Pathogenesis of Haemophilus influenzae. Respiratory infection in COPD patients
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CHAPTER 1

GENERAL INTRODUCTION
Chronic obstructive pulmonary disease (COPD)

Prevalence and risk factors
Chronic obstructive pulmonary disease (COPD) is characterized by the presence of progressive and irreversible chronic airflow obstruction [1]. The term COPD comprises three airway disorders which may occur independently or together in varying degrees in the same patient. These three disorders are: i, chronic obstructive bronchitis characterized by obstruction of small airways due to mucus hypersecretion; ii, emphysema with enlargement of air spaces and destruction of lung parenchyma, loss of lung elasticity, and closure of the small airways; iii, small airways disease (see for recent reviews [5,46,62]). COPD is a common disorder occurring in 14 million persons in the United States [1]. The prevalence of COPD has increased 41% since 1982 and is still increasing [1]. In Europe, COPD, asthma and pneumonia are together the third most common cause of death [82]. In North America, COPD alone is the fourth most common cause of death [1].

Various risk factors are involved in the development of COPD. The most important risk factor accounting for 80-90% of the cases of COPD is cigarette smoking. Environmental factors, including pollutants, dust, and tobacco smoke are also important risk factors. The only well-established genetic risk factor in the development of COPD is the deficiency of α1-antitrypsin (α1-AT, also referred to as α1-proteinase inhibitor). Among the patients with α1-AT deficiencies, more than 95% is homozygous for the Z allele, designated PiZZ. Their α1-AT level is only one sixth of the normal value [1]. Another factor possibly involved in the development of COPD is the occurrence of viral respiratory infections in early childhood [113]. Viral infections influence the cytokine response of airway epithelial cells [40] and enhance the adherence of bacteria to respiratory epithelial cells [47].

Inflammation and bacterial infections
COPD is characterized by the occurrence of chronic inflammation of the lower respiratory tract. It is thought that this inflammatory process plays an important role in the pathogenesis of COPD itself. Increased numbers of neutrophils, macrophages, and T-cells have been found in bronchoalveolar lavage fluid (BALF) and sputum of COPD patients [5]. Also high levels of inflammatory mediators, such as IL-8 and leukotriene B4 (LTB4), both chemoattractants for neutrophils, are present in the
airways of COPD patients [5]. The granules of neutrophils contain a variety of compounds, such as neutrophil defensins with antimicrobial activity as well as neutrophil elastase. The latter enzyme is likely to contribute to the pathogenesis of COPD by degrading proteins, including components of the extracellular matrix.

Patients with COPD suffer from exacerbations of their disease, that are associated with an increased production of purulent sputum. Especially, patients with chronic obstructive bronchitis have such exacerbations which are often associated with bacterial infections, most frequently due to *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae* [28]. The role of these bacterial infections in COPD in general, and in the occurrence of exacerbations in particular, is controversial. Even in patients with stable COPD, bacteria are present in the lower respiratory tract [63].

The bacterial species that frequently cause infections in COPD patients, are present in the microbial flora of the upper respiratory tract. The bacteria enter the lower respiratory tract by inhalation and aspiration. Normally, bacteria are removed from the lower respiratory tract by mucociliary clearance due to the coordinated beat of cilia of the ciliated respiratory epithelial cells. This defense mechanism prevents that in the airways of healthy individuals the presence of small numbers of bacteria will lead to tissue injury or bacterial colonization.

The chronic inflammation in patients with COPD is predisposing for bacterial infections [45] (figure 1). Neutrophil associated enzymes and other compounds damage the epithelium and impair the mucociliary clearing by which bacterial colonization may be facilitated. The damage of the epithelium results in an increased inflammatory state of the lower respiratory tract. The presence of bacteria in the lower respiratory tract also enhances this inflammatory process, since bacteria and their released products induce the production of pro-inflammatory cytokines by epithelial cells [7,51,96]. For *H. influenzae*, the lipid A part of lipoooligosaccharide, an outer membrane constituent, is responsible for this cytokine production [96]. In addition, bacteria themselves contain formylated peptides, which are potent chemoattractants for neutrophils [9]. In healthy individuals, the inflammatory process leads to eradication of the bacterial infection. In COPD patients, however, inflammation will result in infection contributing to ongoing inflammation (figure 1), a process which has been proposed as the “vicious circle hypothesis” [81].
Figure 1. Inflammation and bacterial infections in COPD patients. The predisposed airways of COPD patients are chronically inflamed. The inflammatory process facilitate bacterial colonization and infection, which leads to further amplification of the inflammatory process. See text for details.

**Neutrophils**

Neutrophils are attracted to sites of inflammation and infection by various chemotactic factors [39]. These factors include bacterial products, complement split factors, lipid mediators and chemokines such as IL-8. During the migration processes of the neutrophils from the blood vessel to the airway lumen, the neutrophils might contribute to the tissue injury. In the granules of the neutrophils several components are stored, which are released upon stimulation. Such components, including elastase, cathepsin G, and non-enzymatic polypeptides such as defensins may cause tissue injury [37]. Neutrophil elastase is a neutrophil-derived serine protease, which induces emphysema in laboratory animals [44]. Normally, the effects of neutrophil elastase are counteracted by proteinase inhibitors such as α1-antitrypsin. A persistent imbalance between proteases and antiproteases may lead to airway damage and subsequently to COPD [37,93].
Neutrophils are an important component of the innate immune system, a host defense mechanism providing the first barrier against pathogens. The production of antimicrobial peptides is one of the mechanisms employed by the neutrophil to contribute to this host defense mechanism. These peptides are cationic and display large structural diversity. In the human neutrophil, the most abundantly present antimicrobial peptides are the neutrophil defensins.

**Defensins**

**Source and characteristics**

The defensin family is comprised of molecules present in plants, insects, and mammals [42,54,80]. In mammals, two structurally different types of defensins have been described, α-defensins and β-defensins [23,53,107]. Recently, a third and cyclic type of defensins was isolated from monkey neutrophils [95].

The α-defensins have been found in human, rabbit, guinea pig, rat, and hamster neutrophils, in rabbit alveolar macrophages, and in human and rodent small intestinal Paneth cells. These peptides contain 29-35 amino acids residues, including six cysteines and five other residues that are highly conserved. In human neutrophils, four α-defensins (or neutrophil defensins) have been described (also referred to as human neutrophil peptides, HNP-1, -2, -3, and -4), of which HNP-1, -2, and -3 are the most abundant. Other human α-defensins are human defensins (HD) -5 and -6 which are expressed by small intestine Paneth cells. In addition, HD-5 is also expressed in the human female reproductive tract [76] and in the respiratory tract [20].

β-Defensins are found in bovine tracheal mucosa, in bovine neutrophils, in bovine tongue epithelium, and in mouse and human epithelia. At present, four human β-defensins (hBD) have been described, hBD-1, -2, -3, and -4, which are expressed by epithelial cells from various sources, including those from the respiratory tract [2,10,25,33,34,83]. β-Defensins are longer peptides than α-defensins (38-42 amino acid residues), also having six conserved cysteines, but with different cysteine linkage.

Both α-defensins and β-defensins have similar tertiary structures [38,41,72,73,115] and display a broad spectrum of antimicrobial activities, as they kill Gram-negative as well as Gram-positive bacteria, fungi and some enveloped viruses [61,79,107]. Defensins are considered to display their antimicrobial activities mainly
inside the neutrophil, but when the neutrophil is activated, defensins are released and in the sequestered area between the neutrophil and the target cell to which it is adhering, high concentrations of defensins may be present [22]. In general, defensins require low salt concentrations for their antimicrobial activity and metabolic active targets.

**Effects of neutrophil defensins on epithelial cells**

Neutrophil defensins are cytotoxic for mammalian cells [48,55,57,58,105]. The defensins bind rapidly to mammalian cell membranes [58], and prolonged incubation leads to multimerization of defensins in the cellular membranes [114]. Defensins are internalized into the target cells and subsequently lysis of the cells occurs [105]. Lower concentrations of defensins than required for the cytotoxic effects, stimulate the proliferation of epithelial cells by enhancing DNA synthesis [69]. Since defensins also induce the IL-8 production by airway epithelial cells, defensins contribute to the inflammatory responses [106]. The defensin-induced IL-8 expression is probably the result of increased transcription, since defensins increased the level of mRNA for IL-8 without affecting the stability of this mRNA [106].

**Antimicrobial effects of neutrophil defensins**

Neutrophil defensins interact not only with negatively charged membranes of mammalian cells, but also with membranes of micro-organisms [21]. Upon binding, defensins form multimers which are inserted in the membranes to form voltage dependent channels. These channels are suggested to cause leakage of the target cells [49,52,114]. In Gram-negative bacteria, defensins interact with lipopolysaccharide (LPS) (endotoxin), an outer membrane constituent [56]. This interaction leads sequentially to permeabilization of the outer and the inner membrane. The permeabilization of the inner membrane is lethal for the bacteria [52]. Various studies indicated that modifications of the lipid A part of LPS result in resistance of the bacteria to antimicrobial peptides. Defensin-resistant *pmrA* and *phoP-phoQ* mutants of *Salmonella typhimurium* have aminoarabinose or palmitate additions to the lipid A part of the LPS [30,31,32]. *Pseudomonas aeruginosa* isolates from CF patients have also modifications in the lipid A, which are associated with resistance to antimicrobial peptides [17]. The interaction of defensins with the LPS leads to neutralization of the endotoxic activities of LPS [56]. For Gram-positive bacteria, it was shown that
interactions of defensins with lipoteichoic acid are essential for the antimicrobial activity of defensins [74].

**Haemophilus influenzae infection and COPD**

**The bacterium**

*H. influenzae* is a Gram-negative rod-like bacterium. *H. influenzae* species are common inhabitants of the human nasopharynx, being present in approximately 75% of healthy human adults [97,81]. This species occurs with a polysaccharide capsule or nonencapsulated [75]. Six encapsulated *H. influenzae* serotypes have been identified based on the composition of their polysaccharide capsule (designated a to f). Although capsules in general are important virulence factors [65,64], systemic diseases, such as meningitis, cellulitis, epiglottitis, and pneumonia are mainly caused by encapsulated *H. influenzae* type b [66,97]. Nonencapsulated species lack the genes required for expression of the capsule and they fail to agglutinate with antisera elicited against these capsular serotypes. Therefore, nonencapsulated species are also referred to as nontypeable. Infections with nontypeable *H. influenzae* are limited to the respiratory tract. In children, they are a frequent cause of otitis media [70]. In adults, nontypeable *H. influenzae* is associated with lower respiratory tract infections, especially in patients with COPD [28,70,81,98]. Patients with cystic fibrosis (CF) are highly susceptible to *H. influenzae* infections at all ages [67]. So, although the capsular polysaccharide is an important virulence factor by protecting the bacteria against killing by complement and neutrophils, it does not contribute to the infection in COPD patients.

**Outer membrane proteins (OMP)**

Analysis of the outer membrane of *H. influenzae* on SDS-polyacrylamide gelelectrophoresis revealed various major outer membrane proteins (MOMP). Nontypeable *H. influenzae* show strong heterogeneity in their MOMP patterns [59,100]. This heterogeneity is used to biotype the nontypeable *H. influenzae* isolates. Characterization of isolates is based on MOMP P2 and P5.

**Lipooligosaccharide (LOS)**

LPS or endotoxin is the major glycolipid in the outer membrane of Gram-negative bacteria. Nonenteric pathogens, including *H. influenzae*, express a LPS molecule
lacking the long repeating O-specific polysaccharide side chains, which are characteristic for enteric Gram-negative bacteria such as *Escherichia coli* [8]. Therefore, the endotoxin of *H. influenzae* is often called lipoooligosaccharide (LOS).

LOS is composed of lipid A which is anchored in the outer leaflet of the outer membrane and a heterogeneous surface exposed oligosaccharide core. Heterogeneity of the oligosaccharide core of *H. influenzae* LOS is observed between strains, but also within a clonal population derived from one single strain. LOS is the end product of a complex biosynthetic process encoded by phase variable genes [64]. Variation in phosphate substitutions and saccharide branching chains have been identified. Due to additions of sialic acid or phosphorylcholine to LOS, the LOS resembles structures present in the human host [112].

**Persistence and antigenic variation characterize the *H. influenzae* infection**

In COPD patients, nontypeable *H. influenzae* cause recurrent infections at least for periods up to two years [26,28]. *H. influenzae* has several mechanisms to escape the host defense mechanisms, thereby contributing to persistence in the lower respiratory tract. The bacteria persist despite the presence of specific antibodies, complement and professional phagocytes. Between epithelial cells, *H. influenzae* bacteria hides from host defense mechanisms. During the persistence of nontypeable *H. influenzae* in the lower respiratory tract of COPD patients, the electrophoretic mobility of MOMP P2 and P5 of distinct *H. influenzae* isolates varied [15,16,27,29]. These variants are caused by accumulation of single non-synonomous point mutations, indicating selective pressure probably by antibody mediated defense mechanisms. Antigenic variation has also been observed using a rabbit model with subcutaneous tissue cages [108]. In this model, vaccination with the homologous strain did not eradicate *H. influenzae* [108]. Instead, more antigenic variation was observed after the vaccination with a homologous strain than with a heterologous strain. Whereas this indicated the importance of antigenic variation as escape mechanism, also poor opsonophagocytosis of viable nontypeable *H. influenzae* bacteria in the presence of specific antibodies and complement, contributes to the persistence of *H. influenzae* in COPD patients [109].

The capacity of *H. influenzae* to alter its LOS contributes to colonization and persistence by *H. influenzae*, since it will decrease the recognition of bacterial surface antigens by the host defense mechanisms [18,60,64,111]. Furthermore, *H. influenzae* strains persisting in the lower respiratory tract elicited a lower IL-8 production than
strains isolated only on one occasion [7], indicating that the persistence of 
*H. influenzae* might be associated with reduced inflammation, thus allowing survival of
the bacteria in the mucosa of the COPD patients.

**Pathogenesis of *H. influenzae* infection in COPD patients**

**Binding of *H. influenzae* to mucins**
Colonization of the lower respiratory tract with *H. influenzae* is a pre-requisite for a
*H. influenzae* infection. The respiratory mucosa is covered with mucus, which 
*H. influenzae* initially encounters. Mucus is a complex mixture of secreted molecules, 
including mucins, being high molecular weight glycoproteins with O-glycoside-linked 
carbohydrate side chains. *H. influenzae* binds to such mucins through the MOMP P2 
and P5 and fimbriae [6,11,77]. The excessive production of mucus in the COPD 
patients prevents entrapped and adhered bacteria from being killed, since mucus is a 
diffusion barrier for antibodies and complement and is impermeable for inflammatory 
cells. In healthy individuals, binding of *H. influenzae* to mucins is a host defense 
mechanism, facilitating removal of the bacteria by the mucociliary elevator. Since this 
mucociliary elevator is impaired in COPD, especially in chronic bronchitis, in this 
disease binding of *H. influenzae* to mucins allows bacteria to persist in the lower 
respiratory tract and to reach the epithelium, followed by the colonization of the lower 
respiratory tract.

**Adherence of *H. influenzae* to respiratory epithelial cells**
Adherence of *H. influenzae* to epithelial cells requires the presence of bacterial 
adhensins and cellular receptors. Several adhesins by which *H. influenzae* adheres to 
the respiratory epithelial cells *in vitro* have been identified (see for reviews 
[81,85,101]). Fimbriae (pili), long filamentous organelles that extend from the 
bacterial surface, mediate the adherence to oropharyngeal epithelial cells through 
binding to sialic acid containing lactosyl glycolipids. In the presence of purified 
ganglioside GM2 the adherence of *H. influenzae* to oropharyngeal epithelial cells 
*in vitro* is inhibited [99]. Fimbriae-mediated adherence is especially important for the 
encapsulated *H. influenzae* strains. Type b strains not expressing fimbriae showed an 
impaird colonization of the nasopharynx [24,92,110]. Encapsulated *H. influenzae* 
strains have also surface fibrils, a filamentous adhesin distinct from the fimbriae, with 
cellular binding specificities [86,87].
Of the nontypeable *H. influenzae* strains only a minority expresses fimbriae. Therefore, fimbriae may enhance initial binding of nontypeable *H. influenzae*, but are not essential for the pathogenesis of infection by these strains. Nontypeable *H. influenzae* isolates have various non-fimbrial proteins mediating adherence to epithelial cells. The high molecular weight proteins HMW1 and HMW2 are the most common of these adhesins [3,90], present in 70-80% of the nontypeable *H. influenzae* isolates. The HMW1 and HMW2 proteins show homology to the filamentous hemagglutinin (FHA) of *Bordetella pertussis*, a known adhesin which plays a critical role in the colonization of the upper respiratory tract by *B. pertussis* [3,78]. HMW1 and HMW2 mediate adherence of *H. influenzae* to distinct subtypes of epithelial cells, suggesting that the two adhesins have different receptor specificities [43,91]. HMW1 recognizes a sialylated glycoprotein and its adherence is inhibited by heparin or dextran sulfate [71,84]. The cellular receptor for HMW2 is not known at present. Adhering strains lacking the HMW-adhesin express the adhesin Hia [4]. DNA sequence analysis of the *hia* gene revealed that Hia is the nontypeable variant of the fibrils expressed by the encapsulated strains. Recently, it was shown that Hia also displays autotransporter activities [88]. In contrast to other known autotransporter proteins, Hia undergoes no processing event and remains cell-associated in the full length form. Finally, the Hap protein of *H. influenzae* was identified in all isolates tested; it mediates adherence which leads to invasion of *H. influenzae* into epithelial cells [89]. The Hap protein shows homology with IgA1-proteases of *H. influenzae* and *Neisseria meningitidis*. Hap is unable to cleave IgA1, but displays similar processing and secretion as the IgA1 proteases, having a membrane bound C-terminal domain (Hap$_\beta$) and an internal serine protease domain (Hap$_\alpha$) and a N-terminal signal sequence [35]. The Hap$_\beta$ inserts into the outer membrane and translocates Hap$_\alpha$ through the outer membrane, where the Hap$_\alpha$ gains autoproteolytic activity and is released extracellularly. This release results in loss of the adhesion promoting properties of the Hap protein, since adherence is mediated by the Hap$_\alpha$ domain. Interestingly, secretory leukocyte protease inhibitor (SLPI), locally produced by epithelial cells, blocks Hap autoproteolysis, resulting in increased amounts of Hap$_\alpha$ on the bacterial surface and as a consequence in augmented bacterial adherence [36].

The LOS of *H. influenzae* also contributes to the adherence to epithelial cells. An interaction between LOS of *H. influenzae* and the platelet activating factor (PAF)
receptor leads to adherence and invasion of *H. influenzae*. These processes were suggested to be dependent on the incorporation of phosphorylcholine in its LOS [94].

In summary, multiple adhesins mediate adherence of *H. influenzae* to epithelial cells. These adhesins are responsible for adherence to certain cell types. Some *H. influenzae* strains express multiple adhesins contributing to adherence, whilst other strains express no known adhesins and display no adherence *in vitro*. In primary epithelial cell cultures, nontypeable *H. influenzae* adhered and invaded injured epithelial cells which lost their cilia [113]. Since the airway epithelium of COPD patients is often injured, the preference for *H. influenzae* to adhere to the injured epithelial cells might facilitate the colonization of the lower respiratory tract of these patients. The preference of bacteria to adhere to damaged epithelial cells was also observed for *P. aeruginosa*, which adhered to the asialo GM1 receptors on damaged and migrating epithelial cells [12,13,14].

**Passage of *H. influenzae* through the epithelial cell layers; paracytosis**

Several *in vitro* and *in vivo* studies have revealed that nontypeable *H. influenzae* not only adhered to the epithelial cells, but that the bacteria were also present in the interstitium of the submucosa and within some of the epithelial cells. *H. influenzae* bacteria have also been found in close contact with macrophage-like cells in explanted lungs of COPD and cystic fibrosis (CF) patients [68]. In *in vitro* studies using cultures of epithelial cells on permeable supports, clusters of nontypeable *H. influenzae* between the epithelial cells were observed [103]. The bacteria penetrated the confluent epithelial cell layer by paracytosis, without affecting the permeability or viability of the epithelial cells. *H. influenzae* bacteria in between the epithelial cell layers were protected from the bactericidal activities of several antibiotics and antibody-mediated bactericidal activity [102]. Recently, the protein involved in the paracytosis of *H. influenzae* has been cloned and was designated paracytin [104]. Besides the passage between the epithelial cells, intracellular *H. influenzae* bacteria were found in subepithelial macrophages of adenoid tissue from young children [19]. This invasion of *H. influenzae* was associated with cytoskeletal rearrangements [50].

Summarizing, the pathogenesis of *H. influenzae* infection in COPD patients involves various steps by which the colonization of *H. influenzae* is enhanced. Furthermore, various mechanisms exist by which *H. influenzae* escapes the host defense mechanisms, allowing the persistent infections which have been identified.
However, initial steps for the onset of infection due to *H. influenzae* and the persistence of *H. influenzae* have not been fully clarified.

**Outline of this thesis**

The chronic state of inflammation in the airways of COPD patients is thought to predispose to *H. influenzae* infection, but the mechanism by which inflammation facilitates bacterial infection is not known. The interaction of *H. influenzae* with airway epithelial cells is important for the bacterial infection process as well as for the defense process against the infection, since the bacteria induce the production of pro-inflammatory mediators and antimicrobial peptides by epithelial cells. In the sputum of COPD patients *H. influenzae* as well as the antimicrobial peptides neutrophil defensins are found. Therefore, the effect of neutrophil defensins on the interaction of *H. influenzae* with respiratory tract epithelial cells was the subject for this thesis.

Initially, we determined whether neutrophil defensins had an effect on the adherence of *H. influenzae*. The defensin-enhanced adherence of *H. influenzae* to airway epithelial cells is described in chapter 2. The host cell specificity of this enhanced bacterial adherence phenomenon was examined in chapter 3. The bacterial species showing defensin-enhanced adherence are also described in chapter 3. The identification of the bacterial component involved in the defensin-enhanced adherence is presented in chapter 4.

Neutrophil defensins as well as *H. influenzae* have pronounced effects on the production of pro-inflammatory cytokines. Therefore, the effect of defensins in combination with *H. influenzae* on the production of interleukin-6 (IL-6) and interleukin-8 (IL-8) by respiratory epithelial cells was assessed (chapter 5). The implications of the effect of defensins on the bacterial adherence as well as the implications of the effect of defensins on the pro-inflammatory cytokine production for COPD patients are discussed in chapter 6.

**References**


