The role of Moraxella catarrhalis outer membrane proteins in pathogenesis

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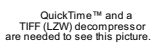
Taras Manolov

Doctoral thesis



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Abstract

Moraxella catarrhalis is an important respiratory pathogen. It is a common cause of otitis media in children, a usual infections agent in sinusitis and makes up to 10% of all exacerbations of chronic obstructive pulmonary disease (COPD). It is known to cause more frequent disease in the colder season, and, generally, its importance is recognised mainly in countries exposed to moderate climate. M. catarrhalis widely colonises infants and children but it is very uncommon for the bacterium to colonise the mucosa in adults.

The present thesis is concentrated on discussing two properties of M. catarrhalis, - binding and inactivation of alpha1-anticnymotrypsin (ACT) and complement component 3 (C3) for which M. catarrhalis outer membrane proteins "ubiquitous surface proteins" (Usp) A1/A2 are responsible. No other pathogenic bacterium is known to bind either ACT or C3.

The ACT is a serine protease inhibitor playing important part in antiprotease lung protection and anti-inflammatory signalling. We established that M. catarrhalis is able to bind and inactivate ACT through the two active domains on both UspA1 and A2 proteins. The phenomenon is proposed to be important for a) protease-antiprotease balance in the lung, for b) exacerbating inflammation and for c) inhibition of phagocytic clearance of M. catarrhalis.

The localization of ACT binding inside the UspA1 and A2 proteins provided us with means to develop a simple and quick method for purification of ACT from plasma or serum based on ACT affinity to a fragment of UspA2 protein of M. catarrhalis. The purification can be carried out practically in one step and the biological properties of ACT are retained.

In the last part of the thesis the C3 binding to M. catarrhalis Usp A1 and A2 was studied. The binding has been attributed to a single fragment in the both UspA1 and A2. Importantly, C3d fragment of C3 has been found responsible for the binding to UspA1/A2. The UspA2 has been demonstrated to inhibit both the classical and the alternative pathways of the complement. The C3 binding by M. catarrhalis strongly correlated with serum resistance of the bacterium, as well as with the expression of UspA1/A2.

Key words: Moraxella catarrhalis, antichymotrypsin, complement, C3, serum resistance, inflammation, cathepsin G, purification, affinity chromatography

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I **Taras Manolov**, Thuan Tong Tan, Arne Forsgren, and Kristian Riesbeck.
Moraxella–dependent α1-antichymotrypsin neutralization – a unique virulence mechanism.
American Journal of Respiratory Cell and Molecular Biology, 2008, 38: 609-617

II **Taras Manolov**, Arne Forsgren, Kristian Riesbeck.
Purification of α1-antichymotrypsin from human plasma with recombinant *M. catarrhalis* ubiquitous surface protein A1.
Journal of Immunological Methods, 2008, 333: 180-185

III Teresia Hallström, **Taras Manolov**, Peter F. Zipfel, Anna M. Blom, and Kristian Riesbeck.
Moraxella catarrhalis Confers Serum Resistance by Ubiquitous Surface Protein A1/2 – Dependent C3d Binding.
Manuscript

Abstract

Moraxella catarrhalis is an important respiratory pathogen. It is a common cause of otitis media in children, a usual infections agent in sinusitis and makes up to 10% of all exacerbations of chronic obstructive pulmonary disease (COPD). It is known to cause more frequent disease in the colder season, and, generally, its importance is recognised mainly in countries exposed to moderate climate. *M. catarrhalis* widely colonises infants and children but it is very uncommon for the bacterium to colonise the mucosa in adults.

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In the last part of the thesis the C3 binding to *M. catarrhalis* Usp A1 and A2 was studied. The binding has been attributed to a single fragment in the both UspA1 and A2. Importantly, C3d fragment of C3 has been found responsible for the binding to UspA1/A2. The UspA2 has been demonstrated to inhibit both the classical and the alternative pathways of the complement. The C3 binding by *M. catarrhalis* strongly correlated with serum resistance of the bacterium, as well as with the expression of UspA1/A2.

List of abbreviations

ABRS	acute bacterial rhinosinusitis
ACT	alpha-1 antichymotrypsin
AOM	acute otitis media
AT	alpha-1 antitrypsin
ATP	adenosine 5′-triphosphate
BAL	broncho-alveolar lavage
BSA	bovine serum albumin
CEACAM	carcinoembryonic antigen-related cell adhesion molecule
CG	cathepsin G
COPD	chronic obstructive pulmonary disease
fMLP	N-formyl-L-methionyl-L-leucyl-L-phenylalanine
Kd	dissociation constant
LOS	lipooligosaccharide
MID	*Moraxella catarrhalis* IgD-binding protein
MMP	matrix metalloprotease
NE	neutrophil elastase
NF-kappaB	nuclear factor kappaB
OMP	outer membrane protein
pAb	polyclonal antibody
PAR	protease-activated receptor
PD	Parkinson disease
PMNL	polymorphonuclear leukocyte
PRM	pattern recognition molecule
RCL	reactive centre loop
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulphate – poly acrylamide gel electrophoresis
TNF-alpha	tumour necrosis factor alpha
TLR	Toll-like receptor
Usp	ubiquitous surface protein (of *Moraxella catarrhalis*)

Introduction

Moraxella catarrhalis

Classification and characteristics

Moraxella catarrhalis is a Gram-negative non-motile capsule-less aerobic diplococcus. Metabolically it can be characterised by the lack of fermentation of glucose, lactose, sucrose and maltose; it is able to carry out the positive oxidase reaction, it is able to reduce nitrate and nitrite; performs hydrolysis of tributyrin (an isomeric glyceryl ester of butyric acid) and does not utilise 5% sucrose to form polysaccharide.

M. catarrhalis grows well on rich standard laboratory media like brain heart infusion broth/agar or tryptic soy digest media although supplements like boiled blood, vitamin and amino acid enrichment (chocolate agar) will result in better growth. Optimally *M. catarrhalis* is grown at 35-37^{0}C in the atmosphere of 3-7% CO_2, but it can tolerate a wider range of temperatures (20-42^{0}C) and ambient air [1].

On the solid media *M. catarrhalis* forms 2-4 mm big grey or non-pigmented, smooth, convex and opaque colonies that can be pushed undamaged on the surface of agar with a loop ("hockey puck" effect).

Currently the taxonomic position of the genus *Moraxella* is as follows: family Moraxellaceae, order Pseudomonadales, class Gammaproteobacteria in the phylum Proteobacteria.

After initial discovery in the end of the nineteenth century as the suspected agent behind disease of respiratory tract (hence the name – *Mikrokokkus catarrhalis*) the bacterium (named successively *Neisseria, Branhamella* and finally *Moraxella (Branhamella) catarrhalis*) was deemed a harmless commensal until the 1970s, when opinion started to strengthen for it being a cause of respiratory distress. The incidence of *Moraxella* increased non-dramatically during that time, so the awareness must have risen mainly due to more accurate identification. It is known that during merely the 1980s the occurrence of beta-lactamase activity among some laboratory isolates of the

bacterium had risen from 36 to 90% [2], thus it can be argued that in the late twentieth century *Moraxella catarrhalis* became more virulent [1, 3, 4].

Diseases, connected with Moraxella catarrhalis

General pathogenic traits of M. catarrhalis

Specificity

M. catarrhalis is a strict human pathogen and is known to colonise (without causing disease) nasal and oral cavities, and the oropharynx. There are no known cases of *M. catarrhalis* causing disease in any other organism than humans. The mostly used mouse pulmonary clearance model for studying of *M. catarrhalis* disease is inescapably fundamentally flawed because untreated mice usually clear the bacterium in 24 hours [4], or, as reported by [5], the bacterium is largely cleared from the site in 6 hours and almost completely eradicated from the organism in 24 hours. Chinchilla nasopharyngeal colonization model was better, so that colonising *M. catarrhalis* was able to persist in modest numbers for at least two weeks [6].
M. catarrhalis is mainly involved in diseases of the upper and lower respiratory tract.

Biofilm formation and its importance

M. catarrhalis is able to form biofilm in the host organism, in vivo [7] as well as in vitro [6], which may increase the resistance of the bacterium to antibiotics [8] and to the immune system of the host. Production of biofilms by pathogenic bacteria may induce the release of anaphylatoxins and protect the bacteria from the deposition of immunoglobulins, components of complement, and finally from the phagocytosis [9]. Bacteria in a biofilm are tightly bound to one another and to the epithelium, resisting engulfment and frustrating phagocytosis. Frustrated phagocytosis may result in an extracellular degranulation of neutrophils and subsequent overwhelming of antiprotease and antioxidant protection in the immediate vicinity. At the level of the whole respiratory system the increase of concentrations of proteolytic enzymes may be unnoticeable, but substantial local damage may be done even after a single burst of proteolytic activity, due to the kinetics of proteolytic enzyme diffusion and inhibition [10].

Seasonal occurrence of disease

A unique, atypical feature, uncommon to other respiratory pathogens, of *M. catarrhalis* is the apparent seasonally increased incidence of disease, with winter and spring months showing higher rates of isolation [11, 12]. Although this seasonality may be put down to seasonal viral respiratory tract infections (by respiratory syncytial virus, but not adenoviruses) that predispose an infection by *M. catarrhalis*, there exists a possibility that the bacterium is particularly equipped for infecting lower temperature-exposed mucosa. An expression of an adhesin of *M. catarrhalis*, UspA1, was shown to be significantly upregulated after a "cold shock" at 26^0C, compared to 37^0C conditions [13]. Another piece of evidence pointing towards "cold" character of *M. catarrhalis* infections is an observation that *M. catarrhalis* strains isolated from healthy Dutch children were showing significantly higher percentage of complement resistance compared with strains isolated from Angolan children [14].

Upper respiratory tract conditions

M. catarrhalis is a more common upper respiratory tract pathogen in childhood than in adults. At 6 months of age 26% of all children have nasopharyngeal colonization of *M. catarrhalis*; by one year 72% infants will have been colonised with the bacterium at least once. This may depend on the fact that during the first 2 years of life anti-*M. catarrhalis* Usp antibodies' levels remain relatively low. Afterwards, the immunoglobulin levels rise, with IgA increasing gradually over the other isotypes, peaking in adult age [15]. This must be due to the possibility that although mucosal colonization with *M. catarrhalis* falls sharply to its nadir in adult age, the bacterium may reside both within and beneath the epithelium as well as inside host cells, as it was shown for pharyngeal lymphoid tissue [16]. It is hypothesised that to possibly avoid the anti-bacterial immunoglobulins *M. catarrhalis* undergoes a shift in genotype and phenotype between strains prevailing in infants versus "adult" strains. The isolates grown from adults demonstrate higher incidence of the *uspA2H* gene compared with lower for *uspA2*, also they show decreased biofilm-forming abilities, lower expression of MID, lower frequency of serum resistance and higher (two fold) prevalence of B type lypooligosacharide (LOS) [17].

Acute otitis media

Acute otitis media (AOM) is an inflammation of the middle ear accompanied by a liquid effusion [1]. AOM is a very common respiratory tract infection that is more often reported in children than adults. It is probably the most common disease seen in children. *Moraxella catarrhalis* is a third major agent causing bacterial otitis media in children, responsible for 10-15% of cases, after *Streptococcus pneumoniae* and *Haemophilus influenzae* [18].

Acute bacterial rhinosinusitis

Acute bacterial rhinosinusitis (ABRS) is a common upper respiratory infection with inflammation of the mucosa of the nose and paranasal sinuses. An estimated 20 million cases of ABRS occur per year in the USA, which costs approximately $3.5 billion [19]. *M. catarrhalis* is isolated in 3-10% adult cases of ABSR; but it is more frequent in ABRS in children. Similarly to AOM, ABRS is mainly caused by *Streptococcus pneumoniae* and *Haemophilus influenzae*; another important bacterium in ABRS is *Staphylococcus aureus*.

M. catarrhalis in the upper respiratory tract - future trends

Both in AOM [20] and in ABRS [21] the respective frequencies of the common respiratory pathogens demonstrate change after introduction of conjugated pneumococcal vaccine [22]. *H. influenzae* is enjoying the main increase at the expense of the *S. pneumoniae* isolates and vaccine serotypes, but *M. catarrhalis'* frequencies are also showing upward trends. The effect of the pneumococcal vaccine introduction is even more evident in the increased *M. catarrhalis*-colonization of children treated with the vaccine [23].

M. catarrhalis was unknown as a cause of AOM as late as in 1950, rising afterwards to approximately 15% in 2000 [15], therefore we may experience the continuation of the trend and further increased importance of *M. catarrhalis* as an AOM infectious agent or generally as an upper respiratory tract pathogen.

Chronic obstructive pulmonary disease and other lower respiratory tract diseases

Moraxella catarrhalis is known to be involved in lower respiratory tract diseases mainly in adults, although the bacterium was implicated as a cause of bacterial tracheitis in children [24]. *M. catarrhalis* is connected with laryngitis: in some studies it was found the most common species of bacteria to be isolated from adult laryngitis patients [24].

M. catarrhalis is not usually a cause of lung and/or bronchial disease in healthy adults but requires predisposing conditions: underlying disease of the respiratory system or immunodeficiency due to chemotherapy, hereditary abnormality or advanced age [3].The bacterium is also recognised in nosocomial outbreaks, indicating that clusters of infections occur in hospitals [4, 25] and has been described as a cause of community acquired pneumonia [26, 27]. *M. catarrhalis* is one of pathogens that has been isolated from bronchiectasis patients' sputa [28-30]. Bronchiectasis – is a chronic disease of the bronchi that substantially decreases the quality of patients' lives. The progressive bronchial dilatation in bronchiectasis is likely to be the result of continued airway matrix destruction [31]; therefore the connection of *M. catarrhalis* with bronchiectasis is relevant.

M. catarrhalis is a major cause of exacerbations in chronic obstructive pulmonary disease (COPD), after *Streptococcus pneumoniae* and *Haemophilus influenzae* [3, 4, 24]. The burden of *M. catarrhalis* caused disease is significant: the bacterium colonises the respiratory tract of 5 to 32% of COPD patients at any given time, is probably causing around 10% of all COPD exacerbations which translates into 2-4 million exacerbations of COPD per year in the USA; the patients experiencing at least one acquisition of *M. catarrhalis* differ from the other patients by about twice as long follow up period [32]. The frequency of *M. catarrhalis* isolation from sputa of COPD patients has risen during the last quarter of the twentieth century; worrying fact considering that took into account the increased awareness of this bacterium [3, 24]. More contemporary studies tend to find higher *M. catarrhalis* frequency than before [24, 33, 34]. As it was mentioned earlier, *M. catarrhalis'* trend to cause disease at a

relatively colder time of the year is also relevant for the lower respiratory tract [4, 24]. Interestingly, *M. catarrhalis* is not mentioned as a significant cause of bacterial exacerbations of COPD in the studies produced in some South-East Asian countries, suggesting possible importance of colder climate for the ability of the bacterium to cause disease [35, 36].

Rare pathological conditions connected with M. catarrhalis

M. catarrhalis is known to cause other diseases; although it happens seldom, but some of those conditions are rather serious and therefore worth mentioning.

At least five cases of endocarditis caused by *M. catarrhalis* were reported [37]. More often is encountered bacteraemia, with mortality as high as 21% [38]. Already 17 child cases of *M. catarrhalis* bacteraemia are known. Most of the children affected were less than two years old, immunocompetent and had accompanying lower respiratory tract symptoms [39].

Among serious systemic conditions caused by *M. catarrhalis* is even meningitis, more often in children than in adults, although the incidence of it is not frequent [40, 41].

The incidence of ocular disease caused by *M. catarrhalis* is rare, but it was demonstrated as the third most important pathogen after *H. Influenzae* and *S. pneumonia* in conjunctivitis [42]; it can also cause keratitis [43].

M. catarrhalis' *outer membrane proteins*

Outer membrane proteins of *M. catarrhalis* were long a subject of thorough study. The bacterium is not known to secrete any soluble virulence factors; therefore to elucidate the virulence of the bacterium the main attention was directed towards investigation of the factors that determine properties of its surface, of which the proteins are the most important. It has to be mentioned that *M. catarrhalis* along with many other Gram-negative bacteria can produce outer membrane vesicles [44, 45], a property that to a certain extent may emulate secretion of soluble proteins, therefore adding more meaning to the importance of outer membrane proteins in the virulence of the

bacterium. To date approximately 20 *M. catarrhalis*' outer membrane proteins (OMPs) have been characterised, of which 6 to 8 are predominant in a typical SDS-PAGE resolved and Coomassie Blue stained outer membrane preparation.

OMP CD

OMP CD is a porin-like protein that probably has a role in adherence to human lung epithelial cells and is able to bind mucins from the middle ear [46, 47]. The predicted size from sequence for OMP CD is 46 kDa, though it migrates as a 55 kDa protein or as a 60 kDa, if reduced [48]. It is highly conserved and antibodies against OMP CD are bactericidal against *M. catarrhalis* [49].

OMP E

OMP E is a 50 kDa protein that also demonstrates high conservancy among isolates of *M. catarrhalis* [50]. It was shown that the mutant devoid of OMP E is more sensitive to the killing effect of normal human serum [51, 52]

CopB (OMP B2)

This 81 kDa protein's expression can be repressed with iron; therefore it is probably involved in iron acquisition for the bacterium [53]. It is largely conserved but has three major areas of moderate heterogeneity, variable regions that are likely to be exposed on the surface [54].

Lactoferrin binding proteins A and B (LbpA and LbpB)

LbpA (100 kDa) and B (95 kDa) are lactoferrin receptors, that is they are responsible for iron capture from human protein lactoferrin [55, 56]. LbpB, but not LbpA induces production of bactericidal antibodies in the human host [57].

Transferrin binding proteins A and B (TbpA and B)

TbpA and B are homologues of LbpA/B, able to bind transferrin, loaded with iron. As well as for LbpA/B proteins, only anti – TbpB immunoglobulins were detected in the human sera, but they were able to kill the bacterium [58, 59].

M. catarrhalis *adhesion protein (McaP)*

McaP is a 62 kDa protein that confers adhesive properties towards Chang, A549 and polarised human bronchial cells. It also acts as an esterase, cleaving both phosphatidylcholine and lysophosphatidylcholine [60].

M. catarrhalis *IgD – binding protein (MID)*

MID is known for its binding of immunoglobulin D (IgD) in a non-immune way [61]. The synonym – Hag – is derived from its hemagglutination ability [62, 63]. MID is also largely responsible for *M. catarrhalis* adherence to lung alveolar type II epithelial cells (A549) and to human middle ear epithelial cells [63].

OMP J

A major outer membrane protein of *M. catarrhalis*, existing in two forms (J1 – 19 kDa, and J2 – 16 kDa) is probably playing role in bacterial clearance, because two of its J2 mutants were less easily cleared from infected lungs [64].

Type IV pili

M. catarrhalis is known to produces type IV pili which are necessary for natural transformation, and have been proposed to play some role in adhesion to the respiratory epithelium and in biofilm formation [6, 65].

Lipooligosaccharide (LOS)

Although LOS is not a protein, it is mentioned here due to its presence in the outer membrane of *M. catarrhalis* and its importance for the virulence. Similar to other Gram-negative bacteria, *M. catarrhalis* also possesses an endotoxin – lipooligosaccharide (LOS) in its outer membrane. But unlike typical structure of enteric bacterial lipopolysaccharide (LPS), *M. catarrhalis* LOS consists of an oligosaccharide core and lipid A without the repeated O-antigen polysaccharide side chains. The *M. catarrhalis*' LOS plays an important role in adhesion to and invasion into epithelial cells, being neither an adhesin per se, nor solely necessary for invasion [66, 67]. Another property of *M. catarrhalis*' LOS is its ability to induce excessive inflammation through stimulating human monocytes to produce pro-inflammatory

NF-kappaB and IL-8, with the latter stimulating naive monocytes to produce TNF-alpha [68]. On the other hand, LOS can also induce production of antibacterial antibodies by the host [69].

Ubiquitous surface proteins A1 and A2 (UspA1/A2)

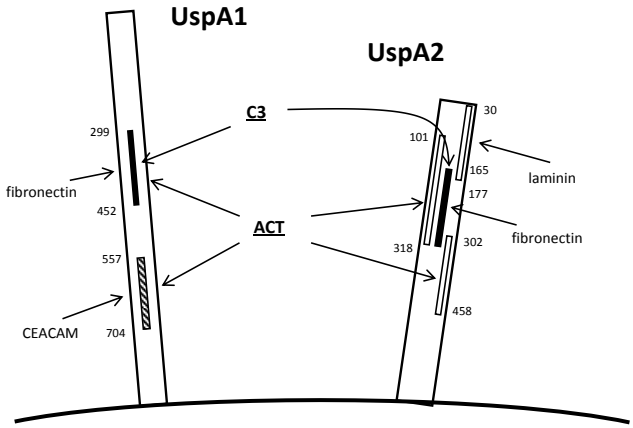
Out of other major OMPs of *M. catarrhalis*, UspA1 and A2 are of particular interest. Ubiquitous surface proteins (Usp) A1 and A2 are multifunctional and interact with the host in several ways [70].

UspA1 is able to bind carcinoembryonic antigen-related cell adhesion molecule (CEACAM) which can be found on epithelial cells [71]. There also exists a "hybrid" form of UspA2 protein, designated UspA2H, sharing adherent properties with UspA1 [72]. The expression of UspA1 is: a) subject to phase variation, changing between high and low levels [73] and b) upregulated upon exposure to cold temperature ("cold shock") consistent with the host inhaling cold air [13], enhancing adherence to the Chang epithelial cells. Due to its binding to CEACAM, UspA1 can induce apoptosis in the alveolar epithelial cells [74]. But besides adhesion, together with LOS, UspA1 can determine invasion of the epithelial cells, although this phenomenon seems to be based mostly on UspA1 interaction with fibronectin and alpha5beta1 integrin, and not on CEACAM binding [67].

UspA1 and A2 of the strain O35E share a 140 amino acid region with 93% identity [75]. Furthermore, Cope *et al*. describe several strains of *M. catarrhalis* that share (not only between UspA1 and A2 proteins but also between strains) several highly conserved repetitive amino acid motifs [76]. Logically, that may mean that whatever properties are determined by such fragment of one of the proteins, they must be shared by the other. That is true in several cases. UspA1 and A2 bind the extracellular matrix protein fibronectin [77], although in the report of McMichael *et al* [70] fibronectin binding to UspA2 was not found, which may depend on whether their strain of *M. catarrhalis* possesses as identical UspA1 and A2 as in previous work by Tan *et al*. UspA1 and A2 both bind laminin, which also is probably important for adherence to host cells and therefore - colonization. UspA1 and A2 together probably play important

roles in serum resistance due to binding of C4bp [78]. The binding of C3 was previously thought to depend mainly on UspA2 [79], but our new data (Paper III that is presented in this thesis) suggest that both UspA1 and A2 are responsible for the binding of C3.

Figure 1. A scheme showing domains on UspA1 and A2 proteins that are responsible for interactions with different host proteins. UspA1 and A2 on the scheme correspond to single proteins (monomers). Note the multifunctional and interlapping domains.



The binding of vitronectin although seems to depend solely on the UspA2 protein [70, 80]. Finally, we will briefly mention here a work that can be found as Paper I of this thesis, where we have recently shown that UspA1 and A2 are able to bind and neutralise ACT (Figure 2), which is a truly unique property of *M. catarrhalis*, considering our knowledge of other species of pathogenic bacteria [81] (Figure 1, 2).

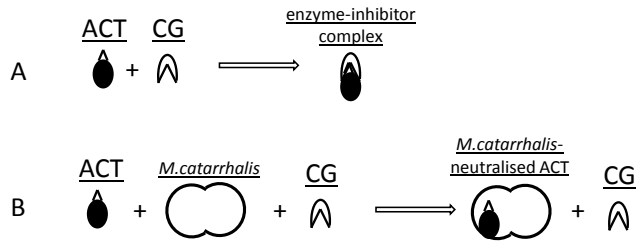


Figure 2.
(**A**) Secreted by PMNL, the protease cathepsin G is quickly inhibited by the excess of ACT.
(**B**) Neutralized by *M.catarrhalis*, the level of ACT is insufficient to control the protease.

M. catarrhalis' *pro-inflammatory properties*

M. catarrhalis is a pathogen, which can induce a strong pro-inflammatory response in human respiratory epithelium. *M. catarrhalis* LOS stimulated monocytes to produce pro-inflammatory cytokines IL-1beta, Il-6, TNF-alpha. LOS-stimulated monocytes secrete high levels of IL-8 and can stimulate adjacent naive monocytes to produce TNF-alpha [68]. Other important evidence that *M. catarrhalis* effects monocyte signalling is the observation that lung epithelial cell culture infected with *M. catarrhalis* induced monocyte recruitment and oxidative burst, the latter being dependant on intercellular contact and not been possible to reproduce with epithelium-derived soluble factors [82].

The presence of bacteria such as *M. catarrhalis* has been found to increase IL-8 release in bronchoalveolar lavage of COPD patients, which is associated with disease progression [83]. The same effect was demonstrated *in vitro* within cell culture. Culture supernatants and heat-killed *M. catarrhalis* induced IL-6, IL-8 and prostaglandin E_2 production from lung epithelial cell culture A549 [84].

Another study found a culture of bronchial epithelial cells (BEAS-2B) responding to *M. catarrhalis* with release of IL-8 and granulocyte-macrophage colony stimulating factor. This effect was associated with activation of p38, extracellular signal-regulated kinase pathways, activation of NF-kappaB, reduction of global histone deacetylase expression and activity, as well as with changes in the nuclear histone acetylation-deacetylation balance in the respiratory epithelial cells [85].

Antichymotrypsin

Antichymotrypsin and serpins

General information on ACT and serpins

Alpha-1 antichymotrypsin (ACT) is a globular glycoprotein of 68 kDa of which approximately 25% comprises carbohydrates. Glycosilation of ACT is heterogeneous and extensive: a molecule of ACT carries four asparagine-linked two- and three-antennary oligosaccharide side chains [86] although sugars are unnecessary for its inhibitory properties [87]. The mature protein contains 394 amino acids [88] forming a single chain [89]. ACT belongs to the family of serpins and is closely related to AT, archetypal serpin, being 42% identical in amino acid sequence [90]. Similarly to ACT, sugars are not necessary for the antiproteinase action of AT [91], probably not for other serpins too.

The name serpins is derived from “serine protease inhibitors”, although not all serpins can inhibit serine proteinases. There are also known serpins that inhibit papain-like cysteine proteases (squamous cell carcinoma antigen-1,2) [92, 93]. Serpins may perform a non-inhibitory role, while retaining similar structure to the inhibitory ones [93]. For example, some human serpins function as hormone transporters, e.g. corticosteroid binding globulin (CBG), thyroxine binding globulin (TBG); angiotensinogen (ANG) is a peptide hormone precursor; and certain serpins function as molecular chaperones (47 kDa heat-shock protein) or tumour suppressors (maspin) [92, 93]. Ovalbumin is a well known example of a non-human non-inhibitory serpin. There are serpins that unlike ACT (and AT) are not secreted but have intracellular functions, e.g. proteinase inhibitor-6 (inhibitor of cathepsin G) or yukopin (inhibits trypsin) [93].

Serpin-like genes have been identified in animals, poxviruses, plants, bacteria and archaea, and over 1,500 members of this family have been identified to date [93, 94].

The three-dimensional structure and reactive centre loop of serpins

The serpins characteristically have mobile reactive centre loops (RCL), which are exposed and susceptible to proteolysis by a variety of proteinases. In human ACT this domain (RCL) is 23 amino acids long and is located near the carboxyl end of the protein. The active inhibitory site of ACT is situated between the leucine 358 and serine 359 residues. The length of the RCL is critical for the inhibitory activity of ACT, as relocation of the active site even by a single residue within the RCL can drastically decrease ACT activity [95].

The three-dimensional structure of ACT is made up of eight alpha helices, three large beta-sheets and an active site situated within the RCL [95]. Serpins exist in the native conformation until binding of a target proteinase to the RCL, which triggers a conformational switch as the RCL inserts into the dominant (A) beta sheet and migrates to the opposite pole of the ACT molecule. This reaction is irreversible and the serpin is no longer able to bind any additional proteinases [95].

Some serpins require binding of cofactors as an additional means to control their inhibitory activity. Antithrombin can efficiently function as inhibitor of proteases thrombin and factor Xa only in the presence of heparin, although it is stable without its cofactor. On the other hand, plasminogen activator inhibitor I in the absence of vitronectin will rapidly (and irreversibly) convert to a latent inactive state. Apparently, heparin binding site of antithrombin is situated in the same region as the vitronectin-binding site of plasminogen activator inhibitor I [93].

Molecular instability of serpins

Serpins that are active as proteinase inhibitors are strained, inherently unstable molecules, that “release” their strain after the binding of a target proteinase, “trapping” it in a stable protease/inhibitor complex. But this instability in the molecule, especially in case of certain even less stable polymorphisms can resolve in a pathological way,

producing polymers that cannot be secreted from cells and thus create problems either due to accumulation in the cell, deficiency outside, or both. These polymeric forms of serpins are considered to be cause of several pathological conditions, e.g. early onset of dementia in case of neuroserpin, emphysema and liver cirrhosis due to AT serpinopathy, and antithrombin-induced thrombosis [96, 97]. Serpin polymers possess considerable stability and form rapidly due to a domain swap of more than 50 residues including two long antiparallel beta-strands inserting into the centre of the dominant beta-sheet of the neighbouring monomer [98].

Serpin molecules can spontaneously undergo transformation to a latent, inactive form; in such cases the RCL apparently inserts itself into the beta-sheet A in the same region where cofactor binding sites of certain serpins are located [93]. Several serpins including antithrombin and ACT [99], are able to switch to the latent conformation. Existence of such latent forms of serpins may present the molecular explanation for a number of pathological situations, where a substantial number of circulating molecules of a certain serpin nevertheless present actual deficiency of its function. For instance, the formation of inactive ACT conformation is behind COPD in patients with Leu55Pro mutation of ACT [100].

Antichymotrypsin deficiency and COPD

Antichymotrypsin is normally found in plasma in the range of 0,3 – 0,6 mg/ml [101]. However, there are conditions when ACT content is lower. Eriksson *et al* described familial deficiency of ACT (around 50% of norm) that in several members of the family results in lung abnormalities involving severe disease [102]. This deficiency was observed at frequencies of 1 to 200/300 in a Swedish population. In this and the study of Lindmark [103] the occurrence of increased residual volume was noted among subjects with the trait. This deficiency was heterozygous and was inherited in an autosomal dominant mode at genetic frequency of 0.003.

Another study established an association between ACT deficiency (59% of norm), COPD and Pro227→Ala substitution [104]. This association seems to be dependent on a particular population, for, as was shown by Samilchuk [105], a geographically distant

(Germany versus Russia) study did not discover the same connection. However, the absence of a Pro227Ala mutation from the cohort of COPD patients may be due to differing gene frequencies in different populations. Faber *et al* [106] described a case of 60% level of plasma ACT coinciding with COPD. Genetic analysis showed that the ACT gene was harbouring the substitution Pro229→Ala. Another patient with COPD was found to have this mutation but it was absent in all control group. The occurrence of the Ala-15 signal peptide allele was not connected with COPD according to this study (70% in both control and COPD patients group versus 30% of Tre-15 allele).

An article by Ishii [107] reports, on the contrary, association of the signal peptide Ala-15 allele (in homozygotes) with COPD (37.7% of Ala-15 homozygotes versus 18.5% in the control group) and no connection to COPD with other polymorphisms. The absence of ACT polymorphisms' association with COPD can be attributed here again to the population differences due to geographic location (Japan).

Once again, another study of a geographically different population (Italy) showed no association of the signal peptide polymorphism with COPD, and no other polymorphisms of the ACT gene were found in the tested population [108]. As in the case of AT deficiency associated with the Z allele [109], there seem to be connection with Northern Europe.

An interesting detail worth mentioning, although not in relation to COPD, is quite telling on the matter of geographical population peculiarities: other ACT gene polymorphisms that seem to be connected with higher risk of developing PD in Japan (AA) have no association in Germany etc. [110].

A different matter is the role of ACT deficiency in CF. The patients having both CF and plasma deficiency of ACT (less than 200 mg/ml) experience significantly less severe lung disease than those with normal and raised levels of ACT [111]. It is worth noting that the -15 signal peptide genotype did not affect plasma levels of ACT, but Ala-15 was overrepresented in the CF patients compared with healthy controls. A similar phenomenon was shown for AT-patients with CF and mild to moderate deficiency phenotypes of AT have preserved lung function compared with those with a

normal AT phenotype [112].This suggests that mild deficiency of the proteinase inhibitor permits down-regulation of the inflammatory response, thereby reducing the severity of the pulmonary damage [113].

Numerous polymorphisms of AT gene are known and the link between certain alleles and significantly increased risk for COPD is well established [109]. Another, less specific inhibitor of a broad range of proteases - alpha 2-macroglobulin (A2M) was found to be heterozygotically altered in a case of A2M serum deficiency and COPD from childhood [114]. These data about proteins related to ACT by function and (in the case of AT) closely related by structure, support the possibility of a connection between ACT genetic polymorphisms and COPD, and underline the importance of ACT for normal lung function. Another interesting detail, quite telling about the importance of ACT is that its plasma deficiency was to our knowledge discovered only in heterozygotes, suggesting a low viability of individuals homozygotic for polymorphisms encoding ACT plasma deficiency.

Cathepsin G - the main target for ACT

Cathepsin G (CG) is a neutral chymotrypsin-like proteinase produced in and secreted by polymorphonuclear leukocytes (PMNL). It may participate in the destruction of phagocytised matter and remodelling of tissue at the sites of inflammation. Due to its relative speed of interaction with ACT compared with other serine proteinases CG is regarded as a primary target for ACT [115].

CG activates airway epithelial cells and induces secretion from airway submucosal glands, and has been shown to cleave components of the extracellular matrix *in vitro*, although less efficiently than NE. Moreover, it can regulate innate immunity and inflammation through cleavage or proteolytic inactivation of collectins, cytokines, complement, and cell surface receptors [116].

CG (as well as NE) is probably very important for battling fungi as CG (and/or NE) - knockout mice are susceptible to fungal infections [117]. CG proteolytic action may be necessary for destruction of fungal spores. On the other hand in the absence of CG (and

NE), mice have increased resistance to the negative effect of endotoxin LPS, despite normal levels of TNF-alpha produced [117]. Thus these serine proteinases may act as effectors in the endotoxic shock cascade downstream of TNF-alpha.

In contrast to the fungal data, CG does not seem to be necessary for antibacterial defence, at least not against *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *E. coli*. CG-knockout mice survived as long as the wild type ones after intraperitoneal injection with these pathogens [118]. There was even a (not statistically significant) trend toward fewer deaths of CG deficient mice that were inoculated with *S. aureus* or *K. pneumoniae*.

Another study established that mice deficient in CG are able to clear *Pseudomonas aeruginosa* more efficiently than wild type. Higher antibody concentrations were also found in the respiratory epithelial lining fluid from mice lacking CG. These data suggest interference of CG either with opsonisation or phagocytosis or both [116]. Similarly, elastase inhibits neutrophil phagocytosis of *P. aeruginosa* by cleaving both CR1 and C3bi, creating an opsonin receptor mismatch [119].

CG is also known to obstruct the process of clearing of apoptotic PMNL by macrophages – the uptake of apoptotic neutrophils that were pre-treated with CG may be practically switched off [120]. The precise nature of this interaction is as yet unknown, but proteolytic digestion of pro-apoptotic ("eat me") signal protein is proposed; although it was established that conventional apoptotic receptor - phospatidylserine receptor was not involved. Earlier studies showed that NE acts in a similar way, impairing the clearance of the apoptotic cells in vitro [121]. In the case of NE, the cleaved apoptotic receptor seemed to be phospatidylserine. Clearance of apoptotic neutrophils by alveolar macrophages (efferocytosis) plays an important role in the resolution of lung inflammation. If not disposed of, apoptotic neutrophils may release granular contents in the epithelial environment which is likely to result in epithelial tissue damage. Indeed, early apoptotic neutrophils under transition to secondary necrosis were shown to induce detachment of lung epithelial cells (A549) in vitro, which happened together with accumulation of NE in the supernatant [122]. This confirms an earlier study that established the role of NE in detachment of lung

epithelial A549 cells [123], and so the importance of removal of apoptotic PMNL was confirmed. Due to the similarity between CG and NE effects' on the efferocytosis, one might propose that CG inflict similar damage on epithelial cells.

The other key outcome of phagocytosis of apoptotic cells by macrophages is the suppression of production of inflammatory mediators, both in vitro and in vivo. Also in this process the interaction between protein pro-apoptotic ligand ("eat me") on apoptotic cells and corresponding receptor on macrophages stimulates the production of anti-inflammatory mediators (IL-10) and quenches the production of pro-inflammatory TNF-alpha [120]. The most recent data on the failed apoptotic cell removal (efferocytosis) are reviewed here [124].

Functions and importance of antichymotrypsin

General inhibitory properties of ACT

After tissue damage the plasma concentration of ACT may double or even quadruple within 8 h, and it is therefore regarded as an acute phase reactant [125]. In fact, during the acute phase the concentration of ACT rises faster than AT. Thus ACT may have a more important role in the initial stage of inflammation than AT, whose concentration is usually four times the concentration of ACT in the plasma. ACT is generally thought to play an important role in the regulation of chymotrypsin-like proteinases. ACT has the highest affinity towards the neutrophil enzyme CG, i.e. it is with CG that the formation of the enzyme/inhibitor complex is the fastest (k_{ass}=5 x 10^7 $M^{-1}sec^{-1}$). The reaction with chymotrypsin (pancreatic) progresses at least 1000 times slower [126]. Other targets for ACT include mast cell chymases, human glandular kallikrein 2, kallikrein 3 (prostate specific antigen), pancreatic cationic elastase and many more [95]. ACT does not inhibit either neutrophil elastase or trypsin [127], rather is a substrate for both.

Formation of stable complexes with proteinases and their turnover

The ACT reacts with proteinases in equimolar ratio to form stable complexes, which eventually dissociate although at a very low rate [128]. Physiologically the dissociation of an ACT-proteinase complex is not particularly important, because the complex itself

is cleared from the circulation by liver, quite rapidly [129]. Moreover, the presence of ACT-proteinase may actually further stimulate local ACT synthesis [130]. In other words, ACT is an irreversible, suicide inhibitor (as all inhibitory serpins are).

Synthesis in the liver and the lung, induction of synthesis

ACT as a typical acute phase reactant is synthesised in hepatocytes and secreted into the bloodstream to be distributed throughout the system. It can also be produced by bronchial epithelial cells, which suggests particular importance of ACT in the lung [131]. Alveolar macrophages are also known to synthesise and secrete ACT [132]. Its synthesis is increased by inflammatory cytokines: IL-6 in hepatocytes; IL-1 upregulates the production of ACT in bronchial epithelial cells [95].

Anti-inflammatory properties of ACT

Inhibition of neutrophil chemotaxis, adhesion and spread

ACT when native, complexed or proteolytically cleaved, is able to regulate neutrophil chemotaxis. Native ACT reduces chemotaxis (particularly when stimulated with fMLP) [133]; whereas cleaved [134] or complexed with cognate proteinase [135] ACT promotes neutrophil migration to inflammatory sites. The inhibition of neutrophil chemotaxis apparently works through CG that is bound to the neutrophil membrane and is involved in receptor-mediated cell activation [136]. Besides arriving in place, a neutrophil would need to attach and spread over the inflammatory site. It appears that ACT is able to suppress these stages as well. ACT (and AT) inhibited proteolytic processing of selected adhesion-related proteins (e.g. fibronectin, CD43) by neutrophil proteinases (CG in case of ACT) and thus inhibited neutrophil adhesion or spread [137].

Inhibition of neutrophil production of reactive oxygen species (ROS)

ROS together with serine proteases are important parts of the host antibacterial defence, but if secreted extracellularly, especially when neutrophils are present in substantial numbers, secretion of ROS and proteinases may cause host tissue damage. It was demonstrated that ACT, both native and complexed with a protease, inhibited ROS production in neutrophils stimulated with fMLP. Moreover, this phenomenon did not

depend neither on the proteinase inhibitory activity of the ACT, nor on the glycosylation [138]. Interestingly, it was not degranulation that was suppressed, only the production. There is also evidence that ACT promotes this action via interaction with a component of the active assembled NADPH oxidase complex [139].

Anti-inflammatory signalling

ACT, as well as AT, inhibits synthesis and secretion of PAF in the leukocytes that were induced by TNF-alpha and IL-1. ACT was able to inhibit PAF synthesis at concentrations 10-100 fold lower than in serum. Therefore, it is unlikely that any significant amount of PAF will be synthesised and secreted when there exists such a serpin-rich environment. Synthesis of PAF may require inactivation of those protease inhibitors or it may be limited to the zones of close cell to cell contact, where antiproteases are absent. On the other hand, CG (and neutrophil elastase) was able to stimulate the synthesis of PAF as well as the autocrine stimulation [140].

ACT in the wound

ACT plays an important role in cell spreading that is part of the process of wound healing. It is one of the factors needed to protect fibronectin to enable cells (e.g. smooth muscle cells) to spread in the three-dimensional fibrin gel. Without ACT (or AT either alpha2-macroglobulin) fibronectin is degraded [141].

ACT inhibits CG-catalysed maturation of matrix metalloproteinase-9 MMP-9 (i.e. TNF-alpha – induced conversion of proMMP-9) in skin wound healing [142]. The effective concentrations of ACT were 5-10 μg/ml which suggests pathophysiological relevance of the inhibitor in attenuation of MMP-9. This fact points towards important role of ACT in wound healing through limiting the excessive proteolysis in the wound.

Importance of ACT in the lung

In bronchiectasis, free and catalytically active NE and CG were detected, which is evidence that antiprotease protective shield does not successfully regulate neutrophil elastase (NE) and CG in the moderate and severe form of the disease [143]. This suggests an important regulatory role for ACT in bronchiectasis. The nature of

protease-antiprotease imbalance in the large bronchi and in the lung parenchyma may differ, because of for example the role of alveolar macrophages.

As previously mentioned AT, the inhibitor of neutrophil elastase, plays a significant role in the development of COPD. The role of ACT deficiency in COPD may be equally as important, since the condition is about 10 times as rare, as is AT deficiency. However, due to the fact that it is found so seldom it is hard to assess. Most likely the ACT deficiency confers a much more decreased viability on the phenotype than AT deficiency, either due to its role in the lungs or because of some not yet elucidated function.

It is established that AT is quite sensitive to oxidation because of the presence of methionine in the active centre [144]. On the other hand, ACT is the most oxidation-resistant antiprotease in the lungs of the ones that were tested with ozone [145]. Thus AT may serve as a backup inhibitor against CG in case the capacity of ACT is overwhelmed, but not vice versa, because ACT is quite specific towards chymotrypsin-resembling enzymes and does not inhibit NE. Therefore, the anti-NE inhibitory capacity has no backup and is vulnerable in the cases when the AT pool is depleted by oxidation or deficient from the beginning.

The imbalance between proteinases and their inhibitors is thought to be responsible for the destruction of the lung parenchyma and the development of emphysema in COPD. The present understanding of the issue is centred around the role of neutrophil elastase and matrix metalloproteases, produced by alveolar macrophages [146, 147], i.e. the role of CG and its inhibitor – ACT is hardly considered. However as mentioned above, CG may play a serious role in the inhibition of efferocytosis, in what may be a positive feedback loop, increasing its own concentration, as well as the concentration of neutrophil elastase because of the release of the necrotic neutrophils content. On the other hand, CG is a chemotactic agent for monocytes, which, transforms them into macrophages, the source of another very important enzyme for the development of emphysema, MMP-12 (macrophage elastase). Importantly, CG can interact with protease activated receptor (PAR) -1 on the overexpressing PAR-1 oocytes [148]. PAR-1 needs to be activated to induce the production and secretion of active MMP-12

in macrophages [149], an enzyme, crucial for the development of emphysema [150]. This receptor can be activated by the serine proteases thrombin and plasmin [149]. Since cathepsin G is also a serine protease, one could suggest the possibility that it can also activate PAR-1, but there is no data at present to support this hypothesis. Finally, CG can activate matrix metalloproteinases (MMP) -2 (in the presence of membrane type-MMP-1 – expressing cell) [151] and MMP-3 [151, 152].

Based on the above, ACT may play an important role in the development of the COPD, a disease that cannot be explained without mentioning proteolytic enzymes and lung damage inflicted by them. The understanding of the nature of the COPD cannot be done without involving the phrase "excessive inflammation", because this is where the misbalance between proteases and their inhibitors originates. If we want to deal with a disease that is the fourth major cause of death globally [153], we will have to, sooner or later, among other important issues, consider studying ACT.

Antichymotrypsin and pathogenic bacteria

Proteases are well-known virulence factors of bacteria. These enzymes damage host tissue s and provide bacteria with soluble substrates and extracellular matrix proteins for adhesion. In addition, proteases may inactivate the host defence system. The ability of *M. catarrhalis* to bind and neutralise antichymotrypsin functions similarly to production of an endogenous enzyme, although in the case it is the host who synthesises proteases (i.e., cathepsin G), which bacterium subsequently uses.

An array of pathogens can inactivate antichymotrypsin by endogenously produced proteases, suggesting that inactivation of antichymotrypsin by microbes is an important virulence mechanism. For example, *Legionella pneumophila* cleaves ACT without digestion [154]. Both elastase and proteinase of *Pseudomonas aeruginosa* inactivated ACT with slight decrease of the molecular weight of the cleaved product [155, 156]. *Streptomyces griseus'* protease mix "pronase", *Bacillus subtilis* subtilopeptidase A, a neutral protease of *Bacillus polymyxa*, subtilisin BPN' from *B. amyloliquefaciens* and *B. thermoproteolyticus'* thermolysin inactivated ACT in catalytic amounts by limited proteolysis [157]. Selected periodontal pathogens, e.g. *Porphyromonas gingivalis* and

Treponema denticola, also demonstrated inactivation of ACT of a near specific degree [158], both with whole cells and with purified bacterial proteinases. Thus bacterial proteases specifically degrade antichymotrypsin. This property of bacterial pathogens to enzymatically destroy antichymotrypsin, and the specificity of the proteolysis, suggests that this property of microbes has evolved particularly to counteract antichymotrypsin at a site of infection.

Purification of antichymotrypsin

The methods of purification

Several methods for purification of ACT have been described. Travis *et al*. [159] purified ACT by ammonium sulphate fractionation followed by chromatography on Cibacron Blue Sepharose at pH 7.0 and chromatography on SP-Sephadex C-50 at pH 5.5. Later Travis and Morii published [160] a modified method, based on the earlier work [159]. This is a traditional method the usage of which can be traced through scientific literature. It is suitable for obtaining individual ACT in intact native condition, but it presupposes generous supply of time and effort. A laboratory whose interests lie beyond mere purification of proteins may not be perfectly prepared or, have the skills and experience needed for mastering the art. One cannot help but notice that in certain cases researchers had to complete the method of Travis and Morii with quite possibly more expensive but executively simpler affinity chromatography with specific "anti-impurities" antibodies [161]. Naturally, researchers coming to work with ACT were pressed to look for more economical methods in relation to time- and work volume.

The DNA-affinity chromatography of ACT has been reported in several articles with variants:

A chromatography on DEAE Sephadex A-50, followed by DNA cellulose column and ammonium sulphate fractionation [162];

A QAE-Sephadex column, followed by a smaller one with the same matrix, a chromatography on DNA-cellulose and a polishing on Sephadex-200[163];

An ammonium sulphate fractionation followed by successive chromatography steps on QAE-Sephadex, DNA-cellulose, and Sephacryl S-300[164].

Apparently not all researchers were satisfied by existing protocols, judging by the number of different variations of the same method. Laine and Hayem [165] employed not less than four different chromatographic steps to obtain pure ACT. Even affinity purification based on Sepharose-coupled anti-ACT immunoglobulins has also been described [166].

Dubin *et al*. published report on purification of ACT together with eight other plasma proteins on a system consisting of seven different columns, though it was only Cibacron Blue-Sepharose, which was specific for ACT. They required one more purification step, using Mono Q ion-exchange chromatography [167].

Rubin *et al*. used ion-exchange chromatography on Sepharose fast Q, continued by chromatography on DNA-cellulose and sometimes on Mono Q [87].

In Paper II of this thesis we describe a method for affinity chromatographic purification of ACT, based on an inexpensive recombinant bacterial protein and quite modest in terms of time necessary for its execution.

Complement

The antimicrobial human defence of the lung

The respiratory system is constantly in contact with the outer environment and must therefore have vigorous defences against invading airborne pathogenic bacteria. The host defences can kill bacteria by employing humoral or cellular means. The most important first step is the recognition of a pathogen. The cells of the innate immunity of the lung, including phagocytes, dendritic and epithelial cells use pattern recognition molecules (PRM) to detect pathogenic bacteria by their conserved molecular patterns [168].

The most important PRM are so called Toll-like receptors (TLR). Dendritic cells as well as epithelial cells of the respiratory system are known to express a variety of TLR. Activation of the TLR can induce the release of chemokines and cytokines into the submucosa, which would initiate an inflammatory reaction and therefore recruit phagocytes (PMNL and macrophages) and dendritic cells and lymphocytes that would start mounting an adaptive immune response [168, 169].

At the same time airway epithelium would release so called antimicrobial peptides into the lumen of the airways. Antimicrobial peptides are small proteins that are usually positively charged and have both a hydrophilic and hydrophobic side that enables these molecules to be soluble both in aqueous solutions and to penetrate lipid-rich membranes. The examples of such peptides are defensins and cathelicidins [170]. There are also larger antimicrobial proteins, which possess antimicrobial activity of different kinds. For example, lactoferrin, lysozyme and secretory leukocyte proteinase inhibitor [168].

Complement, its functions and importance

Complement is part of the innate immune system and underlies one of the main effector mechanisms of antibody-mediated immunity. It has three general physiologic roles: defending against bacterial infection, bridging innate and adaptive immunity, and disposing of immune complexes and the products of inflammatory injury [171].
Complement plays a major role in the defence against infection by microorganisms. It consists of a group of plasma proteins that, when activated by antibodies or cellular surfaces, interact in cascade fashion to produce a membrane attack complex capable of direct cytolysis [169].

Complement protein C3

The third component of complement (C3) is indispensable, because it functions in both the classical and alternative pathways of complement activation. Individuals affected by homozygous C3 deficiency suffer from recurrent pyogenic infections such as pneumonia, septicaemia, otitis media, and meningitis, and the absence of C3 is frequently lethal [171, 172]. Expression of the human C3 gene is tissue specific, with liver hepatocytes being the main site of C3 synthesis [172]. C3 is an acute phase reactant, increased synthesis of which is induced during acute inflammation [172]. A single chain precursor (pro-C3) is found intracellularly, which is processed by proteolytic cleavage into two subunits, the a and P chains. In the mature protein, these are linked by disulfide bonds. Cleavage of C3 by C3 convertases produces two activated fragments, the anaphylatoxin C3a - a vasoactive peptide and a mediator of

inflammation - and C3b. In activated C3b, a highly reactive thiolester group becomes exposed, which allows the fragment to bind covalently to the surfaces of, for example, pathogenic bacteria by a transacylation reaction. Surface - bound C3b acts as a cofactor in the formation of C5 convertase and thus can complete activation of the complement cascade. It is also recognized by C3b receptor - expressing B lymphocytes and facilitates phagocytosis of the foreign particles by C3b receptor - expressing macrophages and neutrophils (Figure 3A). Activity of C3b is limited by specific proteolytic cleavage involving factors I and H [169].

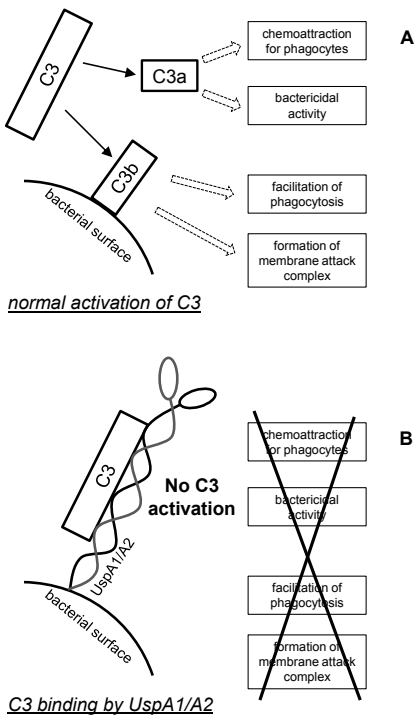


Figure 3.
(**A**) Normal activation of complement component C3. C3 is cleaved in two parts. C3a is secreted while C3b binds to bacterial surface and may participate in the subsequent reactions of the complement cascade or in the phagocytosis.

(**B**) C3 binding by *M. catarrhalis* UspA1 and A2, and its possible significance.

It has been proposed that *M. catarrhalis* binding and inactivation of C3 contributes to the serum resistance of the bacterium [79]. However, in their work Attia *et al.* [80] suggest that C3 binding does not play important role in serum resistance, arguing that the main responsibility for that lies with *M. catarrhalis*' interaction with vitronectin. It must be mentioned however that Attia *et al.* [80] admit they could not fully inhibit

serum resistance of *M. catarrhalis* with vitronectin depletion [80], therefore vitronectin binding cannot totally account for the serum resistance of the bacterium.

In Paper III of this thesis we present more information on the C3 binding by *M. catarrhalis*. It has been established, for example, that C3 bound by the bacterium is not cleaved, that is the C3a fragment is not produced. It must mean that C3 bound at the surface of *M. catarrhalis* does not act either as a component of complement, or as an opsonin for facilitation of the phagocytosis. It is therefore convincing that C3 binding of *M. catarrhalis* may be beneficial for the bacterium both as a contribution to serum resistance, inhibition of bactericidal action of C3a and as a means to inhibit phagocytosis by the PMNL and macrophages expressing C3 phagocytic receptor (Figure 3B).

THE PRESENT INVESTIGATION

Aim

The general aim of this work was to further study the role of outer membrane proteins of *M. catarrhalis* in pathogenesis with particular emphasis on UspA1 and UspA2.

Specific aims of the present work

Paper I

To investigate if *M. catarrhalis* can interact with ACT and to characterise such interaction.

Paper II

To study the possibility to exploit the phenomenon of *M. catarrhalis* ACT binding in a method of ACT purification from plasma or serum.

Paper III

To get further insight into UspA1/A2 proteins interaction with C3 complement component, its importance for the serum resistance of the bacterium, to localise the protein regions, responsible for the binding.

RESULTS AND DISCUSSION

Paper I - Moraxella–dependent α1-antichymotrypsin neutralization – a unique virulence mechanism

To determine if common respiratory pathogens can interact with ACT, we tested binding of ACT by *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* with flow cytometry. Surprisingly, only *M. catarrhalis* bound ACT. Then we tested more species of pathogenic bacteria, including *Moraxella* subspecies both with flow cytometry and direct binding of [^{125}I]-labelled ACT, but only *M. catarrhalis* was able to efficiently bind ACT.

Our laboratory benefits from possessing UspA1/A2 deficient mutants of *M. catarrhalis*, therefore we decided to check if ACT binding was dependent on Usp expression. It turned out that UspA1/A2 – deficient *M. catarrhalis* completely lost binding of ACT, as we checked with flow cytometry and confirmed with whole cells pull-down and elution assay. To assess the correlation between Usp expression and ACT binding, we have run a panel of *M. catarrhalis* strains and corresponding UspA1, A2 or double mutants (when such existed) in a flow cytometry experiment, separately stained with anti-ACT and anti-Usp pAbs. We obtained a strong correlation between Usp expression and ACT binding by *M. catarrhalis*, therefore concluded that UspA1/A2 are solely responsible for the binding of ACT.

Tested with radiolabelled ACT, two strains of *M. catarrhalis* provided us data for the estimation of the dissociation constant (Kd). The Kds for the two tested strains were approximately 25 and 45 nM, meaning that the affinity of *M. catarrhalis* for ACT is rather strong.

To check the ability of *M. catarrhalis* to bind ACT from relevant environments, bacteria were treated with bronchoalveolar lavage (BAL) samples and tested by flow cytometry for the presence of bound ACT on the surface. *M. catarrhalis* has been found to successfully bind ACT from the samples of BAL.

To find out if the ACT, immobilised by *M. catarrhalis* is still active or not, we have run an enzymatic-inhibition assay with bovine chymotrypsin and discovered that *M. catarrhalis* neutralises ACT at its surface.

Already possessing corresponding sets of truncated recombinant fragments of UspA1 and A2 from *M. catarrhalis,* we have set about pinpointing the regions in the

bacterial proteins that were responsible for the ACT binding. The results of the ELISA-based assay suggested that both UspA1 and A2 contain two domains that determine ACT binding. Not completely surprising, the analysis of the proteins' sequences' pointed out the existence of a highly conserved short sequence that, with certain variation, repeats twice per UspA1 and A2 protein. A synthetic peptide based on one of the four such sequences was able to bind ACT when coated on a micro titre plate. However, the recombinant "source" truncated fragment of the UspA2 was more efficient in ACT binding than synthetic peptide, which may be an indication of a less than native conformation of the peptide.

The importance of our finding lies in the fact that ACT is a quite important player in the antiprotease defence of the lung, and even small local decrease in its concentration can set off a cascade of events leading to local damage of the alveolar or bronchial tissue and increased inflammation. On the other hand, *M. catarrhalis* is a bacterium which is known to benefit from inflammation, as it often requires predisposing conditions to start an infection. The possibility that neutrophil cathepsin G (which should accumulate in the lung precisely in such a case of exacerbated inflammation) may interfere with phagocytosis would also be useful for the bacterium.

Paper II - Purification of α1-antichymotrypsin from human plasma with recombinant **M. catarrhalis** *ubiquitous surface protein A1*

Having obtained information on binding of ACT and on location of ACT binding regions on *M. catarrhalis* UspA1/A2, we decided to explore the possibility to use a recombinant truncated fragment of UspA1 or A2 to purify ACT from plasma o serum.

The smallest stable ACT-binding fragment of UspA1/A2 proteins - $UspA1^{557–704}$ was conjugated to CNBr Sepharose and the $UspA1^{557–704}$ - resin was used subsequently in purification of ACT. Plasma or serum was applied; the column was washed consecutively with high and physiological saline and eluted with guanidine-HCL.

The buffer-exchanged and concentrated eluate was proven to contain ACT without significant impurities; the ACT obtained was native: it reacted with anti-ACT pAbs, retained its inhibitory activity against bovine chymotrypsin and bound to *M. catarrhalis*.

The simplicity and short time needed for this method should help it find usage. Compared to other ACT purification methods known from the scientific literature, affinity purification on recombinant truncated UspA1 fragment UspA1$^{557–704}$ is very straightforward, simple and consuming much less time.

Paper III - Moraxella catarrhalis *Confers Serum Resistance by Ubiquitous Surface Protein A1/2 – Dependent C3d Binding.*

To get deeper understanding of the relation of C3 binding by *M. catarrhalis* and serum resistance of the bacterium, a number of isolates of *M. catarrhalis* were analysed on serum resistance and C3 binding. C3met binding by flow cytometry showed a tendency to correlate with serum resistance. The expression of UspA1/A2 and C3met binding correlated strongly.

To check if C3 binding was specific, *M. catarrhalis* was exposed to different concentrations of EDTA-inactivated normal serum. The bacteria effectively bound C3 until saturation at 50% of serum.

It had to be established which part of the C3 protein was binding to *M. catarrhalis.* A direct binding assay showed binding to *M. catarrhalis* of C3d fragment. The binding of C3d to *M. catarrhalis* correlated with UspA1/A2 expression likewise binding of whole C3. When recombinant truncated fragments of UspA1 and A2 were used for binding assay with C3d, a single C3d-binding fragment for both UspA1 and UspA2 proteins was found.

To study in detail UspA1 and A2 inhibition of complement, a haemolytic assay was run with sheep erythrocytes, which demonstrated no effect of whole recombinant UspA1, but UspA2 significantly inhibited all complement pathways.

To confirm that C3 binding was strictly human-related phenomenon, *M. catarrhalis* was incubated with EDTA-treated murine serum. Surprisingly, *M.catarrhalis* bound murine C3 directly from serum, in dose-dependent manner. To check if murine complement was inhibited similarly to human, a haemolytic assay with murine serum

was performed, which showed a significantly decreased haemolysis after pre-incubation with recombinant UspA1 and no effect with UspA2, unlike inhibition of the human complement.

Therefore, UspA1 can inhibit the alternative pathway of the complement in the both mouse and human, whereas UspA2 is able to inhibit all pathways of complement in the human serum.

An interesting finding was that both UspA1 and A2 inhibit C3a generation. It most probably means that C3, which is bound by *M. catarrhalis,* is not activated; therefore C3a fragment is not produced. This phenomenon may have direct implication on the phagocytosis of the bacterium, because inactivated C3 will not opsonise *M. catarrhalis* and therefore will not facilitate its engulfment by phagocytes.

Conclusions

In this thesis we present data on the novel unique property of *M. catarrhalis* – binding and neutralisation of ACT. The information assembled up to date on both the bacterium and the serine protease inhibitor leads us to propose that it is a particular virulence mechanism for *M. catarrhalis*, very well suited for its niche as a respiratory pathogen.

The knowledge gained during the study of ACT binding localisation allowed us to develop an inexpensive and rather straightforward method for the purification of ACT which can significantly cut the time and effort needed for obtaining of the protein.

We also present here recent data on C3 binding to *M. catarrhalis*. More detailed information is provided on the C3 binding and on the correlations between C3 binding and UspA1/A2 expression and serum resistance. These correlations and the ability of recombinant UspA2 to inhibit both hemolysis and the inhibition of C3a generation by UspA1 and A2 strengthen our understanding that UspA1/A2 contribute to the serum resistance of *M. catarrhalis* via non-covalent C3 binding.

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