Bacterial Outer Membrane Vesicles. Mediators of virulence and antibiotic resistance.

Schaar, Viveka

2013

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Bacterial Outer Membrane Vesicles

Mediators of virulence and antibiotic resistance

Viveka Schaar

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DOCTORAL DISSERTATION
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To be defended at the main lecture hall of the Pathology building, Skåne University Hospital Malmö, on Friday October 18th 2013 at 13:00.

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<tr><td>Organization
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Author(s)</td><td>Document name

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<tr><td colspan="2">Title and subtitle</td></tr>
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<tr><td colspan="2">Key words</td></tr>
<tr><td colspan="2">Classification system and/or index terms (if any)</td></tr>
<tr><td colspan="2">Supplementary bibliographical information
Language</td></tr>
<tr><td colspan="2">ISSN and key title 1652-8220
ISBN 978-91-87449-83-3</td></tr>
<tr><td>Recipient’s notes</td><td>Number of pages
Price
Security classification</td></tr>
</table>

Signature _____Date _____

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LUND
UNIVERSITY

Lund University, Faculty of Medicine
Department of Laboratory Medicine, Malmö
Doctoral Dissertation Series 2013:111
ISBN 978-91-87449-83-3
ISSN 1652-8220
Cover photo: Manipulated TEM image of OMV-secreting *Moraxella catarrhalis*, reprinted with permission from Oxford University Press and Springer Science
Printed in Sweden by Media-Tryck, Lund University
Lund 2013

Till Absent Friends

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Schaar, V., Nordström, T., Mörgelin, M. and Riesbeck, K. (2011). *Moraxella catarrhalis* outer membrane vesicles carry beta-lactamase and promote survival of *Streptococcus pneumonia* and *Haemophilus influenzae* by inactivating amoxicillin. *Antimicrobial Agents and Chemotherapy* **55**(8): 3845-53.

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Schaar, V., Uddbäck I., Nordström T., and Riesbeck, K. (2013) Group A Streptococci are protected from amoxicillin-mediated killing by vesicles containing β-lactamase derived from *Haemophilus influenzae*. *Journal of Antimicrobial Chemotherapy*, Aug 2, in press.

Abbreviations

AOM	Acute Otitis Media
APC	Antigen-Presenting Cell
BLNAR	β-lactamase Negative Ampicillin Resistant
BLPACR	β-lactamase Positive Ampicillin-Clavulanate Resistant
BLPAR	β-lactamase Positive Ampicillin Resistant
CEACAM	Carcinoembryonic Antigen-related Cell Adhesion Molecule
cOME	Chronic Otitis Media Effusion
COPD	Chronic Obstructive Pulmonary Disease
CPR	C-reactive Protein
ECM	Extracellular Matrix
E-test	Epsilometer test
ELISA	Enzyme-linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorting
FITC	Fluorescein Isothiocyanate
GAS	Group A Streptococci
Ig	Immunoglobulin
IL	Interleukin
kDa	kilo Dalton
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MBL	Mannose-Binding Lectin
MHC	Major Histocompability Complex

MIC	Minimal Inhibitory Concentration
MID	Moraxella IgD-binding Protein
NAD	Nicotinamide Adenine Dinucleotide
NTHi	Non-typeable *Haemophilus influenzae*
NLR	Nod-like receptor
OME	Otitis Media Effusion
OMP	Outer Membrane Protein
OMV	Outer Membrane Vesicles
PAMP	Pathogen-Associated Molecular Patterns
PBP	Penicillin-Binding Protein
PRR	Pathogen-Recognition Receptor
rAOM	Reccuring Acute Otitis Media
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TEM	Transmission Electron Microscopy
TLR	Toll-Like Receptor
Usp	Ubiquitous surface protein

Populärvetenskaplig sammanfattning

Luftvägsinfektioner som orsakas av bakterier och virus är en av de ledande orsakerna till sjukdom i världen. De kännetecknas av inflammation i svalg, hals, näsa, öron eller i lungorna. I näsa och hals finns en normalflora av bakterier som lever i samspel med sin värd och som normalt sett inte orsakar infektioner. Ibland kan dessa bakterier ändå orsaka sjukdom, som då immunsystemet är försvagat eller då de skyddande ytskikt som finns hos kroppens egna celler förstörts. *Moraxella catarrhalis* är en sådan bakterie som främst orsakar öroninflammation hos små barn samt andra infektioner hos vuxna, bland annat är de med KOL, kronisk obstruktiv lungsjukdom, mer utsatta.

För att kunna kolonisera oss människor och orsaka infektion har bakterier utvecklat imponerande mekanismer för att kunna fästa och överleva inuti sin värd. Bakterier har t ex specifika molekyler på ytan som gör att de kan fästa vid kroppens celler, samma molekyler som känns igen av kroppens celler som främmande och sätter igång immunförsvaret. Bakterierna har därför utvecklat mekanismer för att undvika att bli upptäckta. De kan t ex ”gömma” sig inuti kroppens egna celler, eller locka cellerna att skicka ut ett immunsvar som är ospecifikt för bakterien i fråga. Dessutom kan vissa bakterier skicka ut små blåsor, eller vesikler, från sin yta med bakteriens egen kroppsfrämmande ytstruktur. Vesiklerna är mycket små och kan färdas långt bort ifrån området där bakterien koloniserat och därmed lura kroppen att skapa inflammation på ett annat ställe än där bakterien befinner sig. *Moraxella catarrhalis* är en av många bakterier som bildar dessa vesikler.

I detta arbete har vi undersökt sammansättningen av vesikler från *Moraxella catarrhalis*, och hur de kan interagera med kroppens celler. Vi har funnit att de binder till kroppens celler och därmed skapar inflammation, samt att de faktiskt kan reglera inflammationen genom molekyler som finns på dess yta. Vi har observerat samma fenomen i experiment med möss och kan därmed bekräfta att det inte bara är ett fenomen som sker i provröret.

Vi har även funnit en molekyl i vesiklerna, β-laktamas, som bryter ned vanlig antibiotika, t ex penicillin. När vi odlar andra antbiotika-känsliga bakterier från luftvägarna tillsammans med dessa vesikler så överlever bakterierna antibiotika-behandlingen. På det här sättet tror vi att bakterier som lever i symbios tätt inpå varandra i kroppen inte bara kan hjälpa varandra att orsaka infektion, men också

skydda varandra från kroppens försvar. Vi fann också att vesiklerna skyddade β-laktamaset från inaktiverande antikroppar som finns i blodet hos vissa vuxna.

Vi undersökte slutligen vesikler som härstammar från en annan luftvägsbakterie, *Haemophilus influenzae*, och fann att även de bär på β-laktamas, och kan skydda normalt känsliga Streptokocker från antibiotika. I kliniska studier har man sett att dessa bakterier ibland är svårbehandlade hos patienter med infekterade halsmandlar. Vi föreslår att en bidragande orsak till att dessa bakterier överlever kan vara de små vesikler som frisätts från antibiotika-resistenta bakterier i omgivningen, som t ex *Haemophilus influenzae* och *Moraxella catarrhalis*.

Introduction

Respiratory Tract Infections

The air around us may appear clean, but comprises the most common source of infections for humans. In fact, air contains massive amounts of microparticles, deriving from the earth, water, plants and animals, as well as from us humans. These microparticles, in turn, contain microorganisms, most of which are harmless, but some that constitutes as pathogens and cause airway disease (1).

Respiratory tract infections are among the leading causes of death in the world, according to the World Health Organization (WHO). In low income countries, lower respiratory tract infections cause more than 10% of all deaths, and more than one third of deaths occur in children under fifteen years of age. Furthermore, in developed countries it is the leading infectious cause of death (2). This further stresses the importance of characterizing and understanding the ways microorganisms cause disease in the human respiratory tract.

Anatomy of the respiratory tract

The human airways are usually divided into two parts: the upper respiratory tract which consists of the nasal cavity, sinuses, middle ear, pharynx and larynx; and the lower respiratory tract that consists of the trachea, bronchi and lungs. The upper respiratory tract has a rich flora of bacteria, fungi and protozoa. The lower respiratory tract on the other hand is essentially sterile, as it has no direct contact with the external environment. Most infections thus occur in the upper respiratory tract when pathogenic bacteria compete with the normal flora, and are by nature short and localized. Bacteria from the normal flora can also be opportunistic and cause infections if the immune system is weakened. In contrast, infections in the lower respiratory tract are less common, but when they occur are often more persistent and potentially serious (1, 3).

Upper respiratory tract infections

Sinusitis, pharyngitis, tonsillitis, pharyngitis, epiglottitis and otitis media are all examples of local inflammations caused by viruses or bacteria. Common symptoms for these infections may be nasal discharge or congestion, coughing, sneezing, sore throats or fever, and can differ in severity (1, 3). Several levels of the respiratory tract can also be involved in a single infection.

Pharyngo-tonsillitis

The highest incidence of tonsillitis occurs in children between five and 15 years of age (4). Pharyngo-tonsillitis is characterized by fever, throat pain, redness and enlarged tender lymph nodes. Viruses cause about 50% of all infections (5), while the major bacterial causative agent is *Streptococcus pyogenes*, or group A streptococci (GAS) (15-30%). However, polymicrobial infections can also cause tonsillitis, suggesting the involvement of various pathogens (6). These bacteria bind amongst other proteins to fibronectin in the extracellular matrix (ECM) of the host cells, and some can invade and survive inside the epithelial cells of the tonsils (7, 8).

Acute Otitis Media

Acute otitis media (AOM) is an inflammation of the middle ear, often leading to effusions, or a collection of fluids in the ear (otitis media with effusion: OME). AOM is characterized by pain, fever, and on occasion a negative pressure in the ear caused by inflammation and swelling of the tympanic membrane (9). It is one of the most common diseases in young children, and a major cause for health care consultations and antibiotic prescriptions (10). In fact, approximately 200,000 cases of AOM are diagnosed per year in Sweden, and 70% of children aged below two have had this infection (11). Approximately 10-20% of cases become recurrent AOM (rAOM) or chronic OME (rOME). Viruses may occasionally be the cause of AOM, although this infection is most frequently bacterial. The three most common pathogens that cause AOM are *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* in order of frequency (12).

Lower respiratory tract infections

Lower respiratory tract infections include bronchitis and pneumonia. In addition, chronic obstructive pulmonary disease (COPD) is a chronic disease of the lungs which is in parts characterized by exacerbations due to bacterial and viral infections.

Pneumonia

Pneumonia is defined as acute inflammation of the alveoli, or infiltration of inflammatory cells in the lungs, causing the accumulation of exudate in the bronchi. However, the symptoms vary between children and adults, and depending on the cause of the infection. Examples of symptoms are cough, chest pains, fever and headache (13). Pneumonia is most commonly caused by pathogens like *Streptococcus pneumoniae*, *Haemophilus influenzae* or viruses such as influenza, rhino and corona viruses. Furthermore, up to 45% of community-acquired pneumonia cases in children are actually mixed infections of bacteria and viruses (1, 14).

Bronchitis/bronchiolitis

Bronchitis is an inflammation of the airway mucosa and cell walls and can be either acute or chronic. It is characterized by dry or mucoid cough, chest pain and fever. Bronchiolitis is inflammation in the bronchioles, the smallest bronchial tubes, and mainly occurs in small children. It can lead to the development of serious breathing difficulties as well as fever, cough and mucous production (1). The most common endogenous agents causing bronchitis/bronchiolitis are *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* from the normal flora of the upper respiratory tract. *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* may also cause bronchitis, as well as influenza and RS-virus (1, 15, 16).

COPD

Chronic obstructive pulmonary disease is a chronic airflow limitation disorder characterized by dyspnea, chronic cough and sputum production (17). According to the GOLD (Global Initiative for COPD) definition, COPD is a progressive, enhanced inflammatory response of the lungs and airways to noxious particles or gases, where exacerbations and comorbidities contribute to the severity in each individual patient. A population survey of adults in Spain between 40-69 years of age showed that 9.1% of the population had COPD, of them 15.0% were smokers, 12.8% ex-smokers, and 4.1% nonsmokers (18). The Swedish medical association for lung diseases (SLF) estimates that 400,000-700,000 people have COPD in Sweden (19). Exacerbations are characteristic of COPD infections, and occur once or twice annually on average and the frequency increases with time. Approximately 50% of exacerbations in COPD are caused by bacteria such as *H. influenzae*, *S. pneumoniae* and *M. catarrhalis*, in order of frequency (17, 20).

Pathogens & Host Immunity

Viruses, fungi, and bacteria all cause infections in the respiratory pathways. In order for bacteria to colonize, they first need to adhere to the host epithelial cells. Specific adhesion proteins found in the bacterial membranes are thus of great importance. However, they also need to persist within the host and consequently avoid detection of the host immune response.

The human immune system consists of the innate and the more specific adaptive immune system, each comprised of a cellular and a humoral part. Although these two systems have a distinct set of cells and different mechanisms of action interplay between the two branches results in a diverse and broad line of defense.

Bacteria

Following the invention of the microscope in 1676 by Antonie von Leeuvenhoek, the father of microbiology, discovered the first bacteria. Several major revelations of the microbiological world followed in the coming centuries, including Louis Pasteur's discovery that fermentation was caused by microorganisms and that bacteria cause disease. Later Robert Koch established techniques to isolate and propagate pure cultures of bacteria, and formulated important postulates to determine if bacteria are the causative agents of a disease in 1890 (21, 22). Today we know much more about these microbes causing disease.

Bacteria are small prokaryote organisms that do not have a membrane-bound nucleus. Instead, the nucleoid of the bacteria is a supercoiled molecule of double-stranded DNA found inside the cytoplasm. The cytoplasm is surrounded by an elastic and semi-permeable plasma membrane, consisting of phospholipids and proteins. This is surrounded by a more rigid but permeable cell wall. In Gram-positive bacteria the cell wall consists of a thick layer of cross-linked peptidoglycan, intercalated with teichoic acid which has antigenic properties (Figure 1). In contrast, Gram-negative bacteria have a thinner layer of peptidoglycan surrounded by a second outer phospholipid membrane which contains antigenic lipooligosaccharides (LPS) and proteins that act as porins and adhesins. Both Gram-positive and Gram-negative bacteria may also have a protective anhydrated capsule that protects the organism from phagocytosis and enhances the capacity of the bacteria to cause disease (21).

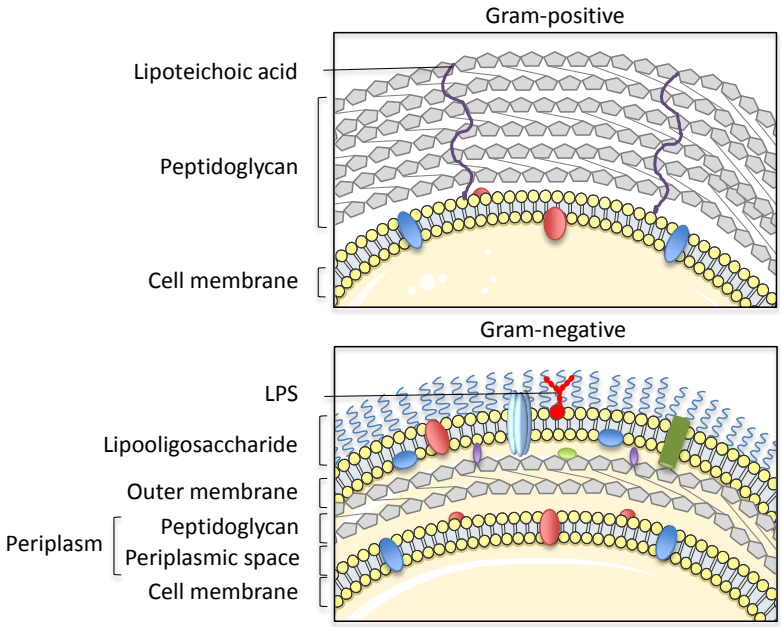

Fig 1. The envelope of Gram-positive and Gram-negative bacteria.
A schematic illustration showing the envelope of Gram-positive (upper panel) and Gram-negative (lower panel) bacteria. Gram-positive bacteria have a thick peptidoglycan layer in contrast to Gram-negative bacteria which have a thinner peptidoglycan layer and the additional outer membrane creating a periplasmic space. The outer membrane contains various porins and transmembrane proteins.

In order to colonize and cause infection, it is imperative that bacteria can attach to the host epithelium. Therefore, adherence factors are essential for bacteria, either in the form of pili or fimbriae extending from the cell surface, or as strain-specific adhesion proteins. In order to cause infection, bacteria also need to resist discovery and destruction by the host immune system.

Innate immunity

The innate part of the immune system is in place before onset of infection, and is largely unspecific in its targeting of microbes. In fact, most pathogens are removed before they have a chance to colonize and cause infection (23).

The first line of defense is the anatomical barriers of the body. The mucous membranes found in the nasopharyngeal tract or in the lungs have cilia to expel foreign microorganisms out of the body, as well as sticky mucoid that trap

pathogens. Furthermore, the normal flora of the mucous membranes competes with pathogens for nutrition and sites of attachment, and secretions of saliva and tears have antimicrobial properties. Regulation of temperature, pH and chemical mediators also play an important role in preventing infections by microbes (23).

Pathogen-associated molecular patterns (PAMPs) are highly conserved structural motifs that are unique to microbes. Examples of these are complex lipids and carbohydrates like LPS and lipoteichoic acid, or unmethylated DNA motifs (CpG) that are not found in human cells. Other PAMPs include flagellin, peptidoglycan as well as double-stranded DNA found in viruses. Since PAMPs are usually molecules that are essential for survival, they are difficult for the pathogen to alter and therefor often conserved in the species. PAMPs are ligands for pathogen-recognition receptors (PRR) found soluble in tissue fluids and in the blood stream, or bound to cells. Soluble PRRs like the mannose-binding lectin (MBL) and C-reactive protein (CRP) act as opsonins and activators of the complement cascade. Cell-bound PRRs are found both on the pathogen surface and intracellularly, and includes both endocytotic PRRs like scavenger receptors that promote attachment and destruction of microbes, and signaling receptors such as membrane-bound toll-like receptors (TLR) and NOD-like receptors (NLR).

The largest group of PRRs is the TLRs, a family of 13 glycoprotein receptors of different ligand specificities that are expressed on immune cells like macrophages, dendritic cells, or non-immune cells of the epithelium. TLRs recognize foreign surfaces of both bacteria, viruses and fungi (24). There are two major groups of these receptors; TLRs 1, 2, 4, 5, 6 and 10 are surface exposed binding extracellular spaces, while TLRs 3, 7, 8 and 9 are found in intracellular compartments such as the lysosome. The earliest discovered Toll-like receptor was TLR4, which binds LPS in complex with other proteins. TLR2 binds multiple glycopeptides and glycoproteins, frequently in complex with TLR6. Furthermore, the intracellular TLR9 binds unmethylated CpG motifs that are characteristic of bacterial and viral DNA. In all TLRs, PAMP recognition triggers an extracellular domain leading to signal activation of a Toll/Interleukin-1 (TIR) domain inside the cell. Although each TLR has its specific intrinsic signaling pathway consisting of kinases and adaptor proteins, all pathways finally lead to the activation of nuclear factor NF-κB in the cell nucleus. NF-κB binds to promoters of target genes, thereby regulating gene expression which leads to the production of a pro-inflammatory response consisting of cytokines, chemokines and DC maturation.

There is also a family of intracellular PPRs called NLRs that face the cytosol and can sense intracellular microbial components (25). There are about 20 genes coding for NRLs in humans, and a number of these are involved in the recognition of intracellular microbes. These cytosolic receptors are found on for example DCs,

macrophages, monocytes and epithelial cells (26). NOD-1 and NOD-2 bind different forms of peptidoglycan, found on most Gram-negative cells or motifs conserved in all peptidoglycan molecules, receptively. The activation of PPRs leads to activation of transcription factors and the production and secretion of cytokines promoting an inflammatory reaction.

The release of cytokines from damaged or activated tissue cells increase permeabilization of the tissue capillaries, leading to an influx of exudate containing pro-inflammatory mediators like antibodies, CRP and complement factors. The complement system is composed of approximately 35 circulating and membrane-bound proteins, which are activated through cleavage of pro-peptides in a proteolytic cascade leading to the insertion of a membrane-attack complex (MAC) in the bacterial membrane, causing cell lysis. The increased permeability of the blood vessels also leads to an influx of phagocytes, such as neutrophils and tissue macrophages, which phagocyte and destroy any microbes present (23). These mechanisms are often able to clear invading pathogens, but if inflammation persists the adaptive immune response becomes activated.

Adaptive immunity

In contrast to innate immunity, the adaptive immune response is highly specific. Although it is slower to respond, the resulting response is a longstanding immunological memory which can differentiate between self and non-self.

The adaptive immune response consists of two groups of cells: antigen-presenting (APCs) and lymphocytes. Generally, all cells present antigens, but dendritic cells, B-cells and macrophages are considered to be "professional" APCs. APC's have MHC class II molecules on their surface, on which they present small antigen peptides and subsequently activate helper T-cells (T_H). Activated T_H cells act as a screening system to co-activate B-cells and cytotoxic T-cells (T_C) which have previously encountered peptides presented by MHC class I molecules on infected cells. Activated T_C's act as effector cells and destroy the infected cells, while B-cells secrete specific antibodies. In serum the first antibody response consists mainly of the high avidity immunoglobulin (Ig) M. However antigen binding leads to a class switch and the secretion of IgG, the most abundant antibody isotype. All these mechanisms together form a specific and highly enhanced immune response at second encounter (21, 23).

Immunity and bacterial infections

The progression of a bacterial infection is a constant battle between the host and bacteria. While the host has several systems to discover and destroy the foreign microbe (Figure 2), in return the bacteria have also developed several strategies to evade the host immune system. This is a complex process that entails hiding the antigenic structures that make up the outer membrane of the bacterial surface, whilst still exposing key molecules such as adhesins in order to cause infection (27).

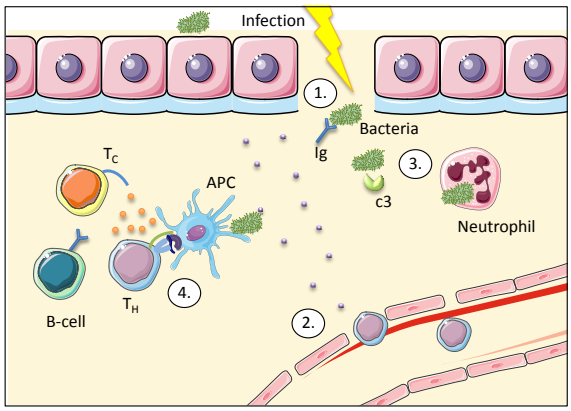

Figure 2. Immune reaction during bacterial infection.
(1) Bacterial adhesion to the epithelium causes tissue damage, and leads to the release of various cytokines. (2) Vasoactive substances increases the permeability and blood flow to the infected area (3) An influx of exudate containing opsonising serum proteins and phagocytes destroy the bacteria. (4) Antigen presenting cells activate T helper cells which in turn activate cytotoxic T-cells and B-cells, which secrete immunoglobulins producing an immonologic memory.

Bacteria have evolved various mechanisms to evade discovery by the immune system. One way is by down-regulating the expression of antigen surface molecules or through mimicry of host surface molecules. Another is through the secretion of PRRs inhibitors and proteases that destroy antimicrobial peptides. Several bacteria have proteins on their surface that bind complement inhibitors, in order to evade opsonization and activation of the complement cascade. Furthermore, bacteria can also express so called superantigens, which stimulate the production of non-specific immune responses in the host, allowing the pathogen to escape (27-29). Finally, bacteria can secrete nanoparticles designated outer membrane vesicles (OMV), which will be further discussed later in this thesis.

The Pathogens

Moraxella catarrhalis

The respiratory pathogen today acknowledged as *Moraxella catarrhalis* has had many names throughout the century. Known as *Micrococcus catarrhalis* when first isolated in the early 1900s due to its morphology and certain biochemical characteristics, it was soon thereafter transferred to the *Neisseria* genus. After a period of less frequent isolation in infections, the bacteria reemerged as a common cause of AOM in the 1960s. However, using the new techniques that had become available at the time it was determined that *N. catarrhalis* actually had little genetic resemblance to the rest of the *Neisseria* species. In fact, it had a higher similarity to the *Moraxella* genus but since *Moraxella* consisted of rod-shaped bacteria that were non-human colonizers instead a new genus was created in 1970, *Branhamella* (30). This name was short-lived as *B. catarrhalis* was finally renamed *Moraxella catarrhalis* in 1984 after much debate, making it the first genus containing both cocci and rods (31, 32).

General characteristics

The respiratory pathogen *Moraxella catarrhalis* is a Gram-negative diplococcus, which exclusively colonizes humans. *M. catarrhalis* is an aerobic catalase positive bacterium, which grows easily at temperatures between 22-37°C with or without 5% CO_2. On chocolate agar it forms small, opaque white colonies of 1-3mm in diameter that are often described as "hockey pucks" since they can easily be moved across the agar. *M. catarrhalis* is unencapsulated, non-motile and is variably piliated (33, 34).

Pathogenesis

Moraxella catarrhalis is often referred to as an opportunistic commensal, meaning that it is frequently found in the normal flora of the nasopharynx, but can cause infections when opportunity arises. This might occur in patients suffering from predisposing medical conditions, or when damage is caused to the respiratory epithelium by viral infections (32, 35). On the other hand, *M. catarrhalis* has also been shown to cause infections in healthy adults (36). Therefore, *M. catarrhalis* is both a commensal and a mucosal pathogen.

According to clinical studies, *M. catarrhalis* can cause a broad spectrum of respiratory diseases including pneumonia, bronchitis, laryngitis, sinusitis and persistent cough (15, 37-41). However, *M. catarrhalis* is most frequently isolated in children with AOM or in adults with COPD. In fact *Moraxella* is the third most common cause of AOM, and is estimated to be responsible for approximately 10%

of acute inflammatory exacerbations in COPD patients (42). In a study of 120 children, *M. catarrhalis* was found to be the most common colonizer of infants under the age of one, and after two years of age 77.5% of all children had become colonized with *Moraxella catarrhalis* in the nasopharynx. The *M. catarrhalis* strains isolated showed a high degree of heterogeneity, as the children acquired and eliminated a number of different strains (43). In a study by Heiniger *et al.* it was found that 91% of adenoids and 85% of pharyngeal tonsils were reservoirs of *M. catarrhalis* in children undergoing tonsillectomy (44). These studies all suggest *M. catarrhalis* is an important pathogen in a clinical setting.

Virulence factors

In order for *M. catarrhalis* to colonize the host and cause infections, adhesion to the respiratory epithelium is essential. Lipooligosacchardies (LOS), pili and fimbriae are involved in *M. catarrhalis* adhesion as well as a range of specific proteins on the bacterial outer membrane (45-47).

The most extensively characterized family of *Moraxella* adhesin proteins are the ubiquitous surface proteins (Usp) which are lollipop-like structures that protrude from the surface of the bacteria (48-51). The two main types, UspA1 and UspA2, are involved both in adhesion and in regulation of host immunity. These surface proteins bind fibronectin and laminin found in the extracellular matrix (ECM) of epithelial cells which may be exposed during infection (52, 53). UspA1 also binds carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1) motifs expressed on epithelial cell surfaces (54, 55). In 2008 Slevogt *et al.* showed that binding of CEACAM-1 by proteins such as UspA1 inhibits the activation of TLR2 on epithelial cells when binding PAMPs (56). Binding of CEACAM-1 thus prevents the activation of transcription factors and consequently leads to suppression of the pro-inflammatory response. In this way, *M. catarrhalis* can efficiently evade detection and the subsequent activation of the immune system.

The expression of UspA1 is also essential for internalization of *M. catarrhalis* by epithelial cells and in pharyngeal lymphoid tissue (57, 58). Hiding inside cells is another efficient way by which bacteria are protected against the immune system. Moreover the UspA proteins are involved in regulation of the complement system. UspA2, and UspA1 to a lesser extent, bind vitronectin and C4BP, regulators of the complement cascade (59-62). Binding of these proteins to the surface allows the bacteria to prevent formation of the membrane attack complex and subsequent lysis (61). A hybrid UspA2H protein also exists, which similar to UspA2 has a conserved ability to bind vitronectin despite extensive sequence variances between isolates (50, 63). In addition, UspA2H binds fibronectin and is involved in cell adhesion (50, 51). Furthermore, a rare variant of UspA2 called UspA2V has more

recently been discovered, which binds epithelial cells via CEACAM-1, suggesting an exchange between functional regions of the two UspAs (64).

The *Moraxella* IgD-binding protein (MID; also known as the human erythrocyte agglutinin (Hag)) is another important virulence factor and *Moraxella* adhesion (65). MID is an autotransporter as well as a superantigen, as it binds surface-bound IgD outside the antigen-binding site independent of antibody specificity, and activates B-cells in a T-cell independent manner (66-69). *M. catarrhalis* thereby induces a polyclonal immune response and can consequently avoid complement activation and phagocytosis. MID and the UspA proteins are essential for *M. catarrhalis* colonization of human respiratory epithelial cells, but are complemented by various other adhesins such as McaP, OmpCD, OmpE, OmpM35 and MhaC/B (70-74).

Iron-acquisition proteins are also crucial for *M. catarrhalis* persistence. Since iron is toxic in its free form it is mostly found in complex with host proteins such as hemoglobin, transferrin, lactoferrin and heme. However, bacteria need iron for optimal growth and fitness and thus express proteins on their surfaces that compete for these complexes. *M. catarrhalis* expresses the lactoferrin-binding proteins (Lbp), transferrin-binding proteins (Tbp) and CopB which bind and utilize these iron complexes (75-77).

Cold shock & biofilm formation

An interesting discovery was made by Heiniger *et al.* in 2005, when it was revealed that *M. catarrhalis* upregulates certain virulence factors like UspA1 at 26°C, which is the temperature of the nasopharynx at colder air temperatures (78, 79). This is called a cold-shock response, and was most likely due to a longer half-life of UspA1 mRNA at 26°C, leading to a higher expression level of UspA1 on the bacterial surface (80). As a result *M. catarrhalis* adhere better to the epithelium, leading to an enhanced activation of the cells (80). Cold shock in *M. catarrhalis* also leads to the upregulation of genes like UspA2, Lbp and Tbp, involved in serum resistance, iron acquisition as well as immune evasion (81).

In various studies, *M. catarrhalis* has been proposed to form biofilm, which is a complex matrix of proteins, DNA and pathogens. *In vitro* assays have shown that OMPs UspA1/A2 and type four pili are involved in biofilm formation (46, 82, 83). Furthermore, *M. catarrhalis* biofilm could be detected in the middle ear of children with OME and occurring AOM (84). However, more studies need to be performed in order to fully elucidate the role of biofilm formation in disease progression of *M. catarrhalis* infections.

Haemophilus influenzae

H. influenzae is a Gram-negative aerobic cocobacillus, which consists of two general types: the encapsulated classified by their capsular antigens (type a-f) and the non-encapsulated (non-typeable *Haemophilus influenzae*; NTHi) (85). Historically, *H. influenzae* type b (Hib) have been a major cause of invasive disease in children, causing up to 2.2 million infections and 520,000 deaths per year (86). However, with the introduction of a vaccine against this serotype, Hib disease and carriage rate has dramatically dropped. In the United States for example, Hib disease has been reduced by more than 95% (87). However, in countries where this vaccine has yet to be introduced, Hib infections are still a major concern. After Hib, *H. influenzae* type f is the most common encapsulated cause of invasive disease, and this infection has increased in frequency since the introduction of the Hib vaccine (88, 89).

NTHi on the other hand is commonly considered to be a commensal of the nasopharynx, and shares the same niche as *M. catarrhalis* (90). NTHi is also an opportunist, and is one of the leading causes of respiratory infections in humans and causing AOM as well as sinusitis, pneumonia, and exacerbations in COPD patients (85, 91-93). Furthermore, NTHi has been found to invade respiratory epithelial cells and tissue macrophages, and accumulate in the tonsils (94-97).

Streptococcus pneumoniae and group A streptococci

Streptococcus is a Gram-positive species which requires rich media like blood agar plates in order to grow. As the name suggests, streptococci are cocci-shaped and can be found either in pairs or as long chains. Even though most streptococcus species are facultative anaerobes, some cannot grow in the presence of oxygen making them obligate pathogens (98). Streptococci can be classified through three different overlapping schemes by their serological or biochemical properties. In addition, streptococci are classified into groups based on their ability to break down red blood cells. While β-hemolytic strains perform a complete hemolysis, α-hemolytic bacteria only partially break down the blood cells, and γ-hemolytic do not perform lysis at all (99, 100). Streptococci are a part of the human normal flora, but are also a diverse group of bacteria that are associated with a range of different diseases. *S. pneumoniae* and *S. pyogenes* are two major human pathogens that cause disease given the right circumstances (98).

Currently more than 90 serotypes of the encapsulated *Streptococcus pneumoniae*, also known as pneumococci, have been recognized. *S. pneumoniae* are usually found as diplococci or in short chains. *S. pneumoniae* are described as α-

hemolytic if grown aerobically on plates, but can become β-hemolytic during anaerobic conditions (99, 101). *S. pneumoniae* primarily colonizes the nasopharynx but has the ability to spread to the lungs causing pneumonia or to the upper airways causing sinusitis and otitis media (102). Historically, penicillin has been the drug of choice for treating *S. pneumoniae* infections. Although penicillin resistance in *S. pneumoniae* is increasing around the world, due to decreased affinity of the penicillin-binding proteins (PBP) to penicillin, resistance is still quite low in Sweden at approximately 6.8% (2009) (99, 103).

Streptococcus pyogenes, or group A streptococci (GAS), are β-hemolytic diploccoci that have been extensively studied and characterized throughout the years. Certain *S. pyogenes* strains have a hyaluronic acid capsule, which allows the bacteria to evade immunity due to its similarity to human hyaluronic acid. These strains are also more likely to be responsible for cases of invasive disease (99, 104). *S. pyogenes* commonly colonizes either the skin or the upper respiratory tract, and although it can be found as the normal flora of the nasopharynx, this occurs less frequently compared to *S. pneumoniae* (100). In the airways, *S. pyogenes* is the leading cause of bacterial pharyngitis and tonsillitis, and can also cause other respiratory infections such as sinusitis, OM and pneumonia. Furthermore, *S. pyogenes* is associated with scarlet fever, impetigo, necrotizing fasciitis, rheumatic fever and in extreme cases, streptococcal toxic shock syndrome (100, 105). In contrast to *S. pneumoniae*, all *S. pyogenes* clinical isolates are completely susceptible to penicillin (105, 106).

Nasopharyngeal co-infections

Polymicrobial infections are created when combinations of pathogens colonize a certain niche, and may comprise a mixture of different microorganisms such as virus, bacteria, fungi and parasites. In a symbiotic polymicrobial infection one pathogen generates a beneficial niche that supports the colonization of another pathogen, making it easier for the co-colonizer to cause infection. For instance, virus infections can lead to the destruction of host epithelial cells which increases bacterial adherence. In addition, a prior virus infection induces the upregulation of certain surface receptors that bacteria can bind to, or the suppression of the host immunity facilitating bacterial infections (107). In addition, polymicrobial infections in biofilms generate advantages such as metabolic cooperation, quorum sensing signaling, more efficient DNA sharing as well as passive resistance (108).

Several studies have aimed at trying to investigate how pathogen survival and their infectious potential is affected by polymicrobial infections. For instance, *M. catarrhalis* has been found to increase the incidence rate, bacterial load as well as

the duration of infection of *S. pneumoniae* (109). A study of a continuous culture biofilm *M. catarrhalis* could protect *S. pneumoniae* in the presence of amoxicillin (110), and Matejka *et al*. found that *M. catarrhalis* were less sensitive to antibiotics in a continuous flow model of biofilm compared to batch-grown cells (111). Another effect of polymicrobial infections is that two infecting pathogens can have an additive effect on infection development. For example, a combined infection of *H. influenzae* and *S. pneumoniae* lead to the synergistic increase of the production of inflammatory cytokine interleukin (IL)-8, the recruitment of phagocytic neutrophils, and the amplification of a pro-inflammatory response (112).

The nasopharynx is often colonized by several microorganisms both of commensal and pathogenic nature, and infections such as OM have been associated with polymicrobial infections (109). A study by Verhaeg *et al.* of more than 1,000 healthy children showed that co-colonization with *H. influenzae* and *M. catarrhalis* are in fact more common than single-species infections (113). Furthermore, in a study by Skovbjerg *et al.* of 664 health day care children under the age of two, the carriage rate for *M. catarrhalis* and *H. influenzae* was 54% and 22%, respectively (114). Evidently, these bacteria are often found in the nasopharynx as opportunistic pathogens, and may affect other colonizing bacteria also *in vivo*.

Several studies have also aimed at investigating polymicrobial infections involving group A streptococci. Firstly, a study from 2004 showed that *M. catarrhalis* co-aggregates with GAS, increasing the ability of the bacterium to adhere to human epithelial cells. Furthermore, Brook *et al*. investigated the correlation between GAS treatment failure and the co-colonization of respiratory pathogens. In a study of 548 children with acute pharyngotonsillitis, the authors found that a significant portion of *M. catarrhalis* or NTHi were associated with GAS-carriage (115). Brook *et al.* suggested that the secretion of free β-lactamase from these co-infecting bacteria in the respiratory tract could allow for the protection and subsequent survival of the susceptible bacteria (116). Co-culturing GAS with *M. catarrhalis* changed the virulence gene expression of GAS, showing how polymicrobial infections can actually affect virulence (117).

Previously, clinical diagnostics have focused on identifying the most abundant and disease causing pathogen, ignoring the apparent co-pathogens. However, more research is showing the importance of characterizing each individual member of a microbial community (108). Polymicrobial infections are an important emerging area of research.

Outer Membrane Vesicles

In 1965, D.G. Bishop and colleague observed what appeared to be extracellular lipopolysaccharides in the supernatant of a lysine-requiring *Escherichia coli* mutant grown without lysine (118). Using electron microscopy, Work *et al.* could find what they described as a "mass of globules" measuring approximately 12-200nm in diameter (119). First it was thought that these nanoparticles were only created under iron-limiting conditions, but it was later shown that they were also produced during normal growth *in vitro* (120). These nanoparticles, or outer membrane vesicles (OMV), are formed when parts of the outer membrane of the bacteria start bulging out, creating a small sphere that pinches off from the membrane (Figure 3).

Since OMV production and secretion is an energy-demanding process, it has been hypothesized that OMV have evolved for a reason. The envelope of bacteria contains proteins involved in adherence, nutrient acquisition, secretion, signaling, quorum sensing, horizontal gene transfer and protection from the extracellular environment. Outer membrane vesicles are reflections of the cell surface, and consequently OMV are important actors in pathogenesis and survival of bacteria. OMV are also an alternative way for protein secretion, allowing the bacteria to interact with its environment at a distance, protecting it from the possible disadvantages of close contact (121-123).

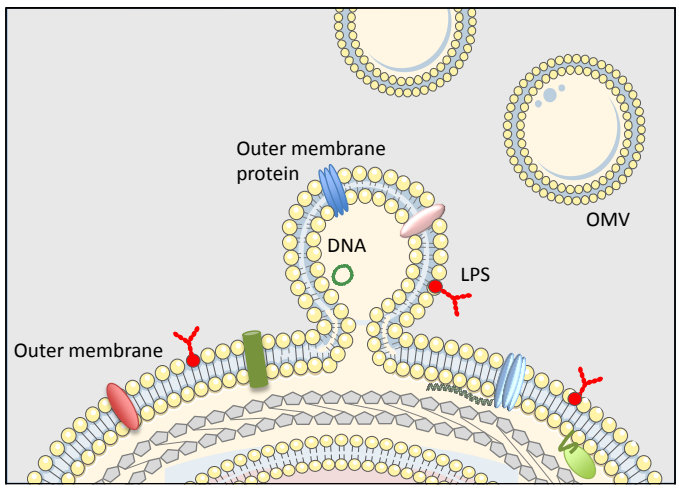

Figure 3. Biogenesis of outer membrane vesicles (OMV).
OMV are formed as the outer membrane of Gram-negative bacteria bulges out and pinches off. The composition of the OMV thus reflects the composition of the bacterial outer membrane, containing lipids, proteins, DNA and specific virulence factors.

Biogenesis

OMV are generally described as spheres of 50-250nm in diameter that are formed at all stages of bacterial growth, and are a common feature for all Gram-negative bacteria studied up to date (121, 124). OMV are secreted by bacteria both in liquid and on solid media, as well as *in vivo*. The first report of OMV found in humans came in 1982, where OMV were found in the cerebrospinal fluid of a child with a *Neisseria meningitides* infection (125). In 1992 endotoxin was found in complexes with lipoproteins in plasma from a patient with meningococcal septic shock, and in another study OMV were found in urine, blood and internal organs of both rats, dogs and humans (126, 127). In 2005, *M. catarrhalis* OMV were identified by transmission electron microscopy (TEM) in a nasal sample from professor Riesbeck's daughter, an at the time eight-year old girl with a sinusitis infection (52).

The aim of many OMV studies has been to understand how OMV are formed and how this process is regulated, yet no definite model has been established. This is partly due to the fact that no mutant completely devoid of OMV production exists, making it difficult to determine the exact mechanism involved in the generation of OMV (121, 128). Wensink *et al.* hypothesized that the detachment of the outer membrane from the underlying peptidoglycan layer needs to occur as an initial step for the formation of OMV, which might happen where there is an imbalanced overproduction of outer membrane lipids and proteins (129, 130). This was supported by a study where the Lpp protein involved in linking peptidoglycan to the outer membrane was mutated, leading to hypervesiculation (131). Another model suggested that an imbalance occurs in the turnover of peptidoglycan, creating turgor and bulging of the membrane, which would suggest that certain proteins might be enriched in OMV (132). In contrast, a third theory advocated that a buildup of integral membrane proteins or small molecules found on the inside of the outer membrane causes an inherent curvature of the outer membrane and consequently leads to the OMV production (123). One study on *Pseudomonas aeruginosa* supported this theory as the authors found that the OMV were composed of mostly B-band LPS, compared to the bacterial outer membrane which contained both B-band and A-band LPS. The B-band LPS is longer and more negatively charged than the A-band, leading the authors to hypothesize that an accumulation of these negative charges lead to a repulsion force and subsequent curvature of the outer membrane (133). However, this theory also suggests that different bacteria have certain conserved proteins involved in OMV production, but to find possible candidates we need better genetic studies as well as comparative analysis of OMV (134).

OMV biogenesis is often considered to be stress regulated. This further implies that OMV secretion might be essential for Gram-negative bacteria to deal with environmental stress. A study by McBroom *et al.* supported this hypothesis; OMV from underproducing *E. coli* mutants could not withstand lethal envelope stress, while overproducing mutants survived better compared to the wild type (135). Other factors that affect OMV production and composition are certain antibiotics, oxygen stress, and the availability of iron or nutrients (136, 137). For example, ciprofloxacin, gentamicin and mitomycin all affect the secretion and composition of OMV in Gram-negative bacteria (138-141). Furthermore, it has been suggested that pathogenic bacteria in general produce more OMV than non-pathogenic bacteria (142, 143). In conclusion, OMV biogenesis is a regulated mechanism that is essential for bacteria to prevail and persist in the human host.

Characterization and composition

Bacteria can transfer material into the extracellular environment at a distance from the site of colonization through the secretion of OMV. OMV are smaller in surface area and thus interact with environments that are inaccessible to the whole bacteria, as well as with host cells and other bacteria within the niche (144). OMV also act as protective vesicles in protein secretion, where soluble material may be released from the cell in complex with other proteins or surrounded by insoluble material (145).

As mentioned previously, OMV secretion occurs during all stages of bacterial growth. The composition of OMV reflects the surface of the parent bacteria, containing phospholipids, LPS and proteins. These proteins are mainly derived from the outer membrane and periplasm, although DNA and cytoplasmic proteins have also been identified in OMV (141, 146-148). Two-dimensional gel electrophoresis, western blot and mass spectrometry analysis revealed that OMV contain outer membrane proteins that are specific virulence factors for the bacteria. For instance, OMV can act as carriers of active bacterial toxins for *Campylobacter jejuni*, *Salmonella enterica*, and *Vibrio cholera* (149-151). Vesicles from *Helicobacter pylori* contained not only specific adhesins BabA and SabA, but also proteases and ureases (152). OMV can also contain heme-binding proteins as well as hemolysins (153-156). Furthermore, studies of *M. catarrhalis* OMV found that the vesicles contain specific virulence proteins UspA1/A2 and MID (157, 158). In addition, there are reports that OMV can be enriched for certain virulence factors. The *Borrelia burgdorferi* Oms28 porin, the enterotoxigenic *E. coli* (ETEC) enterotoxin LT, *P. aeruginosa* B-band LPS, as well as *P. aeruginosa* aminopeptidase were all shown to be enriched in vesicles secreted from the parent bacteria (159-161).

Since the establishment of OMV as vehicles for proteins and molecules, several studies have aimed at investigating how OMV deliver their cargo to cells. Kardurugamuwa *et al.* showed that OMV fuse with the outer membrane of other Gram-negative cells and become integrated, releasing their antigens (162). TEM analysis determined that OMV from *Salmonella typhi*, *S. enetrica* and *E. coli* could fuse with both *P. aeruginosa* and *V. cholerae.* However, OMV from these bacteria could only attach to the surface of the Gram-positive bacterium *Staphylococcus aureus*, without fusing with the membrane (133). Considering the different composition of Gram-negative and positive cell envelopes, these results might not be entirely surprising. Furthermore, another study showed that *B. burgdorferi* OMV not only fused with the surface of host epithelial cells, but there was also a lipid exchange between bacteria and host cells (163).

Cell interactions

In order to interact with the host, bacteria need to bind host cells, and the same goes for OMV. Consequently, numerous studies have focused on investigating how OMV are involved in host cell binding and the promotion of infection. Binding of virulence factors on OMV by epithelial cells through PPRs like TLRs, leads to the activation of NF-κB and triggers a pro-inflammatory response mediated by cytokines. In a unique way, OMV thus have the possibility to interact with and regulate the inflammatory response of epithelial cells at a site distant from colonization.

OMV from Gram-negative bacteria adhere to the mucosa and epithelial cells of the respiratory tract (164-166). One interesting example is *H. pylori*, which normally stays unattached to the mucosa. However, OMV secreted from this bacterial species containing the OMPs BabA, SabA and cyototoxin bind and invade gastric epithelial cells (152, 167, 168). Attachment of *Legionella pneumophilia* OMV to A549 lung epithelial cells modulate their cytokine release, leading the cells to secrete IL-7 and the anti-inflammatory IL-13 which are normally not produced when whole bacteria bind (169). OMV may also inhibit the fusion of the phagosome with the lysosome of macrophages (170). Furthermore, OMV derived from *H. pylori, N. meningitides* and *P.aeruginosa* bind to lipid rafts of epithelial cells, and are taken up through endocytosis. Binding of peptidoglycan activates the intracellular PPR receptor NOD-1 and induces an IL-8 release from the cell (26).

OMV also directly interact with cells of the host immune system, thereby acting as potent regulators of inflammation. Depending on the bacterial strain or the environmental circumstances OMV can be either pro- or anti-inflammatory

mediators, interacting with phagocytic cells such as neutrophils, macrophages as well as immunity B- and T-cells and the complement system. For instance, OMV from *Brucella abortus* are internalized by monocytes through clathrin-mediated endocytosis, leading to the upregulation of ICAM-1 and the downregulation of MHC class II molecules on the cell surface. OMV treatment of these cells thus led to an increased number of bacteria adhering and being internalized, and a downregulation of the innate immune response which promotes the persistence of the bacteria in host cells (171).

OMV are also involved in the regulation of the adaptive immune cell response. As previously mentioned, OMV from *M. catarrhalis* were found to contain the superantigen MID. In a study by Vidakovics *et al.*, it was shown that *M. catarrhalis* OMV could bind to B-cells through MID, leading to clustering of the B-cell receptor (BCR) in lipid rafts, followed by endocytosis of the OMV (157). Interactions with lipoproteins and DNA found on the surface of the OMV led to a T-cell independent activation of the B-cells, through binding of TLR2 and TLR9. This led to the secretion of polyclonal IgM and the inflammatory cytokine IL-6 unspecific for *M. catarrhalis*, thereby redirecting the immune response. MID could also be found on OMV secreted from *M. catarrhalis in vivo*, implying that this phenomenon occurs in a clinical setting. Another example of OMV interacting with adaptive immune cells comes from a study of the pathogen *Bacteroides fragilis.* OMV were found to contain a capsular polysaccharide (PSA) which induces regulatory T cells to secrete anti-inflammatory cytokines through interactions with DCs (172). The resulting tolerance of the mucosa leads to the prevention of experimental colitis in a mouse model.

Finally, OMV are also involved in regulating the complement system of the human host in models of infection. For instance, OMV from *M. catarrhalis* was shown to absorb complement factor C3 from serum through binding it to UspA1 on the vesicle surface (158). In co-cultures with serum-sensitive NTHi, OMV could thus protect NTHi from complement-dependent lysis, suggesting a new strategy by which co-colonizing bacteria can work together to defeat the host immune response. OMV may also perform molecular mimicry, as shown by *H. pylori* vesicles with LPS. The vesicles express Lewis blood antigens very similar to those found in the gastric mucosa, thereby creating an autoimmune response against the host (173, 174).

Biofilms and vaccines

OMV play a role in biofilm formation and maintenance; mediating adherence, delivering material and competing for growth factors. OMV were found to be

important components of *H. pylori* biofilms, and in fact the addition of OMV to a *Helicobacter* culture triggered the biofilm formation (175, 176). Moreover, 52% of all LPS found in *P. aeruginosa* is derived from OMV, thus making it an important feature of biofilm according to a study by Schooling *et al* (177). The presence of OMV in *Pseudomonas* biofilm was confirmed by transmission electron microscopy (TEM), and the authors suggested that a large majority of the outer membrane proteins found in the biofilm was in fact OMV-derived (178).

Another important role for OMV has been in vaccine research. Considering that OMV are carriers of common virulence factors specific for each bacteria, secreted in complex proteins and lipids of the outer membrane whilst being non-replicating, they are ideal to use as vaccine agents. Many studies have focused on investigating the potential of OMV as vaccines for pathogens including *Neisseria meningitides*, *S. flexneri*, *V. cholera*, *S. enterica*, *B. pertussis*, ETEC and many others (179-184). In fact, vaccines against *Neisseria meningitides* serotype B have been used in several countries like Cuba, Norway and New Zealand. A study from the Cuba showed that the OMV vaccine had a promising efficacy of 83-94% (185). More recently, a vaccine containing three *N. meningitides* surface antigens was developed in order to provide broad protection and minimize the risk of escape through mutations. In the study the authors compared the vaccine incorporating only the antigens, to one containing the same proteins with the addition of OMV. Interestingly, the immunogenicity was enhanced when OMV was added to the vaccine (186). When developing an OMV vaccine it is essential that it is not cytotoxic in itself, for example toxic LPS needs to be removed whilst keeping the vesicles intact. OMV vaccines have a potential as an alternative way of treating bacterial infections, in a world facing the growing problem of antibiotic resistance (121).

Pathogens & Antimicrobial Resistance

The human body has developed several sophisticated strategies to avoid bacterial infections. In cases when the immune system is not successful in eliminating a pathogen we are, however, forced to use antimicrobial drugs. Nevertheless, through natural selection bacteria have also rapidly evolved resistance mechanisms against these antimicrobials.

Antimicrobial drugs

The first antibiotic, penicillin, was discovered accidently by Alexander Flemming in 1928 (22). Since then, several antibiotics have been discovered and developed into semi-synthetic modifications. In general, Gram-negative bacteria are more difficult to treat than Gram-positive, due to their extra lipid membrane. One of the main criteria for an antibiotic is to be toxic for the prokaryote while leaving the host cells intact, targeting molecules and processes exclusive to the bacteria (101). These include inhibitors of cell wall synthesis, protein synthesis, folic acid metabolism, and DNA/RNA synthesis.

Cell wall synthesis inhibitors

The largest group of antibiotics is inhibitors of cell wall biosynthesis. The peptidoglycan-containing cell wall is unique to bacteria, and is therefore an ideal target for antibiotics. β-lactam antibiotics inhibit enzymes that catalyze cross-linking of glycan molecules N-acetylglucosamine and N-acetylmuramic acid, the final step of peptidoglycan and cell wall biosynthesis. These transmembrane enzymes are called penicillin-binding proteins (PBP) and the number of variants differ between bacterial species. As the name suggests these antibiotics have a β-lactam ring, and a side chain that gives specific properties to each antibiotic substance. For example, the side chain determines if the antibiotic is taken up by the cell and how resistant it is against degradation (187-189). Cell wall synthesis inhibitors are bactericidal and thus directly kill the bacteria.

In Sweden, phenoxymethylpenicillin (penicillin V) is the most common β-lactam still used in treating AOM and pneumonia. However amoxicillin, a semi-synthetic derivative of penicillin, has a higher porin penetrance in Gram-negative bacteria. Furthermore, cephalosporins and carbapenams bind PBP-3 and PBP-2 respectively, and are frequently used with bacteria resistant against extended spectrum antibiotics. Vancomycin is another cell wall synthesis inhibitor that act on earlier steps compared to the β-lactams, which is mainly used against Gram-positive bacteria (190).

Other antimicrobial drugs

Protein synthesis inhibitors that target the ribosome are aminoglycosides such as tetracyclines or chloramphenicol which binds different parts of the ribosome subunits. These antibiotics can be either bacteriostatic, meaning they slow down growth instead of directly killing the bacteria, or bactericidal.

Folic acid is important in the synthesis of nucleic acids as well as in protein synthesis. Examples of inhibitors are sulphonamide and trimetoprim which are competitive inhibitors and uptake inhibitors, respectively. Folic acid metabolism inhibitors are mainly bacteriostatic.

DNA/RNA synthesis inhibitors such as quinolones and rifampicine block the replication of nucleic acid sequences, through binding and inhibition of unwinding supercoiled DNA or inhibiting polymerases, respectively. These antibiotics are mainly bactericidal (101).

Antibiotic resistance

Resistance to antibiotics can be acquired either as a random mutation in the chromosome of a particular bacterial strain giving it a selection advantage over other strains, or through the spread of a plasmid or transposon carrying a resistance gene. In fact, a single base pair substitution or deletion may lead to a changed protein sequence which can potentially mean the acquisition of resistance to antibiotics. For instance, an alteration in the protein sequence of the PBP-proteins may lead to a lower affinity for β-lactams. The permeability of the cell membrane can decrease, making it difficult for antibiotics to pass, and efflux systems pump out antibiotics.

Furthermore, some bacteria have acquired resistance against β-lactams by expressing enzymes that hydrolyze the β-lactam ring, called β-lactamases. These enzymes were first discovered in the late 1940s, soon after antibiotics had become a common treatment in the clinic. There are currently more than 300 types of β-lactamases, classified into four groups by sequence similarities and their catalytic mechanisms. One option when treating resistant bacteria is by using alternative antibiotics with a different mechanism of action, another is to combine for example amoxicillin treatment with a β-lactamase inhibitor like clavulanic acid that inactivates β-lactamases (191). However, the emergence of new broad-spectrum β-lactamases is a major problem across the world, and is one of the pre-eminent issues modern health care currently faces (101, 188, 192).

Testing for antibiotic susceptibility and resistance

In order to make sure the patient receives the correct antibiotic patient samples are grown in the clinical laboratories, and tested for susceptibility. Bacteria are thereafter classified as sensitive, intermediate or resistant (the SIR system).

Minimal Inhibitory Concentration (MIC) determination

The MIC for a certain bacterial strain is evaluated through broth or agar dilution methods. The bacteria are grown with varying antibiotic concentrations, and the MIC is the lowest antibiotic concentration which inhibits its growth (101). E-tests are commonly used to determine antibiotic MIC-values on agar plates. It consists of a plastic strip which has a predefined antibiotic concentration gradient, that is placed on a plate with growing bacteria. The MIC value can be identified at the point on the strip where the growth inhibition zone ends (193).

Disk diffusion

Disk diffusion methods are used in order to measure the sensitivity of a certain bacterial strain to an antibiotic on agar plates. Perforations are made in agar plates with the bacteria growing on them, and antibiotic samples added and diffuse into the agar. The size of the zones where the bacteria do not grow indicate the susceptibility of a certain bacteria to the antibiotic (194).

β-lactamase analysis

The chromogenic substance nitrocefin is used to analyze the presence of β-lactamase in bacteria. Hydrolysis of nitrocefin by the enzyme changes the colour of the substance from yellow (380nm) to red (500nm), and this change in absorbance can be measured using spectrophotometry.

M. catarrhalis and *H. influenzae* resistance against β-lactam antibiotics

Moraxella catarrhalis

The unique *M. catarrhalis* β-lactamase enzyme BRO was first described in 1977, and is encoded by the chromosomal gene *bro* (195, 196). Within just a few years after its discovery the enzyme was found in up to 75% of all *M. catarrhalis* isolates in the United States (197). This has led to speculation that this dramatic effect was due to an interspecies horizontal gene transfer, however this topic is still up for debate (198, 199). Today, studies report that between 90-97% of all *M. catarrhalis* strains are β-lactamase positive (199-201).

Two variants of the β-lactamase gene exist: the more common *bro-1* and the less prevalent *bro-2*. The *bro* genes code for proteins that differ by only one amino acid as well as a deletion of 21 base pairs in the promoter region of *bro-2*. This results in the proteins having different isoelectric points (202, 203). Compared to β-lactamases originating from other Gram-negative bacteria, *M. catarrhalis* BRO has a significantly different protein sequence (203). After analyzing the gene regions flanking *bro-1/2*, Bootsma *et al.* suggested that the β-lactamase gene was spread through horizontal transfer to *Moraxella.* The gene sequence has a significantly different GC-content compared to the rest of the *M. catarrhalis* genome (31% vs. 41%) (198). BRO has a signal sequence motif LPXTG which is characteristic of Gram-positive microbes suggesting that perhaps this enzyme is derived from a Gram-positive species (202). Further strengthening this hypothesis, it was determined that *M. catarrhalis* β-lactamase is a lipoprotein, which is common in Gram-positive β-lactamases. BRO is synthesized as a precursor protein and the signal sequence is modified by lipidation. Corresponding with this, approximately 10% of β-lactamases in *M. catarrhalis* were found to be membrane-bound on the outer membrane, as well as in the periplasm (202).

Haemophilus influenzae

In 1974, two cases of ampicillin-resistant *H. influenzae* strains were reported (204). Since then, *H. influenzae* resistance has increased worldwide, with 4% resistant strains reported in Russia, 26% in the United States and 31% in France (205). Two major resistant groups exists in *H. influenzae,* those that are β-lactamase positive and ampicillin resistant (BLPAR) and those which have other resistance mechanisms, BLNAR (β-lactamase negative ampicillin resistant) (206). A majority of *H. influenzae* strains are BLPAR, where the β-lactamase is of TEM-1 or ROB-1 type (94% vs. 5%) (205, 207). In Sweden, it has been reported that β-lactam resistance has increased from 11% in 1994 to 23.3% in 2009. In contrast, approximately 4% of strains are BLNAR (208). In these strains ampicillin resistance is generally due to mutations in the PBP-3 proteins, leading to a lowered affinity for β-lactams. However, BLNAR strains are still relatively uncommon globally. Furthermore, BLPACR (β-lactamase positive ampicillin clavulanate resistant) strains have both β-lactamase and chromosomally derived resistance, and are tested by their resistance to cephaclor (206). Despite the increase in resistance, ampicillin is still the first choice of treatment for most *H. influenzae* infections.

The present investigation

Aims

The aim of this thesis was to characterize outer membrane vesicles (OMV) secreted by the Gram-negative pathogens *Moraxella catarrhalis* and non-typeable *Haemophilus influenzae* (NTHi) in the respiratory tract, and to investigate different ways in which OMV interact with both the host immune system, as well as other pathogens in the surrounding area. The specific aims of the thesis were:

- To determine the proteomic composition of OMV from the nasopharyngeal pathogen *Moraxella catarrhalis*

- To investigate if OMV from *M. catarrhalis* bind to and activate respiratory epithelial cells from humans *in vitro* and mice lung cells *in vivo*

- To examine if OMV from *M. catarrhalis* contain active β-lactamase *in vitro* and *in vivo* and if these OMV can protect other antibiotic sensitive bacteria in co-infections from antibiotic-induced killing

- To investigate if healthy adults have antibodies against *M. catarrhalis* β-lactamase and if OMV thus can act as protective vesicles against neutralization by these antibodies

- To establish if OMV from non-typeable *Haemophilus influenzae* likewise contain active β-lactamase, and if these OMV can protect group A streptococci from antibiotic-induced killing in co-cultures

Results and Discussion

Paper I: Multicomponent *Moraxella catarrhalis* outer membrane vesicles induce an inflammatory response and are internalized by human epithelial cells

M. catarrhalis is one of the main bacterial agents causing AOM in children and exacerbations in adults with COPD. Although several studies have focused on elucidating how *M. catarrhalis* causes infection through specific virulence factors, very few studies have concentrated on an important virulence mechanism for Gram-negative pathogens: the secretion of outer membrane vesicles. We know from earlier studies that OMV from *M. catarrhalis* contain virulence factors MID and UspA1, but otherwise very little about the composition of these nanoparticles. Characterizing OMV from *M. catarrhalis* may lead us to discover new biological functions of these vesicles. Consequently, in paper I, we decided to carry out a proteomic study of *M. catarrhalis* OMV.

Following OMV isolation, vesicle proteins were separated according to size and their isoelectric focusing point through a 2D-gel electrophoresis. Through MALDI-TOF mass spectrometry the protein spots were analyzed and the proteins were identified through sequence analysis. We found 85 spots and could identify 58 *M. catarrhalis* proteins, 22 which were originating from the outer membrane or periplasm. Proteins isolated were common outer membrane proteins such as ompCD, ompE, copB, and ompM35 that play roles in adhesion, serum resistance, iron acquisition and antibiotic resistance (73, 77, 209, 210). However, because of a size limitation of the gel, MID and UspA1/A2 could not be isolated this way and had to be identified using western blot. Other proteins identified were involved in cell envelope functions, energy metabolism and transport and binding proteins.

The analysis also revealed the presence of numerous cytosolic proteins in the *M. catarrhalis* OMV, mainly involved in protein synthesis. This has been seen in other proteomic studies as well, and different theories have tried to explain this phenomenon. Recently, a study by Perez-Cruz *et al.* showed that two types of OMV are secreted from bacteria, the majority originating from the outer membrane with just a bilayer membrane, and a minority of double bilayer-type which also contained cytosolic proteins (148, 211). Another study suggested a model where the presence of autolysins in the periplasmic space leads to the development of a gap in the periplasm, allowing cytosolic proteins and DNA to "leak" into the OMV (141). It is apparent that more research needs to be done to establish the full mechanism of OMV biogenesis. Meanwhile, when observing the

major spots of our 2D-gel it clearly illustrated that the main constituents of the vesicles were OMPs (Figure 4). Consistent with this, the highest scores from the MALDI-TOF analysis were almost exclusively obtained by outer membrane, periplasmic and extracellular proteins. In contrast, most cytosolic proteins got relatively low scores.

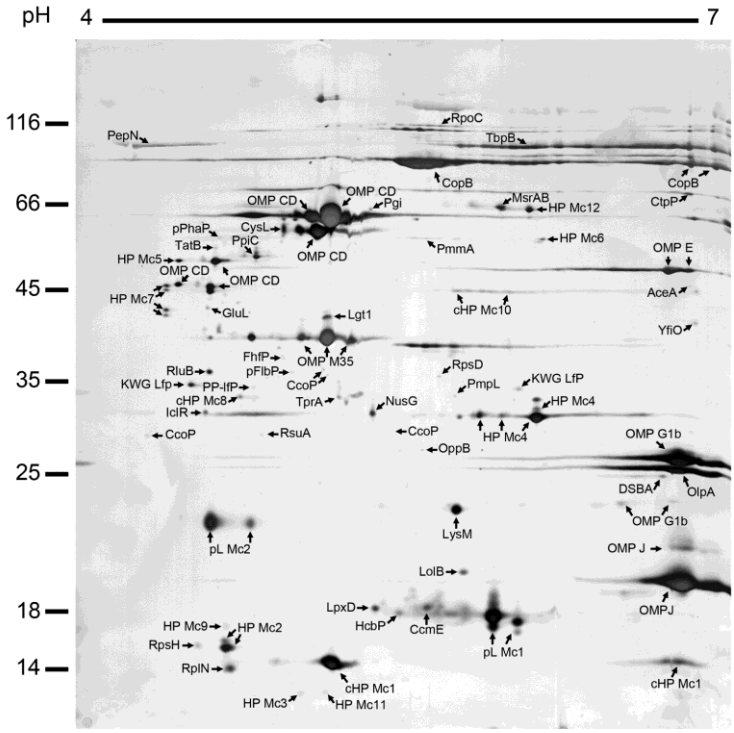

Figure 4. Protein spots from *M. catarrhalis* OMV.
Two-dimensional electrophoresis of *M. catarrhalis* OMV where proteins are separated in size (kDa) vertically and their isoelectric point horizontally.

As shown in several studies, OMV are mediators of inflammation that allow the bacterium to colonize in one site and cause inflammation in another. In this way the bacterium can evade the immune response while staying pathogenic. We knew from a previous study in our group that OMV from *M. catarrhalis* bound to and activated B-cells in a T-cell independent manner, inducing a polyclonal pro-inflammatory immune response (157). As *M. catarrhalis* is a Gram-negative pathogen that primarily colonizes the respiratory tract, we wanted to investigate how OMV secreted from this bacterium interacts with respiratory epithelial cells. Using fluorescence microscopy we showed that OMV bind human alveolar A549

epithelial cells at specific lipid raft domains. After isolating the lipid raft motifs we determined that OMV stimulation of epithelial cells also leads to TLR2 clustering. With flow cytometry analysis we could further observe that binding of OMV to A549 cells lead to the upregulation of the adhesion protein ICAM-1 on the cell surface, as well as the secretion of the pro-inflammatory cytokine IL-8. Because A549 cells have little or no TLR4 expression on their surface they are less responsive to LPS, indicating that this response is mainly due to OMV surface protein binding to TLR4 (212).

Carcinoembryonic antigen-related cell adhesion molecules (CEACAM)-1 is widely expressed on respiratory epithelial cells and implicated as a regulator of infection and inflammation, and is often co-localized with TLR2 (56, 213). Slevogt *et al.* showed that the adhesion protein UspA1 of *M. catarrhalis* binds CEACAM-1 on the surface of epithelial cells, thereby abrogating the TLR2 signaling pathway, resulting in reduced transcription of NF-κB and a decrease in the secretion of pro-inflammatory cytokines (56). In this study, we wanted to investigate if UspA1 on the OMV surface has the same attenuating effect on the TLR2-dependent signaling pathway of A549 cells, thereby allowing the bacteria to dampen the subsequent immune reaction. Using flow cytometry analysis we confirmed that OMV from UspA1-deficient *M. catarrhalis* produced a stronger immune response than the wild-type counterpart. The results of this study show that binding of OMV to epithelial cells creates an immune response characterized by IL-8 secretion and ICAM-1 upregulation, and that OMV can regulate this response through interaction of CEACAM-1 and UspA1. We hypothesize that through OMV secretion the pathogen activates epithelial cells to promote adhesion and infection, while at the same time "fine tuning" this immune response so that the bacteria may still successfully evade the immune response.

In addition, we wanted to confirm that OMV induce a pro-inflammatory response *in vivo*, using a mouse model. When the mice inhaled an OMV solution, we could clearly see the development of inflammation in lung specimens, represented by an influx of neutrophils and a disturbance of the membrane integrity of the alveolar cells. Mice are usually not considered the ideal model for *M. catarrhalis* infections since the bacteria are cleared within a few hours because of the strong immune response they induce in the lungs (214, 215), which we could clearly observe in our results.

In conclusion, the present study established that OMV from *M. catarrhalis* are composed of several major virulence factors and other outer membrane proteins important for survival and pathogenesis. We also confirmed that OMV from *M. catarrhalis* induce inflammation in respiratory epithelial cells from the human host, through binding to the cell membrane and activating cell surface receptors.

However, OMV can also modulate the immune response of these cells through binding of UspA1 on the vesicle surface. It has previously been shown that *M. catarrhalis* OMV are found in nasopharyngeal infections *in vivo* (37, 38, 52), and this study suggests that OMV are indeed important regulators of inflammation.

Paper II: *Moraxella catarrhalis* outer membrane vesicles carry beta-lactamase and promote survival of *Streptococcus pneumoniae* and *Haemophilus influenzae* by inactivating amoxicillin

Since the 1980s, approximately 97% of all *M. catarrhalis* strains are resistant to β-lactam antibiotics, through carriage of the *Moraxella*-unique enzyme β-lactamase. Considering that β-lactamase is a periplasmic enzyme (202), in paper II our aim was to further study OMV secreted from β-lactam resistant *M. catarrhalis* strains and investigate if they carry β-lactamase.

In order to investigate the presence of β-lactamase we cloned and produced a recombinant protein and after immunization of rabbits purified polyclonal anti- β-lactamase antibodies. Screening for β-lactamase in the OMV through western blot and TEM analysis confirmed that OMV from β-lactamase positive strain KR526 contained the enzyme, while the β-lactamase negative control strain Bc5 did not (Figure 5). Using the chromogenic substance nitrocefin, we found that that these vesicles were enzymatically active and that most β-lactamase was protected from proteinase activity inside the OMV. Our results also indicated that β-lactamase was not enriched in OMV. In addition, we could see that OMV can break down amoxicillin in a dose-dependent manner, in contrast to OMV from β-lactamase negative strains.

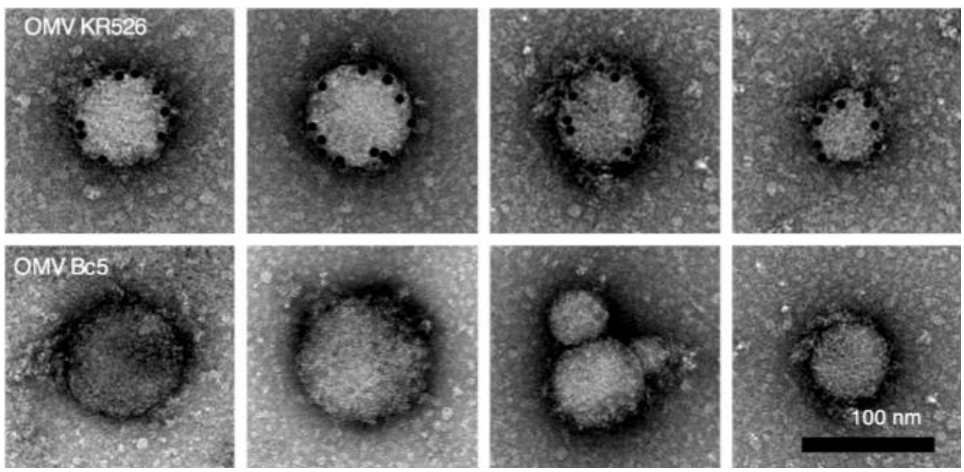

Figure 5. *M. catarrhalis* OMV contain β-lactamase.
TEM showing OMV from the β-lactamase positive KR526 (upper bar) and negative strain Bc5 (lower bar). Black spots represent gold-labeled anti-β-lactamase antibodies.

Previous studies have shown that *M. catarrhalis* is often found in co-cultures with other respiratory tract bacteria such as *Haemophilus influenzae* and *Streptococcus pneumoniae* (109, 110, 216). In addition, we know that OMV are found *in vivo* in patients with *M. catarrhalis* infections (52). Brook *et al.* hypothesized that β-lactamase producing bacteria in polymicrobial infections such as AOM or pharyngotonsillitis can survive and protect other bacteria through the release of free β-lactamase into the environment (116). Since OMV secretion allows bacteria to safely secrete proteins far away from the site of infection, we hypothesize that this might be a mechanism whereby bacteria share β-lactam resistance. We consequently wanted to examine if OMV from *M. catarrhalis* can protect susceptible *M. catarrhalis*, *H. influenzae* and *S. pneumoniae* strains in culture from amoxicillin-induced death. Interestingly, our results convincingly showed that β-lactamase-containing *M. catarrhalis* OMV protected other pathogens from antibiotic killing for up to five hours. The growth of the susceptible bacteria was comparable to that of the control incubated without antibiotics. This could effect could not be seen with OMV from β-lactamase negative strains.

Many studies have discussed the benefits of co-colonization of bacteria, and the advantages for the bacteria in polymicrobial infections (107, 108, 116). This project suggested a novel virulence mechanism for OMV, as mediators carrying and sharing antimicrobial resistance in mixed infections.

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

In a study of patients with cystic fibrosis, Giwercman *et al.* discovered significant levels of β-lactamase activity in patient sputum, with activity increasing after treatment with antibiotics (217). The authors suggested that *P. aeruginosa* secreted β-lactamase freely into sputum as an *in vivo* resistance mechanism. Furthermore, they found anti-β-lactamase IgG in serum and sputum from these patients, which was not present in healthy controls (218). In a later study, Ciofu *et al.* discovered β-lactamase in OMV from *P. aeruginosa*, suggesting that OMV were a possible mechanism for secretion from this bacteria into the extracellular space (219). Since we had previously established that *M. catarrhalis* OMV also contained this enzyme, our aim in this study was to further examine the presence of anti-β-lactamase IgG in serum of health adults.

In order to determine that the discovery of β-lactamase in OMV was not only *in vitro* or a laboratory phenomenon, we first wanted to make sure that OMV also contained β-lactamase in a human host. We established using TEM that a nasal sample from a child with sinusitis due to a *M. catarrhalis* infection was packed with bacteria secreting OMV-containing β-lactamase. This verified that OMV had a clinical relevance as mediators of antimicrobial resistance.

Next, we compared the antibody levels against *M. catarrhalis* β-lactamase in humans to those against *M. catarrhalis* adhesins and virulence proteins MID, UspA1 and UspA2. Since *M. catarrhalis* β-lactamase has a unique protein sequence compared to known β-lactamases from other Gram-negative bacteria, we were confident that these antibodies were specific for the *M. catarrhalis* enzyme. Our results showed that the highest IgG levels were against UspA1 and UspA2, with lower levels against MID and β-lactamase (Figure 6). In a previous study from our group similar results were obtained, although antibody levels against the MID protein were generally higher than those against UspA1/A2 (53). Our analysis of the sera showed that only a small portion had specific antibodies against β-lactamase, about 15%. These antibody levels were significantly lower than against adhesion proteins UspAs, suggesting that this protein is less accessible for the immune system.

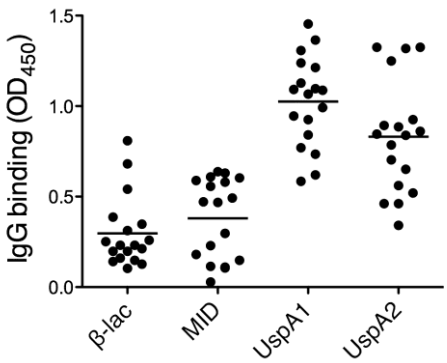

Figure 6. Comparison of IgG levels in human serum against *M. catarrhalis* virulence proteins. Results from enzyme-linked immunosorbent assay (ELISA) showing binding of IgG from healthy adults to common *M. catarrhalis* virulence factors β-lactamase, MID, UspA1 and UspA2 measured as absorbance.

The lack of antibodies against *M. catarrhalis* β-lactamase in most healthy adults indicated that this protein is rarely exposed to the extracellular space. This corresponds with data from our previous study, where we proposed that the majority of β-lactamase was found inside the OMV. However, in the present study

we also found that purified anti-β-lactamase antibodies could bind to and partially inhibit the activity of the β-lactamase in OMV, suggesting that the enzyme may also be exposed to the extracellular space. Put together, these results indicate that OMV act as storage pools for β-lactamase, although the localization of the enzyme can vary between preparations perhaps due to different environmental circumstances affecting OMV production. Similar to the study previously mentioned by Giwercman *et* al., other groups have suggested that β-lactamase can sometimes be found freely secreted from bacteria into the extracellular space (220, 221). However, since extracellular proteases would quickly hydrolyze any freely secreted proteins, we propose OMV secretion as a more probable delivery mechanism of these proteins.

In conclusion, the aim of this project was to investigate if there are antibodies against β-lactamase in human serum, as in patients with *P. aeruginosa*-derived cystic fibrosis. We found not only the presence of these antibodies, but could also determine that these antibodies bound OMV from resistant *M. catarrhalis* strains, and that they could inhibit the activity of this enzyme to a certain degree. We further confirmed the role of OMV not only as vehicles of protein secretion, but also as protective vehicles against serum proteases and inhibitory antibodies. In future studies it would be highly interesting to see how antibody levels vary in children with AOM or in adults with *M. catarrhalis*-derived exacerbations in COPD as compared to healthy adults.

Paper IV: Group A streptococci are protected from amoxicillin-mediated killing by vesicles containing β-lactamase derived from *Haemophilus influenzae*

Finally, in our fourth project, we wanted to investigate the prevalence of β-lactamase in other Gram-negative species in the nasopharynx. NTHi is a common colonizer of the upper respiratory tract and is frequently found to be β-lactamase positive and thus antibiotic resistant (205). Consequently, in paper IV we examined OMV from NTHi and could indeed confirm the presence of β-lactamase in the secreted vesicles (Figure 7).

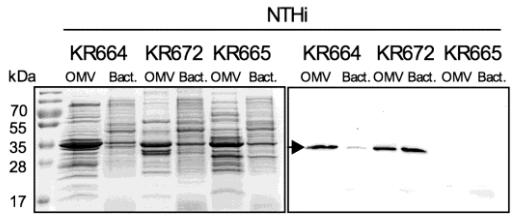

Figure 7. OMV from NTHi contain β-lactamase.
SDS-gel and western blot showing OMV from β-lactamase positive NTHi strains KR664 and KR672 contain β-lactamase, as opposed to susceptible strain KR665.

On another note, several studies have shown that even though group A streptococci are highly susceptible to β-lactams, these bacteria are sometimes inexplicably resistant to penicillin-treatment in patients with tonsillitis (222, 223). In a study by Brook *et al.* NTHi and *M. catarrhalis* frequently co-infected these patients with GAS treatment failures, leading the authors to hypothesize that these bacteria shared β-lactam resistance in a symbiotic relationship (115, 224). Additionally, other reports suggested that cephalosporins were more effective in GAS infections compared to penicillin. Interestingly, most NTHi and *M. catarrhalis* strains are susceptible to later generation cephalosporins, further implicating these bacteria in treatment failures with penicillin.

In light of our earlier studies, the aim of the current project was to investigate if OMV could be potential mediators of resistance in GAS infections. Using similar methods to our previous studies, we found that these OMV were potent hydrolyzers of amoxicillin even at peak plasma concentrations. The β-lactamase-containing vesicles could effectively protect GAS against β-lactam antibiotics which were entirely susceptible to the antibiotic without these vesicles. We also compared OMV from the different species and found that NTHi OMV seemed to be more efficient at breaking down amoxicillin compared to *M. catarrhalis* OMV. This could be due to a more potent or more closely packed β-lactamase.

In conclusion, the results of this study corroborate well with the previous findings indicating that co-infecting antibiotic resistant bacteria may protect susceptible pathogens in polymicrobial infections (116). We suggest that OMV may be mediators of resistance between bacterial strains in the nasopharynx. We also propose that OMV may be a mechanism whereby NTHi and *M. catarrhalis* protect GAS in pharyngotonsillitis infections from antibiotic-induced killing in co-infections. Today, clinical diagnostics focus on recognizing and treating the most abundant and disease causing pathogen, however our results stress the importance of also screening for and possibly treating co-pathogens present in an infection.

Conclusions

The aim of this thesis was to further elucidate the role of OMV secreted from Gram-negative pathogens of the respiratory tract, and their involvement in immune activation or evasion, as well as how they interact with other pathogens found in the same colonization niche.

First, we studied the proteomic composition of *M. catarrhalis* OMV, and were able to establish that the vesicles were composed of major *Moraxella* virulence factors involved in virulence, adhesion and nutrient acquisition. We also found that the OMV bound to and activated respiratory epithelial cells *in vitro* as well as *in vivo* and that OMV could induce and regulate the immune response to a certain degree.

Previous studies have hypothesized that bacteria in polymicrobial infections can share antibiotic resistance factors. We propose that OMV secretion could be a mechanism through which bacteria achieve this in a protected manner. In the papers that make up this thesis we showed that OMV secreted from patients with nasopharyngeal infections contain β-lactamase and that incubation with these vesicles save antibiotic susceptible respiratory pathogens from killing. We also demonstrated that OMV are protective vehicles that shield β-lactamase from neutralizing antibodies in serum.

Respiratory tract infections are a major cause of disease in the world, at a high cost for society. At the same time, antibiotic resistance is emerging as a threat to modern health care. For this reason, we find it essential to investigate pathogenesis and virulence mechanisms of the common microbes found in our respiratory tract.

Future perspectives

Throughout the last 40 years, OMV have been studied from various perspectives; their biogenesis and composition, their roles in bacterial pathogenesis and survival, as well as their interactions with other cells.

In this thesis we have studied the composition of *Moraxella* OMV obtained using standard laboratory settings. For future studies, it would be interesting to study how the vesicle protein composition varies with different environmental circumstances, such as a change in temperature, pH, or during antibiotic treatment. Using electron microscopy it would also be interesting to see how and if the OMV size and shape vary with change s in the environmental. We would also like to investigate the expression of specific proteins in different OMV preparations. For example we could determine if the expression of proteins involved in fine tuning the immune response in vesicles varies when produced under cellular stress. In addition, it could be interesting to investigate if β-lactamase is more frequently packed into these vesicles when bacteria are grown with antibiotics, or perhaps in co-infections with other bacteria. Not many studies regarding the role of OMV in polymicrobial infections have been conducted so far, and we believe this might be an interesting field of research. Furthermore, a central question would be to investigate how much OMV is secreted by bacteria in the normal flora of healthy adults, as well as by pathogens during infection, or in polymicrobial infections.

However, to be able to find the answer to these questions, it is imperative that we discover more precise methods to quantify OMV. Presently, most laboratories use protein content to achieve this. However, since the vesicles vary both in size and protein composition during different stages of growth or methods of isolation, this is an arbitrary unit and a more accurate method is desirable. One way of doing this might be using small particle counters used for nanoparticles such as viruses, or through direct counting in grids using TEM which, although tedious, might be a more accurate and precise method devoid of variation. However, a more simple way might be using flow cytometry analysis, although this might be better as a relative measurement between different preparations.

To date, the genetic trigger for OMV production has not yet been established. The discovery of a bacterium devoid of OMV production might give a clue to how important OMV are in pathogenesis and bacterial survival, or in the case of our studies as vehicles mediating inflammation and antibiotic resistance. It is also feasible to suggest that this trigger is general for most bacterial species; therefore it is desirable to investigate how this mechanism is regulated. Specifically, this might allow us to determine the importance of OMV in an *in vivo* animal model.

In conclusion, though a lot is known about OMV as mediators of virulence and bacterial survival, much still remains to be elucidated in order to determine the true impact of these nanoparticles on other microbes and the human host.

Acknowledgements

Now for the essentials: (I always said I would keep it short…)

Thank you to Kristian Riesbeck for being my supervisor these past four years. You certainly have an endless enthusiasm for science, and never left an email unanswered for more than a minute or so. Thank you for trusting me with my projects, and always pushing me to always do my best!

To my unofficial co-supervisor Therése Nordström. You helped me start off my PhD studies, and then came back just in time to help me finish. Thank you for all the support and guidance and for keeping your door always opens for a chat.

To Arne Forsgren, my co-supervisor, who has been an important presence in the lab, and has often shared good advice and experiences most generously.

To all my lab mates, without whom this past four years would have been so much harder and this thesis considerably thinner. You all made your definite impression on me, and I am forever grateful for your patience and help. In order of appearance: Marta, Kalpana, Yu Ching, Birendra, Laura, Farshid, Fredrik, Micke, Can, Burcu, Tamim, Ida, Klaudyna, Emma, Chrystelle, Christophe, Florence, Magnus, Oindrilla and Kerstin. Thank you for good advice, poker nights, excellent cakes, therapy train rides to Lund, coffee breaks, balcony sessions, disturbing my peace, bad jokes, wedding dinners, happy hours and so much more. And of course, most importantly, for being such great friends! Tack också till Anki och Nasida för all ovärdelig hjälp och uppmuntran dessa år.

Special thank you's to Adriana for being the first to read the thesis and boosting my confidence, and to Matilda for taking your time to help me decrypt the science-talk. Also, thank you to Oindrilla for deciphering the TLRs, to Susanne for being an essential part of pepp-klubben, and to Farshid for acting as an all-around thesis-consultant.

To all my wonderful, inspiring and thoughtful friends outside the lab, I couldn't have asked for better people. From east to west, you mean the world to me, from Obrunis of Uppsala to the Fåúr of Stockholm, from the great Rosenthalers of Lund

to the (real) Chapel Hill, back to the global Apples of Lund and now onwards to Big City Malmö. I hope for many more adventures to come with all of you!

Thank you of course to my ever-expanding family of Schaars, Stenlunds, Stewarts, Johanssons and Alvandis. You ARE the best; I will prove it scientifically in my next thesis. Please visit more often. Or let's all move to Malmö.

To Keyvan: we made it! Thank you for listening, for staying positive, and for always, always being there when I needed you. I really couldn't have done it without your love and support. Love you and your big heart.

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