 (0.74–3.59)	0.28	**5.47 (1.85–16.17)**	**0.002**
Neutropaenia	38	11 (28.9)	1.51 (0.69–3.30)	0.30	1.33 (0.41–4.28)	0.63
AIDS	1	0 (0.0)				
Burn wounds	6	2 (33.3)	1.77 (0.32–9.95)	0.62	4.08 (0.45–36.68)	0.21
Composite comorbidity score ($n = 235$)						
Charlson score ≤4	44	4 (9.1)	1.00			
Charlson score 5–8	119	27 (22.7)	2.93 (0.96–8.94)	0.06	2.91 (0.95–8.92)	0.06
Charlson score 9–12	49	13 (26.5)	3.61 (1.08–12.08)	0.04	**3.67 (1.09–12.41)**	**0.04**
Charlson score ≥13	21	8 (38.1)	6.15 (1.59–23.82)	0.009	**7.05 (1.79–27.86)**	**0.005**
Healthcare-related ($n = 235$)						
Urinary catheter >1 week	88	18 (20.5)	0.87 (0.45–1.65)	0.75	1.09 (0.50–2.39)	0.82
Surgery last month	48	9 (18.8)	0.76 (0.34–1.69)	0.56	0.98 (0.38–2.51)	0.96
Hospitalised >1 week	82	20 (24.4)	1.21 (0.64–2.29)	0.62	1.61 (0.76–3.41)	0.22
Resident at nursing home	33	8 (24.2)	1.15 (0.48–2.72)	0.82	1.16 (0.41–3.32)	0.78
Polymicrobial infection	90	22 (24.4)	1.13 (0.62–2.02)	0.68	**2.52 (1.18–5.40)**	**0.02**
Origin of infection ($n = 235$)						
Urinary tract	82	12 (14.6)	0.37 (0.18–0.75)	0.005	**0.28 (0.12–0.65)**	**0.003**
Respiratory tract	44	17 (38.6)	2.81 (1.38–5.70)	0.003	**2.81 (1.13–7.01)**	**0.03**
Wound	38	12 (31.6)	1.81 (0.84–3.90)	0.13	1.99 (0.81–4.92)	0.13
Central venous catheter	9	0 (0.0)				
Other/unknown	50	11 (22.0)	0.99 (0.47–2.11)	0.98	1.16 (0.47–2.83)	0.75

Patient characteristics correlated to 30-day mortality and presented with odds ratio (*OR*), adjusted odds ratio (*adj. OR*) and 95% confidence interval (*95% CI*). The multivariable model contained age, sex, pulmonary disease, vascular graft, peripheral vascular disease, chemotherapy in the last 6 months, haematological diseases including malignancies, metastasis, diabetes mellitus and neurological paresis. Significant values are in **bold font**

age, CCI and origin of infection (Fig. 3a–c). Importantly also, patients in intensive care units that were treated with ciprofloxacin had a lower 30-day mortality than those receiving other antimicrobials (0 dead of 9 treated: 0% vs. other: 7/19, 36.8%, adj. $p = 0.035$).

Discussion

In this retrospective cohort study, we provide evidence that the choice of antimicrobial treatment affected the 30-day mortality in patients suffering from *P. aeruginosa* bacteraemia.

Table 3 The antimicrobial treatment choice influences 30-day mortality

	30-Day mortality, empirical treatment ($n = 219$)					
	n	Died (%)	OR (95%)	*p*-value	adj. OR (95% CI)	adj. *p*-value
Cefotaxime or cefuroxime	100	23 (23.0)	1.06 (0.56–2.00)	0.87	0.68 (0.31–1.49)	0.34
Benzylpenicillin	8	4 (50.0)	3.51 (0.85–14.42)	0.08	3.09 (0.52–18.38)	0.22
Imipenem or meropenem	34	5 (14.7)	0.55 (0.20–1.51)	0.25	0.84 (0.23–3.12)	0.79
Piperacillin–tazobactam	37	7 (18.9)	0.78 (0.32–1.90)	0.58	0.61 (0.20–1.89)	0.39
Ciprofloxacin	11	1 (9.1)	0.33 (0.04–2.64)	0.30	0.57 (0.06–5.56)	0.63
Combination including tobramycin	40	10 (25.0)	1.20 (0.54–2.66)	0.66	1.10 (0.39–3.11)	0.85
Any other combination	39	6 (15.4)	0.58 (0.23–1.48)	0.30	0.40 (0.13–1.27)	0.12
No empirical antibiotic treatment	15	8 (53.3)	4.52 (1.55–13.20)	0.007	**5.84 (1.43–23.84)**	**0.01**
Adequate antipseudomonal treatment	104	16 (15.4)	0.45 (0.23–0.88)	0.02	**0.37 (0.16–0.89)**	**0.03**
	30-Day mortality, definitive treatment ($n = 203$)					
	n	Died (%)	OR (95% CI)	*p*-value	adj. OR (95% CI)	adj. *p*-value
Cefotaxime or cefuroxime	10	4 (40.0)	3.93 (1.04–14.81)	0.043	5.59 (0.94–33.35)	0.06
Imipenem or meropenem	43	5 (11.6)	0.65 (0.23–1.80)	0.41	1.26 (0.35–4.49)	0.73
Piperacillin–tazobactam	67	11 (16.4)	1.05 (0.47–2.32)	1.00	1.07 (0.40–2.87)	0.89
Ceftazidime	21	1 (4.8)	0.24 (0.03–1.88)	0.18	0.19 (0.02–1.91)	0.16
Ciprofloxacin, monotherapy	25	2 (8.0)	0.43 (0.10–1.92)	0.27	0.32 (0.06–1.83)	0.20
Combination including ciprofloxacin	78	5 (6.4)	0.25 (0.09–0.68)	0.006	**0.16 (0.05–0.55)**	**0.003**
Combination including tobramycin	35	4 (11.4)	0.65 (0.21–1.97)	0.44	1.23 (0.31–4.91)	0.77
Adequate antipseudomonal treatment	174	20 (11.5)	0.17 (0.07–0.41)	<0.001	**0.17 (0.05–0.62)**	**0.007**

The effect of empiric and definitive antimicrobial treatment on 30-day mortality. Correlations to 30-day mortality presented as odds ratio (*OR*) with 95% confidence interval (*95% CI*) and *p*-values. The multivariable model contained age, sex, lung disease, vascular graft, peripheral vascular disease, chemotherapy in the last 6 months, metastasis, haematological disease, diabetes mellitus and neurological paresis, coinfections, treatment in the intensive care unit, tracheal intubation and urinary catheter. Significant adjusted *p*-values are in **bold**. Treatment regimens given to less than five patients are not shown in this table

Despite the average time to culture results being as short as 48 h, inadequate empiric antimicrobial treatment on admission negatively affected mortality rates. Similarly, inadequate definitive treatment after blood culture results was also associated with higher mortality rates. Particularly, definitive combination treatment that included ciprofloxacin favourably affected the 30-day mortality.

In total, we identified 292 patients with *P. aeruginosa* bacteraemia between 2005 and 2012. The overall 30-day mortality in this study was 22.9% and the annual incidence was 8 per 100,000 inhabitants, with higher rates among men and with increasing age. This was in line with previous studies, which have reported 30-day mortality rates ranging from 23% to 36.5% and incidence rates in the range 3.6–10.8 cases per 100,000 inhabitants and year [1, 3–8]. The incidence was higher for males than females, which was accounted for by the higher incidence among males with urinary catheter. Although the number of blood cultures steadily increased during the study period, no increase in the incidence of *P. aeruginosa* bacteraemia was seen over the entire study period. However, in parallel to the increase reported in the UK, the incidence rate in our study rose between 2005 and 2009 [1]. The explanation for the rising incidence during the first several years of the study and the concurrent increase in the UK is, at present, unclear and no epidemiological link is known.

The importance of correct empiric therapy on admission has been debated and, in the present study, the initial antimicrobial choice was important, as patients who received adequate antipseudomonal treatment had significantly lower mortality rates [3, 17, 24]. No individual empirical antimicrobial monotherapy or combination therapy was associated with changes in mortality. The large number of different administered antimicrobials resulted in low statistical power for all but the most commonly used drugs and only five patients received empirical ciprofloxacin combination treatment.

After positive blood cultures with *P. aeruginosa*, adequate definitive antipseudomonal treatment decreased the 30-day mortality. In contrast to ciprofloxacin combinations, combination therapy including tobramycin was not superior to monotherapy, similarly to that previously reported [20]. To find the potential influence of selection bias on our results, a propensity score was calculated to ensure comparability of the cohort receiving ciprofloxacin and those receiving other treatments, but no such bias could be identified. A few previous studies have investigated whether combination therapy was favourable against *P. aeruginosa* bacteraemia. For example, Kim et al.

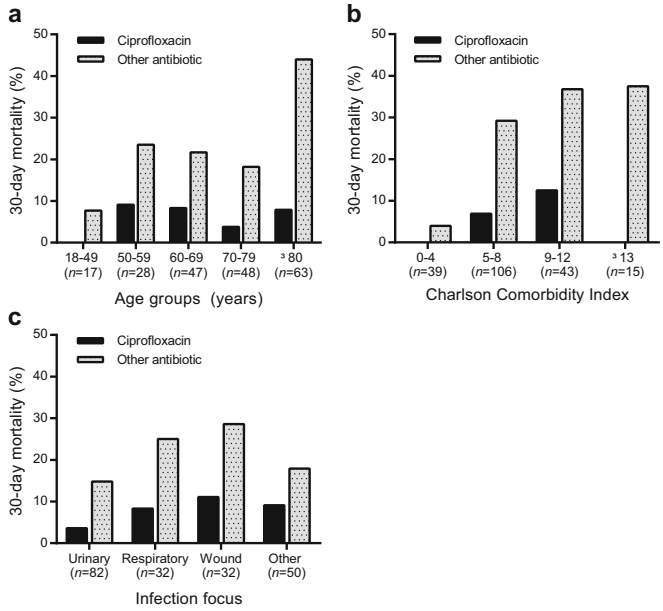

Fig. 3 Ciprofloxacin-treated cases had a lower 30-day mortality. Thirty-day mortality rates in percent after treatment with ciprofloxacin or other antimicrobial drug as definitive therapy when culture results were available. The results were stratified by age groups (**a**), comorbidity as defined by the Charlson comorbidity index (CCI) (**b**) or infection focus (**c**)

showed that adequate combination therapy of any sort was beneficial for a subgroup of bacteraemic patients with febrile neutropaenia [18]. Peña et al. reported, however, that the choice between combination therapy and single-drug therapy did not affect outcome [17]. Furthermore, in a recent meta-analysis focusing on empiric treatment, no difference in mortality was seen between the study groups, but in contrast to the present investigation, no analysis was made separately for aminoglycoside and ciprofloxacin combination therapy [19]. Our results are supported by DiMondi et al., who investigated the short-term outcome of bacteraemia and pneumonia; ciprofloxacin combinations comprised 90% of the combination therapies that were associated with favourable outcome [16]. Both beta-lactams and ciprofloxacin are highly efficient against *P. aeruginosa* in vitro. The reason for the favourable outcome for ciprofloxacin combination-treated patients in the present study is unknown. Combination therapy broadens the antimicrobial spectrum and synergy between antimicrobial drugs has been described, but synergy between ciprofloxacin and beta-lactams has not been reported [25].

In our cohort, 10.0% of the bacterial strains had reduced susceptibility for ciprofloxacin. This should be compared to three BSI studies from the USA that reported varying resistance levels (range 4.7–31%), with the highest levels from Maryland, where a majority of cases had been hospitalised for more than three days at inclusion [3, 4, 24]. Local knowledge of resistance levels is important, but it may be difficult to derive current resistance levels from surveillance programmes, as BSI may not be reported and, often, bacteraemia-causing bacteria are more susceptible. For comparison, the fluoroquinolone resistance in Skåne County of *P. aeruginosa* bacteraemia strains was 9.8% in 2011, whereas in the same year, 88.1% of *P. aeruginosa* strains from the respiratory tract of patients with CF were resistant to fluoroquinolones (unpublished data). Hence, combinations of antimicrobials including ciprofloxacin may be the most effective antipseudomonal treatment against bacteraemia, but the results are not, however, generalisable to pneumonia.

Patients suffering from BSI were of higher age than the general population and had multiple comorbidities, which also correlated to higher 30-day mortality, as did pulmonary disease (and infectious foci in the respiratory tract), malignant disease with metastasis and haematological malignancies. As inadequate antimicrobial therapy increased mortality, the likelihood of *P. aeruginosa* BSI should be considered at the choice of empiric antimicrobial therapy. Not all septic patients need empiric antipseudomonal treatment at admission, but it could be considered for patients with increased risk of *Pseudomonas* bacteraemia and with high risk of mortality. In addition to critically ill patients, these results suggest that patients with neoplasia and the elderly with multiple comorbidities could be eligible for such treatment. However, laboratory tests in vitro have shown that the use of ciprofloxacin at sub-MIC concentrations lead to bacterial mutations, causing resistance against both ciprofloxacin and beta-lactam antimicrobials [26]. Hence, any decisions to use ciprofloxacin should

be made wisely in order to limit further resistance development and monotherapy with ciprofloxacin should probably be avoided.

Our study cohort had a minimised population selection bias and, consequently, allowed for observation of, in total, 4.2 million adult person-years. The records at the microbiology laboratories allowed us to identify every occurrence of *P. aeruginosa* bacteraemia. This population-based approach allowed for a valid analysis of the incidence rate. We were not able to use a scoring system for severity of disease; instead this was determined by treatment at an intensive care unit. In the future, a prospective randomised interventional study to examine the effects of treatment regimens would be of high value, although the relative infrequency of *P. aeruginosa* bacteraemia would make this challenging in practice. An alternative would be a larger retrospective trial with emphasis on markers of acute severe disease.

In conclusion, this study gives an indication that *P. aeruginosa* bacteraemia should be treated with definitive antimicrobial drug combination regimens including ciprofloxacin when susceptible. Inadequate empiric antipseudomonal treatment on admission or inadequate definitive therapy after notification of positive blood cultures was associated with increased mortality. These results are of particular importance to those at the greatest risk for *P. aeruginosa* bacteraemia, the elderly patients with multiple comorbidities and patients with malignancy. Appropriate antipseudomonal treatment should be considered for these patients as early as possible to minimise the risk of death.

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Compliance with ethical standards

Conflict of interest Dr Jonas Ahl has received speaker honorarium from Pfizer, AstraZeneca, Meda and MSD, and research grants from Pfizer for a study not related to the present work. All remaining authors declare that they have no conflicts of interest.

Ethical approval Informed consent was not relevant to this retrospective study according to the Regional Ethical Review Board in Lund, Sweden, who granted approval of the present study (Dnr 2014/10).

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