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Abstract

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Overall, the results from this thesis suggest that HAMLET has dual anti-bacterial roles: first by directly killing bacteria and second by stimulating immune responses to eliminate bacteria. Additionally, in the presence of galactose pneumococcal growth and metabolism is slow, suggesting a role in bacterial pathogenesis (*in vitro*).

Key words: *Streptococcus pneumoniae*, Antibiotic resistance, Alpha-lactalbumin, HAMLET, Biofilms, Bacterial metabolism, Immunomodulatory effects.

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To my parents and sister

Table of Contents

Abstract

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List of papers

Paper I Roche-Hakansson H, **Vansarla G**, Marks LR, and Hakansson AP. The human milk protein-lipid complex HAMLET disrupts glycolysis and induces death in *Streptococcus pneumoniae*. ***J. Biol. Chem***. 2019; 294: 19511–19522.

Paper II **Vansarla G**, Ganganna K, and Hakansson AP. Defining the mechanisms involved and their interrelationships during HAMLET-induced death in *Streptococcus pneumoniae* (In manuscript).

Paper III **Vansarla G**, Håkansson AP, and Bergenfelz C. HAMLET a human milk protein-lipid complex induces a pro-inflammatory phenotype of myeloid cells. ***Eur. J. Immunol.*** 2021; 51: 965-977.

Paper IV De S, **Vansarla G**, Bergenfelz C, and Hakansson AP. The role of niche-associated carbon sources in *Streptococcus pneumonia*e biofilm formation and metabolism (In manuscript).

Introduction

Bacterial infections are one of the major causes of morbidity and mortality worldwide in all age groups. Antibiotics as a treatment strategy is known to be very effective in controlling infections. However, high consumption of antibiotics has led to an evolutionary pressure of bacteria to develop and spread resistance, which has endangered the efficacy of antibiotics and increased the threat of antimicrobial resistance (AMR) [1]. AMR is a condition in which the bacteria causing infection(s) become resistant to the antibiotics used for treatment. Antibiotic resistant and/or multi-drug resistant (MDR) bacteria cause approximately 33,000 deaths in the European Union and 35,000 deaths in the USA each year [2], with a global estimate of 1.27 million deaths annually (in 2019) [3-5]. These deaths are expected to increase to 10 million by 2050, world-wide [6]. WHO published a list of 12 bacterial species posing a threat to human health as 'priority pathogens' (including *Streptococcus pneumoniae*) to guide and promote research and development of new antibiotics or alternative treatment strategies [7]. In this thesis, we used a purified protein-lipid complex from human milk, HAMLET, (human alpha-lactalbumin made lethal to tumor cells) which has been shown to have both tumoricidal and bactericidal properties [8, 9]. HAMLET's bactericidal activity is more effective against respiratory pathogens and uses novel death pathways [9, 10]. It has its highest activity against *Streptococcus pneumoniae* (the pneumococcus). We therefore chose pneumococci as a model organism and studied key molecules and mechanisms involved in HAMLET-induced pneumococcal death and host immunomodulatory effects. Further, to better understand pneumococcal pathogenesis we investigated differences of niche-associated carbon sources on pneumococcal biofilm formation and metabolism. Altogether, this thesis is an attempt to provide an improved understanding of the antibacterial activity and immunomodulatory effects of HAMLET and of pneumococcal pathogenesis, to identify future novel targets for antimicrobial therapy.

Antimicrobial resistance

Antimicrobial resistance (AMR) is one of the principal public health problems of the 21st century, threatening the available treatment strategies for present bacterial infections [4]. The problem of AMR is a major concern, especially due to the increasing antibiotic resistance in bacteria that resulted in an estimated 1.27 million deaths globally in 2019 [4, 5]. The modern era of antibiotics against bacterial infections started after the discovery of penicillin by Sir Alexander Fleming in 1928 [11, 12]. Since then, antibiotics use to treat bacterial infections has saved many lives [13]. However, the issue of AMR was already highlighted by Alexander Fleming in his Noble prize acceptance speech, where he suggested caution in the use of penicillin and antimicrobial drugs for the subsequent ability of bacteria to develop resistance. Antibiotic resistance was first reported in the 1930s (sulfonamide resistance) [14]. Since then, the trend has continued with bacteria rapidly becoming resistant to new antibiotics together with increased spread of resistance. The WHO, in 2014, highlighted the problem of AMR with a report stating that it is a serious and growing threat if not addressed would lead us entering into a post-antibiotic era [6].In 2017, the WHO listed several organisms, based on their high incidence of resistance and global burden of diseases were considered especially threatening to human health. [7]. These reports have spurred more research on epidemiology and alternative treatment strategies.

AMR is a set of processes whereby bacteria become resistant to antimicrobial drugs, by either acquiring resistance genes from other organisms (by horizontal gene transfer; the process of exchanging genetic information among organisms) or by evolving mechanisms to overcome the action of antimicrobial compounds. The resistance of bacteria can be divided into intrinsic, extrinsic/acquired or adaptive resistance. Intrinsic resistance is an evolutionary trait specific to bacterial species and gives bacteria the ability to resist the action of antibiotics [15]. It occurs in bacteria naturally due to the absence of drug targets, due to enzymatic degradation of drugs used for the treatment, or due to bacteria extruding the antibiotics physiologically by using efflux pumps [16]. Acquired resistance is the ability of bacteria to resist the activity of

antibiotics which were previously effective. It occurs due to genetic changes through mutations within bacteria or due to exchange of genes among bacteria in a niche (host site) by horizontal gene transfer or by conjugation [17]. Adaptive resistance is the ability of bacteria to adapt to the environment or to antibiotics temporarily for survival without acquiring mutations [18]. Adaptive resistance is acquired by bacteria in response to niche conditions like stress, pH changes, concentrations of ions, availability of nutrients or sublethal concentrations of antibiotics used for the treatment [19]. In practice, it is important to have knowledge about the resistance of bacteria (intrinsic as well as the ability of specific organisms to acquire or adapt) to avoid incorrect therapy and to decrease the risk of resistance development and spread. Some of the causes of antibiotic resistance are overuse or inappropriate prescription and inconsistence of patients to follow the course of antibiotic therapy.

Overall, to minimise the difficulties of antibiotic resistance, alternative treatment strategies, such as novel antibiotics against novel bacterial targets, efflux pump inhibitors, immunomodulators and adjuvants as well as antibiotic-sensitizers are urgently needed.

Human milk

Human milk is widely acknowledged as the normative and ideal source of nutrition for healthy growth and development of infants [20]. For this reason, the WHO and United Nations Children's Fund recommend breastfeeding infants for at least 6 months and to continue up to 2 years of age [21, 22]. The nutritional content of human milk continuously changes according to the needs of the growing infant [23]. During the lactation period, the milk evolves into 3 kinds of milk: colostrum, transitional and mature milk. The first milk produced is the colostrum. It contains higher concentrations of whey proteins with a lower fat content compared to mature milk [24, 25]. Transitional milk is a produced 2-5 days after childbirth and by the end of 6 weeks postpartum, the milk is considered fully mature.

The nutritional components of human milk are diverse and depend on the maternal diet [26]. Human milk contains about 87-88% of water and it has 124 g/L solid components as macronutrients, such as approximately 60-70 g/L of carbohydrates, 35-40 g/L of lipids and 8-10 g/L of protein [27, 28].

Carbohydrates are the major macronutrient in human milk. In infants, carbohydrate are ingested and digested in the form of lactose, with the help of an enzyme called lactase-phlorizin hydrolase (lactase) [28]. Apart from lactose, the milk contains human milk oligosaccharides (HMO's) and glycoproteins. Nutritionally, they are of minimal use but help to promote a bifidobacterial-dominated gut microbiota, which protects infants from diarrheal disease and promote physiological development and function of the gastrointestinal tract [29, 30]. Carbohydrates are also known for having the ability to block adherence of pathogens to mucosal epithelial cells. [31, 32].

Lipids are the second most abundant macronutrients in human milk and a major source of energy for infants, and consists of approximately 85% saturated (palmitic acid and stearic acid) and monounsaturated (oleic acid) fatty acids with the rest being poly-unsaturated fatty acids (linoleic acid, alpha-linolenic acid, eicosapentaenoic acid (EPA), arachidonic acid (AA) and docosahexaenoic acid (DHA)) [33]. The latter fatty acids also play a key role

in the development of the central nervous system, in inflammatory responses, and immune function of infants.

Whey and casein are the major protein groups in human milk. The ratio of casein: whey varies over the course of the lactation period and becomes 40:60 in mature human milk. Casein, in human milk exists in alpha, beta and gamma forms and aids in intestinal motility and absorption of calcium in infants [34]. Alpha-lactalbumin (ALA), lactoferrin, lysozyme, and secretory IgA are representative of whey proteins. During initiation of lactation, ALA plays a key role in milk production [35]. It alters the substrate specificity of galactosyltransferase from N-acetylglucosamine on glycoproteins in the Golgi apparatus to free glucose, thus forming lactose [36]. In infants, ALA is an important source for supply of essential amino acids (like tryptophan, lysine, cysteine and others) and absorption of minerals [36]. In contrast, IgA, lactoferrin and lysozyme protect the infant's intestinal mucosa against pathogenic bacteria and inhibits spread of pathogens [36].

Thus, in addition to provide bioactive factors for optimal development of the infant, human milk also has several antibacterial effects including blocking adherence and spread of pathogenic bacteria consequently reducing the risk of infectious diseases [23, 37, 38].

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HAMLET

HAMLET is a complex of partially unfolded alpha-lactalbumin (ALA) and human specific oleic acid (OA, C18:1:9 cis) [39], which kills tumour cells and bacterial cells but not healthy differentiated cells. It was discovered by serendipity while investigating anti-adhesive properties of milk against upper respiratory pathogens (*Streptococcus pneumoniae* and *Haemophilus influenzae*) [40, 41]. In this anti-adherence experiment, bacteria were preincubated with fractions of human milk and then added to either primary epithelial cells or cancer cells (the lung cancer cell line A549). Interestingly, bacteria failed to bind to either cell type, however the casein fraction of milk killed cancer cells while healthy cells were spared.

Initial analysis revealed that the casein fraction from human milk, obtained after low pH precipitation, inhibited the adhesion of bacteria and efficiently killed the cancer cells [40, 41]. To isolate the active component, casein was fractionated by ion exchange chromatography and the eluted peaks were analysed. The peaks eluted did not show any cytotoxic activity, suggesting that the active component was still bound to the column due to high affinity towards the matrix. However, after elution with high salt buffer (1M NaCl) an additional peak was eluted that had cytotoxic activity. This peak contained ALA as its major component. Due to its oligomeric nature on SDS-PAGE it was named multimeric form of ALA (MAL). As native ALA did not have cytotoxic activity, it was hypothesized that the oligomeric nature of the eluted fraction was the reason for its cytotoxicity. [41, 42].

Later, after additional characterization, the active component in HAMLET was found to consist of partially unfolded ALA and oleic acid (OA). HAMLET is now produced by an FPLC (fast liquid protein chromatography as described in methods section) method by exposing partially unfolded ALA to a column conditioned with human specific OA (**Figure 1**).

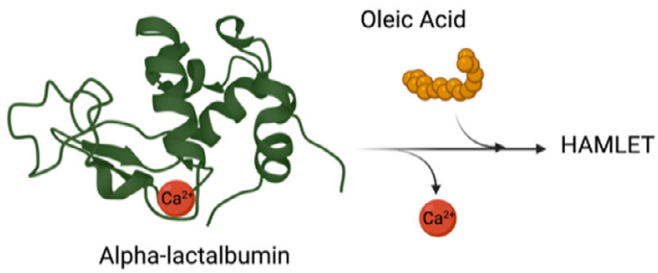

Figure 1. Schematic representation of the HAMLET complex formation. Structure of native ALA was obtained from the Protein Data Bank (PDB), acess number 1A4V (Created in BioRender).

Cellular targets of HAMLET

HAMLET has both tumoricidal and bactericidal activities [41, 43]. In cancer cells, HAMLET's activity has been investigated in some detail and HAMLET is known to induce an apoptosis-like death in cancer cells from 40 different origins, sparing healthy cells [8, 41, 44].

HAMLET also kills bacteria, by a mechanism resembling the apoptosis-like death in cancer cells [9]. HAMLET's bactericidal activity is selective against respiratory pathogens with its highest activity seen against Streptococcus pneumoniae, but HAMLET also has bactericidal activity against other Streptococci, *Haemophilus influenzae* and *Mycobacterium tuberculosis* [45, 46]. HAMLET-induced death in bacteria is not universal as it does not have activity against other Gram-positive organisms such as Staphylococci, Enterococci and *Bacillus subtilis* or Gram-negative bacteria such as *Escherichia coli*, *Klesbsiella pneumoniae* and *Pseudomonas aeruginosa* [9].

Besides its direct bactericidal activity, HAMLET has been shown to sensitize a large number of antibiotic resistant bacterial species to a wide range of antibiotics [43, 47, 48]. This potentiation effect to antibiotics is partially due to increased association of antibiotics with the bacteria [49]. HAMLET's potentiation effect has also been seen in HAMLET-resistant bacteria such as *Staphylococcus aureus*, *Acinetobacter baumannii*, and *Moraxella catarrhalis* [43, 48, 49].

In cancer cells, HAMLET causes influx of calcium and targets the mitochondria to initiate inner membrane depolarization and rupture, resulting in cancer cell death [44, 50]. HAMLET also induces calcium influx in healthy or differentiated eukaryotic cells without causing death [51, 52]. Similarly, in pneumococci it causes a dose-dependent depolarization of the plasma membrane, which induce a sodium-dependent influx of calcium leading to subsequent bacterial death [9]. Inhibitors of ion channels, like ruthenium red (calcium channel inhibitor) and amiloride (sodium channel inhibitor), rescue bacteria from the HAMLET-induced bactericidal activity, suggesting an import role of ion fluxes in HAMLET-induced bacterial death [47, 53].

Previous studies show that in cancer cells, HAMLET interferes with glycolysis by binding and inhibiting the activity of hexokinase contributing in the death of cancer cells [54]. Further, similar to HAMLET-activation of mitogen-activated protein kinases (MAPKs) (are serine/threonine kinases in eukaryotes) during its tumoricidal activity [53], in bacteria HAMLET's activity involves activation of a serine/threonine kinase [43]. Altogether, this suggest that HAMLET-induced mechanisms are different from traditional antibiotics or anticancer drugs. In bacteria, HAMLET-induced death involves multiple targets and pathways, which would make it more difficult for bacteria to develop resistance.

Streptococcus pneumoniae

Streptococcus pneumoniae is a Gram-positive, spherical aerotolerant bacterium from the genus Streptococcus, usually found in pairs (diplococci). Pneumococci are classified based on their virulence-related polysaccharide capsule (the outer envelope of bacteria) and so far, 101 serotypes have been identified [55]. In the last century, the pneumococcus has been the subject of many investigations and provided insights into basic principles of bacterial biology. Some of the important discoveries resulting from investigations on pneumococci includes the discovery of the Gram-staining technique (for identification and classification of bacteria), identifying the capability of bacteria to take up and incorporate exogenous DNA from the environment (natural transformation/competence) [56, 57], and the concept of development of drug resistance in bacteria [58].

The pneumococcus has been, and continues to be among the major causes of mortality and morbidity globally, causing a greater number of deaths compared to most other infectious agents [59]. Children under the age of 5 years, elderly, and immunocompromised individuals are all risk groups for diseases caused by pneumococci [60]. In 2016, pneumococcal lower respiratory infections caused approximately 650,000 deaths in children under the age of 5 years and approximately 1.1 million deaths in adults over the age of 70 years world-wide [61]. Interestingly, this study also suggests that sociodemographic factors such as malnutrition, accessibility to primary health care and hygiene play major roles in the mortality rates of children, however these factors do not seem to have a similar effect on adult mortality rates [61].

Pneumococci are part of the normal flora and common colonizers of the human upper respiratory tract. Approximately 27-65% of healthy children and more than 10% of healthy adults carry the pneumococcus as a commensal in the nasopharynx [62, 63]. A commensal is an organism that lives in the host symbiotically without causing harm [64]. However, the pneumococcus is also considered a pathobiont, an organism which under normal circumstance causes no harm but has pathogenic potential to cause disease under specific conditions (like those caused by environmental changes due to e.g., viral co-infections) [65].

As a pathobiont in the upper respiratory tract, the pneumococcus can, when disseminated to other sites in the host, cause upper and lower respiratory tract infections such as otitis media, pneumonia, chronic obstructive pulmonary disease (COPD), sepsis or meningitis [66]. Pneumococci are commonly observed during co-infections (infection by multiple pathogens) with other respiratory pathogens, such as respiratory syncytial virus (RSV), influenza A virus (IAV) or severe acute respiratory syndrome virus (SARS-CoV-2) leading to higher mortality or morbidity of such patients [67, 68]. Among these, pneumococcal co-infection with IAV is the most well documented, with IAV -induced secondary pneumococcal pneumonia causing close to 50 million deaths in 1918 (Spanish flu pandemic) [69]. The currently available literature on coronavirus diseases-2019 (COVID-19) suggests that the bacterial co-infections in COVID-19 diagnosed patients range from 0-40 % and that the most commonly isolated bacteria from these patients are pneumococci [70-72].

Diagnosis

Diagnostic methods used to detect infections are important for risk stratification and/or evaluation of the patients. Chest radiography or computer tomography (CT) are used to check patterns of infiltration in the lungs during pneumonia (which could be caused by several organisms) and these methods could crudely suggest which pathogens (virus versus bacteria) would be the potential cause. However, X-rays are mostly used in pneumonia for confirmation [73]. CT has higher sensitivity and accuracy compared to radiography. However, due to high cost and radiation exposure this technique is scarcely used for diagnostic purposes.

There is currently no gold standard method for identification of pneumococci and improved diagnostic methods are needed [74]. Classically, diagnosis of pneumococcal infections is done by growing bacteria from suitable patient samples. The patient samples are acquired by collecting respiratory secretions (sputum, bronchoalveolar lavage or pleural fluid), blood or urine [75]. In laboratories and clinical setups, pneumococci are identified through detection by visualizing morphological characters (as mentioned below), molecular detection and by antigen-based detection methods [76].

Pneumococci have specific phenotypical characteristics such as catalase negativity, α-haemolysis after growth on blood agar, optochin susceptibility and bile solubility, which are used to assess the cultured bacteria from patient

samples. The optochin susceptibility test was a mainstay for the identification of pneumococci until the identification of optochin resistant pneumococcal strains. Optochin is a chemical which inhibits the pneumococcal F (0)F(1)-H^+-ATPase that is involved in maintaining the proton motive force in both pneumococci and *viridans* streptococci. However, optochin-sensitivity is a characteristic not seen in other *viridans* streptococci [77]. Very few pneumococcal isolates have been found to be insoluble in bile (a phenotype due to the presence of major autolytic enzyme LytA) [78]. Thus the bile solubility test is more specific than optochin susceptibility test [79].

Molecular detection of pneumococci is primarily done by polymerase chain reaction (PCR) and quantitative polymerase chain reaction (q-PCR). Genes that are unique to pneumococci, like pneumolysin (*ply*), autolysin (*lytA*), pneumococcal surface adhesin (*psaA*), capsular polysaccharide (*cpsA*) or the spn 9802 gene fragment are used in these PCR-based methods [74, 80, 81]. The pneumolysin gene was first used for detection of pneumococci by PCR-based methods [82] and thought to be a specific biomarker for identification. Later studies have shown the presence of ply in non-pneumococcal *viridans* streptococci (*S. pseudopneumoniae* and *S. mitits*) [83, 84], which lead to use of *psaA*, *lytA* and spn9802 genes as biomarkers for the detection of pneumococcal infections.

The antigen-based method is an indirect method for detecting pneumococci from urine samples of the patients. This test is based on detecting the C polysaccharide (CPS) cell wall antigen, common to all pneumococcal isolates [85, 86]. However, the assay has limitations, such as cross-reaction with closely related streptococci and the fact that antigen can be detected for weeks after onset of the disease, making it less accurate for detecting acute disease. Therefore, this test is usually used in combination with other diagnostic methods [87, 88].

Treatment and prevention

Antibiotic therapy is the first line of treatment against bacterial infections. Since the discovery of antibiotics, the mortality and morbidity of bacterial infections have reduced noticeably [89]. Treatment of pneumococcal infections with antibiotics varies based on age, severity of the infection and geographical location. For example, patients diagnosed with low-risk community-acquired pneumonia (CAP) without comorbidities are prescribed

with beta-lactam antibiotics (amoxicillin) or tetracyclines (doxycyckine) and macrolide monotherapy (erythromycin) are used as last choice [90]. Fluoroquinolones (moxifloxacin or gemifloxacin) are used in high-risk CAP patients and in severe cases dual therapy with beta-lactams (penicillin and cephalosporins) plus macrolides (erythromycin) or fluoroquinolones are used [91]. In hospital-acquired pneumonia (HAP), cephalosporines (clindamycin) are used, if the patient has allergies to beta-lactams. In more severe cases combination therapy is used (more potent beta-lactams or clindamycin) [92] .

Vaccines are used as a preventive strategy to reduce the incidence of diseases with pneumococci. Immunogenic proteins and/or the capsular polysaccharides found on the pneumococcal surface, which act as antigens in the host, are the basis for vaccine development [93]. Polysaccharide and conjugate vaccines are two types of pneumococcal vaccines used to date. The current polysaccharide vaccine was developed in 1983 and contains 23 capsule serotypes (PPSV23) that covered 80-90% of the infective serotypes of pneumococci at the time [93]. However, children under the age of 2 years failed to mount efficient immune protection, which led to the development of pneumococcal conjugate vaccines [94]. Conjugate vaccine is a type of subunit vaccine, which combines a weak antigen with strong antigen [95]. In order to overcome the drawback of PSV23 and to protect children under the age of 2, the 7-valent pneumococcal conjugate vaccine (PCV 7) was developed in 2000 [96]. It was introduced based on the seven most frequent serotypes associated with invasive disease at the time and included serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F [97]. Introduction of PCV7, had major impact on reducing the incidence of invasive pneumococcal disease, decreasing the hospitalization of both children and elderly with pneumonia. Additionally, it also had an effect on colonization and reduced the carriage rates of vaccine serotypes [98, 99]. The second polysaccharide conjugate vaccine 10 (PCV 10) was developed to include additional serotypes lent in from other countries than US and Europe such as serotype 1, 5 and 7F and was introduced in 2009. A year later, the 13-valent polysaccharide conjugate vaccine (PCV 13) with the serotypes from PCV10 as well as the additional serotypes 3, 6A and 19A, was developed to protect against a wider range of pneumococcal serotypes that were problematic or emerging [96, 100]. Currently, the 10 and 13-valent pneumococcal conjugate vaccines are in use and pneumococcal 15-valent and 20-valent conjugate vaccines are in the pipeline [101].

Although immunization with PCV vaccines protects against pneumococcal infections, long-term use of these vaccines has been shown to impact the nasopharyngeal microbial flora by a process called serotype replacement and

serotype switching [102, 103]. An increase of non-vaccine type (NVT) pneumococci due to a decrease in vaccine type (VT) pneumococci post vaccination is leading to serotype replacement [104]. Additionally, prior vaccine serotypes are switching their capsules thereby becoming NVTs and can be more virulent again based on their complete genetic background, this process is called serotype switching and is a result of vaccine-induced selective pressure [105]. However, with the growing emergence of antibiotic resistant pneumococci, the antibiotics efficacy is also dropping, suggesting the need for novel antibiotics, vaccines, antimicrobial therapeutics or alternative treatment strategies.

Pneumococcal host interactions

Pathogenesis is the mode of disease/infection development. The pathogenesis of pneumococcal infection is a complex interplay between pneumococcal virulence factors (proteins/enzymes of the bacteria), host factors (physical barriers and immune responses) and the normal flora present in the niche. In the human upper respiratory tract, the nasopharynx is the primary ecological niche of pneumococci [106]. There, they firmly attach to the mucosal surfaces of epithelial cells, colonize and may replicate or form biofilms without affecting the host. However, in response to changes in the niche, due to e.g., viral co-infections and altered host immunity, the bacteria may spread to distant regions of the host resulting in invasive infections.

Nasopharyngeal Colonization and Biofilms

Colonization (i.e., carriage) is a state where micro-organisms enter various niches of a host, grow and multiply/replicate without causing harm to the host. For many organisms, including pneumococci, carriage is a prerequisite for subsequent progression to disease [107]. Colonization serves as a reservoir for bacteria and source of spread between hosts [107], suggesting that pneumococci are transmitted by respiratory aerosols (droplets containing bacteria) or direct contact with the carriers. Pneumococcal colonization may occur with one or multiple strains [108, 109].

The initial step of colonization involves adherence of pneumococci to the epithelial cell surfaces of the host's upper respiratory tract. To be able to adhere to the host nasopharyngeal epithelial surface, pneumococci need to adopt to host immune factors and/or other niche-associated resident microbial flora of the upper respiratory tract [110]. Once attached to the epithelial lining pneumococci continue to establish colonization through formation of biofilms. Biofilms were first observed in the 1970s when Nils Hoiby observed a link between infection and bacterial aggregates in cystic fibrosis patients [111]. Since its observation, the definition of biofilms has evolved and is now defined

by some as ‘complex bacterial communities, attached to surfaces and embedded in own extracellular matrix’ [112]. Biofilms protect bacteria from shear forces, environmental stressors or host immune responses [113]. Moreover, biofilm bacteria are less susceptible to antibiotics and show different phenotypes and gene expression patterns during growth compared to planktonic bacteria (free-living bacteria) [114].

In the upper respiratory tract, biofilms from many bacterial species are present including the pneumococcus [115, 116]. Pneumococcal biofilm bacteria are less virulent, and are more adapted for colonization and persistence in the host compared to planktonic bacteria [117]. Pneumococcal biofilm formation can be divided into 3 stages namely initial attachment, aggregation and matrix maturation [118]. The mature matrix contains extracellular DNA (eDNA), proteins and carbohydrates. These components constitute 90% of the total biomass of the biofilm and helps in linking pneumococcal cells together and attach them to the host cell in a mesh [119]. The eDNA in the biofilm matrix also helps in increasing genetic and phenotypical variation in biofilm bacteria by horizontal transfer and spread of antibiotic resistance and other traits among them [120].

Host-Immune responses

We are repeatedly exposed to micro-organisms (pathogenic and non-pathogenic) present in the environment. The ability of these organisms to invade into the body and to cause infections depends on both the pathogenicity of the organism and the integrity of the host immune system. The immune system is an interactive network of barriers (skin and mucosa), lymphoid organs (such as the bone marrow, spleen, thymus, and lymph nodes), cells, humoral factors (soluble immune factors which respond to danger in the body), and cytokines (cell-to-cell communication signals). The important functions of the immune system are to recognize and neutralize danger from the external environment (e.g., infectious agents), to provide protection from diseases developing inside the body (e.g., cancer), and to maintain normal homeostasis (balance) of the body [121]. Based on the speed and specificity in responding to threats, the immune system can be divided into innate immunity or adaptive immunity.

Innate immunity is the host’s first line of defence [122]. The elements of the host innate immune system include natural physical barriers (skin and

mucosa), phagocytic cell enzymes (e.g., lysozyme), phagocytes (like neutrophils, monocytes and macrophages), serum proteins (such as complement proteins, lectins and ficolins) and antimicrobial peptides (e.g., defensins and cathelicidins) [123]. It recognizes threats with the help of pattern recognition receptors (PRRs, such as Toll-like receptors and complement regulatory receptors) present on/inside the host cell. These receptors distinguish molecular signatures on for example pathogens through pathogen associated molecular patterns (PAMPs), such as polysaccharides, glycolipids, lipoproteins, nucleotides and nucleic acids. They also recognize damage associated molecular patterns (DAMPs), such as endogenous alarmins signaling danger [123-125].

Within minutes of entering the nasal cavity of the host, pneumococci encounter mucus secretions. To evade initial clearance by mucus, pneumococci express polysaccharide capsule. Almost all capsular polysaccharides expressed by pneumococci are negatively charged, which increases their repulsion to mucins and allow them to translocate across the negatively charged mucus layer [126]. During translocation of pneumococci across the mucus layer, the ciliary beating of epithelial cells is inhibited by the expression and release of the pore forming toxin pneumolysin [127]. Pneumococci also express exoglycosidases (NanA, NanB, and NanC) which can cleave sialic acid on mucins and alter mucins adhesive properties [128]. Pneumococci escape antibacterial molecules secreted in mucous secretions, such as the lysozyme cell wall degradation with the help of genes *PgdA* (peptidoglycan *N*-acetylglucosamine deacetylase) and *Adr* (*O*-Aacetyl-transferase) and overcomes lactoferrin (sequester free iron on microorganisms) activity to escape from lactoferrin induced bactericidal activity with the help of *PspA* (pneumococcal surface protein A). [129, 130].

The adaptive immune system cooperates with the innate immune system in the elimination of pathogens. It consists of antigen presenting cells (APCs), T cells and B cells [131].The primary functions of the adaptive immune system are to recognize ‘non-self’ antigens, distinguishing them from self-antigens, generating immunologic mechanisms to eliminate pathogens, and developing an immunologic memory that will facilitate the elimination of the pathogen in case of reoccurring infections [132].

Monocytes/macrophages together with dendritic cells (DCs) are APCs that play important roles in both innate and adaptive immunity. During inflammation, monocytes circulating in the blood reach the site of infection, transform into macrophages and exhibit phagocytosis (the process of ingesting and eliminating pathogens/cell debris or dead host cells) [133]. Depending on their biological functions, macrophages can be divided into classically

activated type-1 macrophages (M1) or alternatively activated type-2 (M2) macrophages. M1 macrophages are induced by microbial products (e.g., bacterial lipopolysaccharides; LPS) and cytokines (interferon gamma; IFN-γ or tumor necrosis factor-alpha; TNF-α) [134]. Once activated, they up-regulate the expression and production of pro-inflammatory cytokines like interleukins (IL-23/12, IL-6, IL-1), macrophage inflammatory protein-1 (MIP-1α), monocyte chemoattractant protein-1 (MCP-1) and major histocompatibility complex (MHC) class II [135, 136]. M2 macrophages, on the other hand, are induced by numerous inflammatory mediators, for example after recognition of Immunoglobulin G (IgG) complex and TLR-ligands. Once activated, they produce anti-inflammatory cytokines (like IL-10) and are involved in tissue rebuilding and resolution of the inflammation process [135, 136]. It should, however, be emphasized that the M1/M2 nomenclature is oversimplified and there are as many macrophage phenotypes as there are stimuli [137].

Similar to monocytes/macrophages, immature DCs phagocytose antigens, but are upon maturation more efficient in activating T-cell responses and induce cell-mediated immunity against pathogens.

Toll-like receptors, like TLR2, TLR4 and TLR9 on epithelial cell surfaces are involved during pneumococcal infections. TLR2 recognizes lipoteichoic acids (LTAs) present in the cell wall of pneumococci [138]. TLR4 recognizes pneumolysin, which regulates the complement system and inhibits phagocytosis by the innate immune system [139]. It is also a pro-inflammatory toxin, which damage the host cells and helps in the spread of bacteria between hosts [140, 141]. TLR9 binds to the bacterial CpG motif on DNA and activate innate immune responses to eliminate them [142]. Additionally, TLR2 and TLR4 together activate macrophages during pneumococcal infections which further leads to phagocytosis [143].

During pneumococcal infections, immunoglobulin A (IgA) antibody is detected on mucosal surfaces and saliva, which helps the host in initiating opsonin-mediated phagocytosis. However, pneumococci have the ability to cleave IgA, by expressing a protease called IgA1 protease, which facilitate escape from phagocytosis [110, 144].

Pneumococcal metabolism

The human pharynx can harbour more than 700 different microbial species [145]. In the nasopharyngeal niche, pneumococci have to compete with the residing microbiota for nutrients, resist host inhibitory metabolites, and face the host defence system to propagate and colonize [146]. To overcome this hostile environment, pneumococci express virulence factors to facilitate colonization, which are highly regulated by the availability of nutrients and host metabolic signals in the niche [147]. Thus, metabolism, nutrition supply and niche space play a key role in bacterial colonization and pathogenesis.

In order to produce energy, pneumococci utilize easily available carbohydrates in a niche, such as glucose. However, in the nasopharynx the concentration of free glucose (that is commonly the dominant carbohydrate source in culture media) is low [148]. So, in order to successfully live in this niche pneumococci, possess multiple transporters and pathways for nutrient acquisition and use. This is exemplified by the expression of glycosyl-hydrolases (enzymes which cleave carbohydrates from N-linked glycan structures present on mucins) that degrade complex polysaccharides available in the niche into easily utilizable oligo-, di- and monosaccharide forms, such as sialic acid, hyaluronic acid, N-acetyl glucosamine and galactose [149, 150]. These carbohydrate acquisition mechanisms to use different sugar sources give pneumococci a selective advantage over other bacterial species present in the niche.

Carbohydrate uptake systems in pneumococci are not well characterized. However, studies predict that there are approximately 21 phosphotransferase systems (PTS) and 8 ATP (Adenosine tri phosphate) binding cassettes (ABC) that import at least 32 distinct carbohydrates used for pneumococcal metabolism [151]. The available mono- and disaccharide carbohydrates in the niche are transported through PTS, where they are phosphorylated with the help of a cascade involving enzyme I (EI) and a histidine-containing phosphocarrier protein (HPr) and using phosphoenolpyruvate (PEP) as a phosphate source [152]. These phosphorylated carbohydrates are then used in central metabolic pathways [152]. On the other hand, the carbohydrates transported through ABC transporters are not modified. ABC transporters

utilizes more bacterial energy than PTS transporters, to modify carbohydrates once intracellular [153].

The pneumococcus has multiple carbohydrate metabolism pathways (**Figure 2**) and lacks a TCA (tricarboxylic acid) cycle and oxidative phosphorylation. The carbohydrates imported into the cells by PTS or ABC transporters are converted to generate ATP primarily by glycolysis (the Embden-Meyerhof-Parnas (EMP) pathway) present in all streptococcal species [152]. The end product of this pathway is pyruvate, yielding a net 2 ATP and 2 NADH molecules for each glucose molecule. Further, NADH is oxidised into NAD to maintain the redox balance by conversion of pyruvate into lactate by lactate dehydrogenase.

Overall, the knowledge of bacterial metabolism in the niche microenvironment is crucial for understanding pathogenicity of infections and development of novel control strategies.

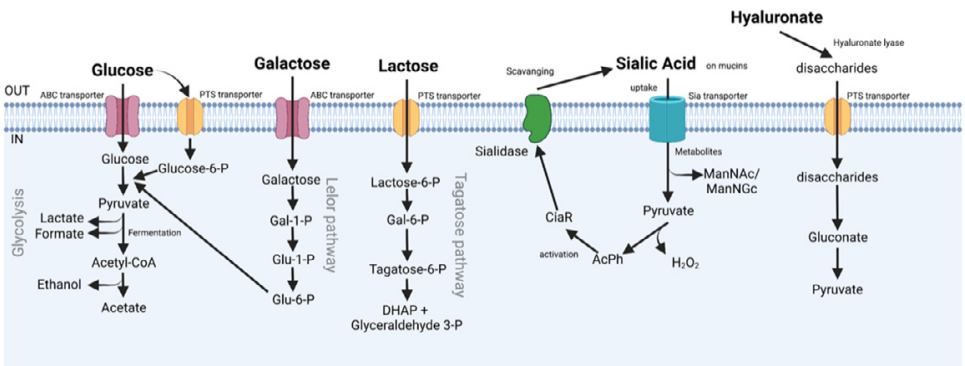

Figure 2. Schematic representation of selected sugars and their pathways in pneumococci. P-addition of phosphate, DHAP-Dihydroxyacetone Phosphate, Gal-galactose, Glu-Glucose, CiaR-two-component response regulator, ManNAc- N-Acetyl-D-mannosamine, ManNGc- N-Glycolyl-D-mannosamine, AcPh- acyl carrier protein phosphodiesterase. (Created in BioRender).

Present investigations

Aims

To identify novel potential targets in pneumococcal infections, and understand the role of HAMLET as an alternative treatment strategy, we divided the objective of this thesis in two parts:

1. To investigate the antibacterial and immunomodulatory properties of HAMLET.
2. To investigate the effects of niche-associated carbon sources on pneumococcal metabolism and its role in pneumococcal biofilm formation and dispersal.

The specific aims were:

I. To study HAMLET-induced glycolytic targets in pneumococci.

II. To study how known HAMLET-induced bacterial targets and pathways interact during HAMLET-induced death.

III. To investigate HAMLET's immunomodulatory effects using human myeloid cells.

IV. To study the role of niche-associated carbon sources in pneumococcal biofilm formation and metabolism.

Methods

HAMLET Production

HAMLET is produced by ion exchange chromatography. The production of HAMLET involves multiple steps and can be divided into 3 phases. (**Figure 3**). Human milk was collected and stored at -20°C prior to use for production of the HAMLET complex.

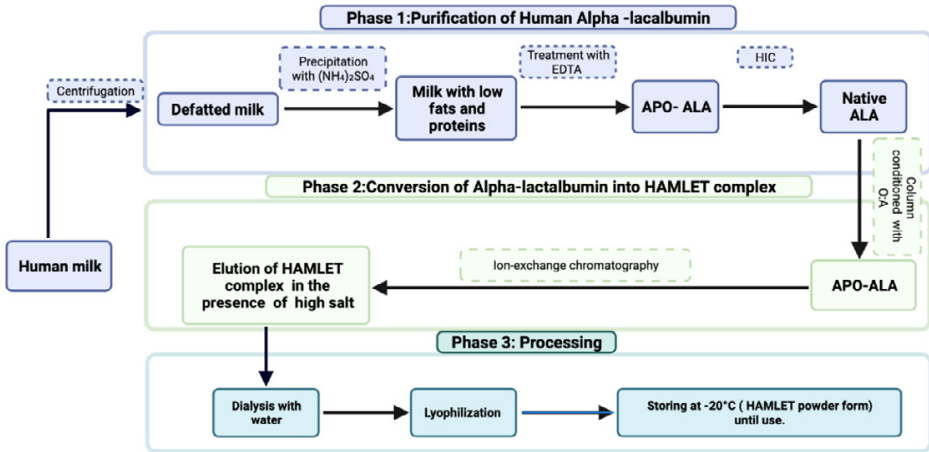

Figure 3. Schematic representation of steps invovled in the HAMLET production. (Created in BioRender)

Purification of human alpha-lactalbumin

One litre of human milk contains about 2 g of ALA that is purified by hydrophobic interaction chromatography (HIC) [154, 155]. First, the collected milk is defatted by centrifugation and precipitated with ammonium sulphate to remove unwanted proteins. Next, the sample is treated with ethylenediamine tetra acetic acid (EDTA) to remove the calcium ions ($Ca2^{+}$), making ALA partially unfolded (apo-ALA) and more hydrophobic. The apo-ALA is then passed through a HIC (phenyl sepharose) column, where it tightly binds to the matrix. On elution with a buffer containing $Ca2^{+}$, the partially unfolded ALA retains its native conformation, detaches from the column matrix, and is collected and used for conversion into HAMLET.

Conversion of alpha-lactalbumin into HAMLET

ALA is converted into the HAMLET complex by ion exchange chromatography using an anion exchange Di-ethyl amino ethyl (DEAE) matrix. Prior to the conversion, the matrix is pre-conditioned with human milk specific oleic acid. To allow interaction with the OA bound to column, the purified ALA from the above step is treated with EDTA again to attain partially unfolded form (Apo-ALA). When passed through the DEAE column, apo-ALA binds to matrix-bound OA and the HAMLET complex is eluted with a buffer containing high concentrations of salt (sodium chloride) [39].

Processing

The obtained HAMLET complex is further subjected to dialysis to remove the salt from the anion exchange chromatography elution buffer. Dialysis is done using large amount of deionized water at 4ºC. The complex is further subjected to lyophilization and stored at -20 ºC until further use. This procedure does not affect the stability of the HAMLET complex [156].

Batch evaluation

All batches were thoroughly evaluated for potential variability in bactericidal activity by performing bacterial viability testing. Bacteria are treated with various concentrations of a HAMLET batch and incubated for 1 h. Further, viability of the bacteria is tested by plating serial dilutions of the HAMLET-treated culture on blood agar plates and counting CFU/ml (method described in paper I). Batch-to-batch variation depends on number of OA molecules bound to ALA when passed through the column in final step of HAMLET production. The concentrations of pure OA or ALA corresponding to the levels in the respective HAMLET batch are used as controls in the experiments.

Main findings of the thesis

HAMLET targets glycolysis and inhibits energy production in pneumococci (Paper I)

To investigate HAMLET's activity on pneumococci, we performed short term bactericidal activity assays (time-kill assay), where bacteria were exposed to HAMLET for 1 h and viability was assessed by viable plate counts. We observed that HAMLET induced dose-dependent pneumococcal death (**Figure 4**). ALA alone showed no activity, but OA alone showed dose-dependent activity, albeit less activity than HAMLET as a complex.

As HAMLET targets and interferes with glycolysis in tumour cells [54, 157], we hypothesized that HAMLET could have a similar effect in bacteria. To investigate HAMLET's role in glycolysis and ATP production, we measured ATP production and lactate secretion and observed that pneumococci produced less ATP and lactate in the presence of HAMLET compared to ALA or OA alone (**Figure 5**). Further, to confirm HAMLET's effect on glycolysis and ATP production, we either stimulated glycolysis by addition of high sugar or inhibited glycolysis with the inhibitor 2-deoxyglucose. As hypothesized, HAMLET had less bactericidal activity in the presence of high sugar and displayed a higher activity in the presence of the glycolysis inhibitor. Thus, these results suggested that HAMLET's bactericidal effect on pneumococci is dose-dependent, and that HAMLET-induced death is accompanied by inhibition of glycolysis and energy production.

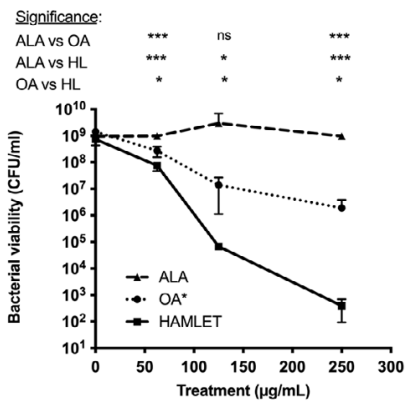

Figure 4. Bactericidal Activity of HAMLET, ALA and OA. Dose-dependent death of D39 pneumococci exposed to HAMLET complex compared to ALA or OA with concentration quivalent to the concentration present in HAMLET complex. (*indicates p <0.05, *** indicates p <0.001; ns indicates nonsignifiant) [10]. (Paper I-Figure 1).

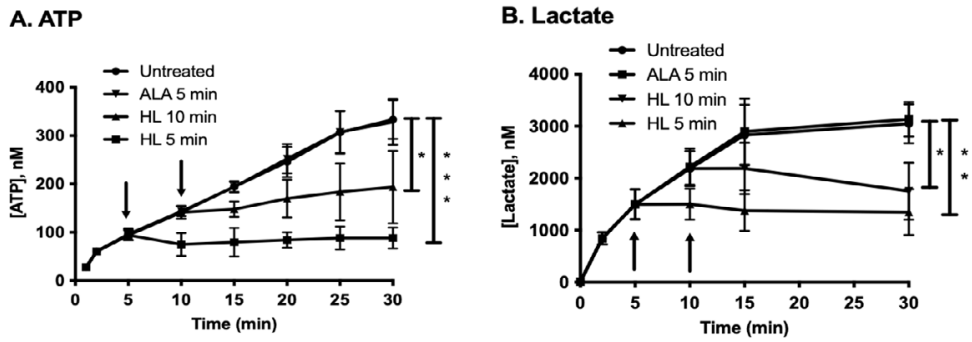

Figure 5. Inhibition of glycolysis and enrgy production. (A) ATP production **and** (B) lactate secretion decreased after addition of HAMLET complex compared to ALA. (*indicates p <0.05, *** indicates p <0.001).[10] (Paper I- Figure 3).

HAMLET binds to and inhibits the activity of two central glycolytic enzymes in pneumococci (Paper I)

We next used a proteomic approach to identify potential targets involved in HAMLET's glycolytic inhibition. Two central glycolytic proteins, fructose bisphosphate aldolase (FBPA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were identified as potential HAMLET targets. To assesses HAMLET's interactions with FBPA and GAPDH, we performed binding and enzymatic assays with recombinantly produced glycolytic proteins. We observed that HAMLET bound to and inhibited the activity of both the enzymes. Interestingly, ALA showed similar effects on the glycolytic enzymes whereas OA showed no or significantly lower inhibition than HAMLET or ALA. However, when we investigated FBPA's activity in whole cells, HAMLET but not ALA or OA inhibited FBPA activity (**Figure 6**). Though, ALA inhibited the activity of recombinantly produced glycolytic enzymes in vitro, it showed no effect on FBPA's activity in whole cells. This suggests that HAMLET binds to and inhibits glycolytic enzymes by gaining access across the membrane whereas ALA does not pass across the membrane.

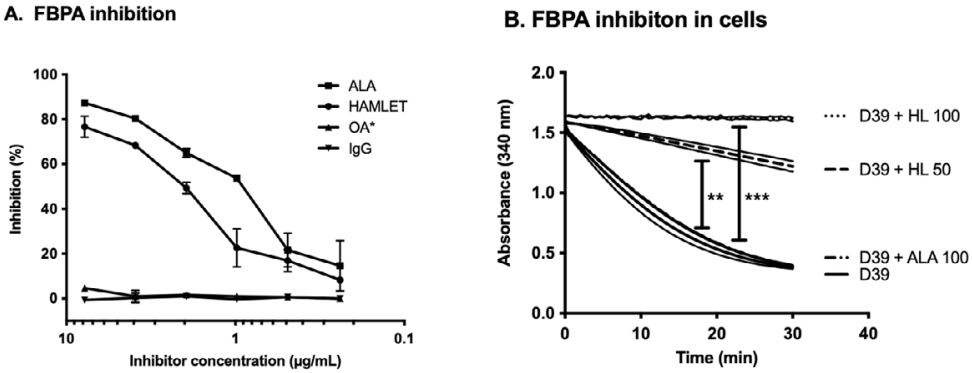

Figure 6. Inhibition of recombinant and intracellular FBPA activity. (A) Inhibiton of recombinant FBPA activity with both HAMLET and ALA *in vitro*, (B) Inhibiton of FBPA activity in whole cells is onolyl observed with HAMLET. (*indicates p <0.05, *** indicates p <0.001; ns indicates nonsignifiant). [10] (Paper I – Figure 5 A and 6).

HAMLET-induced energy production and membrane depolarization are associated (Paper II)

Previous studies have shown that HAMLET-induced pneumococcal death is also accompanied by membrane depolarization and rupture that requires a sodium-dependent calcium influx and activation of a serine/threonine kinase [43, 47, 49]. First, to investigate the relationship between HAMLET-induced ion transport with energy production in bacteria, we measured depolarization and rupture of the plasma membrane in the presence and absence of HAMLET and a glycolysis inhibitor (2-deoyglucose). HAMLET stimulated a dose-dependent depolarization and rupture that was higher in the presence of the glycolysis inhibitor, (**Figure 7**). Second, to address the relationship between ion transport and serine/threonine kinase, we used the kinase inhibitor staurosporine. Measuring depolarization and rupture of the membrane we observed that, inhibition with kinase inhibitor partially influenced HAMLET-induced membrane depolarization and rupture. These results suggest that HAMLET-induced glycolysis inhibition facilitate membrane depolarization and rupture and activation of kinase is partially linked with ion transport in the membrane.

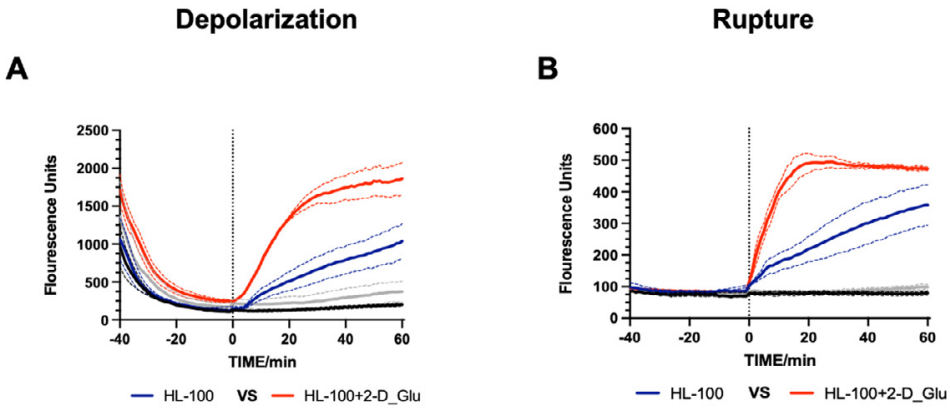

Figure 7. Depolarization and rupture after inhibiting glycolysis (A) Membrane depolarization or (B) membrane rupture were measured in bacteria treated with HAMLET in the presence (red line) or absence (blue line) of glycolysis inhibitor and the signal was compared to untreated control bacteria (black line). (Paper II-Figure 3)

Similarly, by measuring glycolytic activity through intrabacterial ATP production in the presence or absence of Na^{+} or $Ca2^{+}$ inhibitors or facilitators, which will inhibit sodium-dependent calcium influx or facilitate it by causing pores in membrane, we observed that more ATP was produced in HAMLET-treated bacteria preincubated with ion transport inhibitors compared to bacteria treated with HAMLET alone. On other hand, less ATP was produced in the presence of ion transport facilitators (**Figure 8**). Additionally, to assess the link between HAMLET-induced activation of kinase and glycolysis, we measured ATP production in the presence of kinase inhibitor. More energy was produced in presence of kinase inhibitor compared to HAMLET alone. This suggests that HAMLET-induced ion transport and membrane depolarization and kinase activity inhibits ATP production in the bacteria.

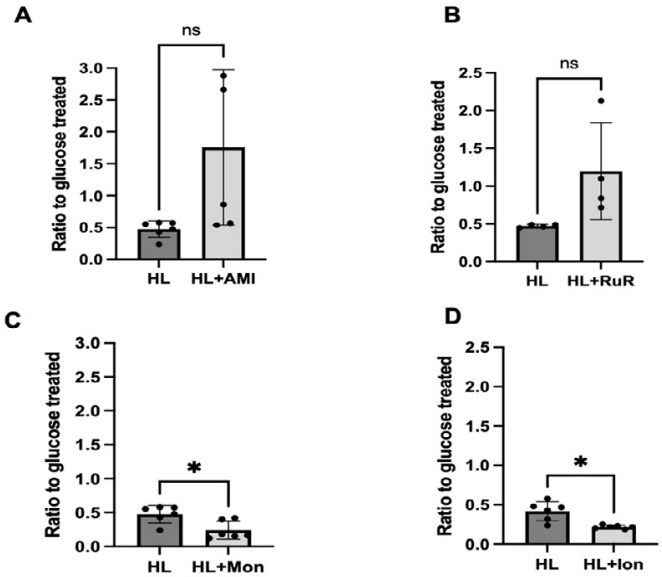

Figure 8. ATP production in presence of HAMLET and ion transport inhibitors/facilitators ATP release in the presence of HAMLET and with (A) sodium inhibitor - more ATP release (C) sodium facilitator - less ATP release (B) calcium inhibitor - more ATP release (D) calcium facilitator - less ATP release. (*indicates p <0.05 ; ns indicates nonsignifiant. (Paper II -Figure 4).

HAMLET induce maturation of monocyte-derived dendritic cells and macrophages (Paper III)

Previous studies have shown that HAMLET induces signals involved in innate immunity in healthy, primary kidney cells [44, 51, 52, 158]. Therefore, we hypothesized that HAMLET, besides killing bacteria and cancer cells, have immunomodulatory effects. To understand HAMLET's potential immunomodulatory role, we first cultured primary human monocytes and differentiated them into macrophages and dendritic cells and stimulated them with HAMLET. By light microscopy we observed that HAMLET induces dose-dependent morphological changes in both macrophages and dendritic cells (**Figure 9**), suggesting that HAMLET has an effect on monocyte-derived macrophages and dendritic cells. To address these morphological changes, we investigated the surface phenotypes using flow cytometry. In macrophages, HAMLET-stimulation increased the percentage of cells expressing the M1-like macrophage associated co-receptor CD86, whereas in dendritic cells HAMLET increased the expression of the dendritic cell maturation marker CD83 compared to control cells stimulated with either ALA or OA. These results suggested that HAMLET induce surface phenotypes similar to activated M1-like macrophages and mature dendritic cells.

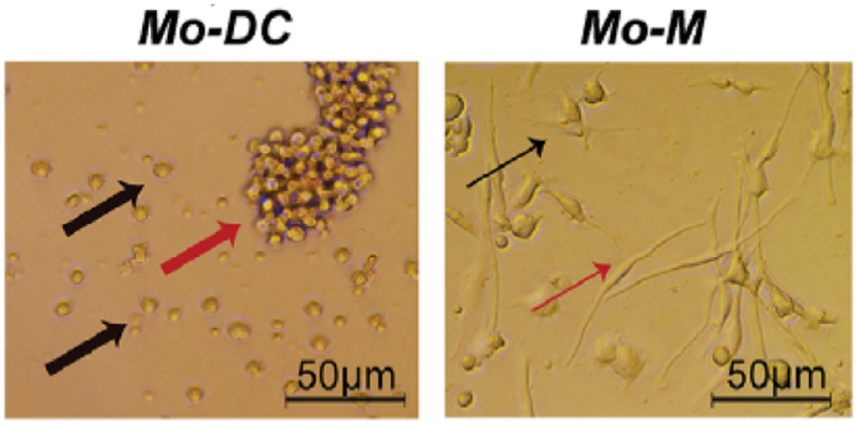

Figure 9. Morphological changes induced by HAMLET on Monocyte derived macrophages and Denritic cells Left pannel represents monocyte derived dendritic cells (Mo-DC) and right pannel represents monocyte derived macrophages (Mo-M). The cells were treatd with HAMLET and difference were observed. Thick black arrow indicate Mo-DC protrusions and thick red arrow indicates aggregates. In Mo-M, thin balck arrow indicates small protrusions and thin red arrow indicates elongated morphology. (Paper III-Figure 1) [159].

HAMLET induces functional changes in macrophages and dendritic cells **(Paper III)**

Further, we measured the cytokine release from macrophages and dendritic cells after HAMLET stimulation and tried to delineate the immune mechanisms involved. Our results suggested that the HAMLET-induced immunomodulatory effects are partially mediated by calcium-, NFκB-, and p38-signaling pathways. To investigate the effect of HAMLET treatment on the functionality of macrophages or dendritic cells, we performed a phagocytosis assay (for macrophages) and a mixed lymphocyte reaction assay (MLR, for dendritic cells). HAMLET-stimulated macrophages were more efficient in phagocytosis of pneumococci compared to untreated macrophages. Further, we observed that HAMLET-stimulated dendritic cells had an increased capacity to stimulate T-lymphocyte proliferation compared to untreated control cells or cells stimulated with ALA. Altogether, these results suggest that HAMLET-stimulated monocyte-derived macrophages or dendritic cells are functionally affected (**Figure 10**).

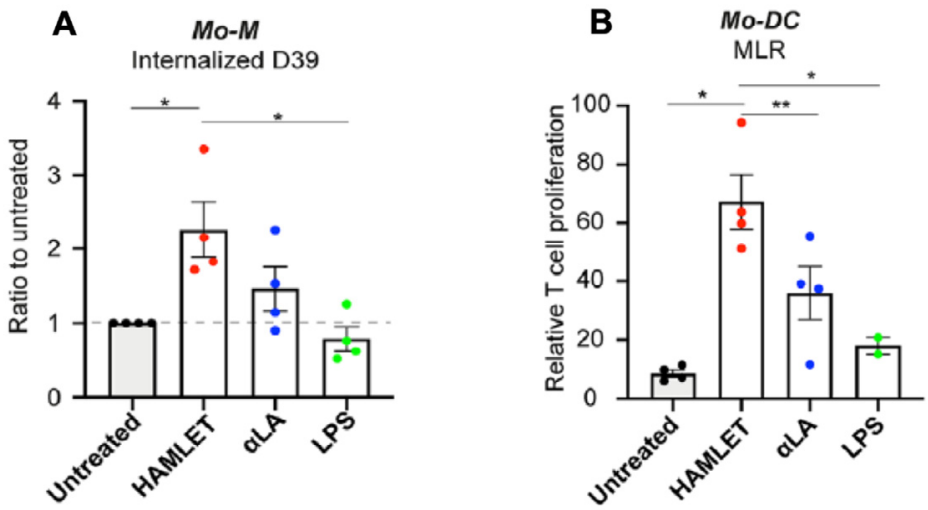

Figure 10. HAMLET-induced functional changes. (A) Monocyte derived macrophages (Mo-M) stimulated with HAMLET show more phagocytosis of pneumococci compared to control stimulations (ALA, LPS) and unstimulated cells. (B) Proliferation of T-lymphocytes in the repsence of HAMLET-stimumated DCs compared to control-stumulated DCs (ALA, LPS) or unstimulated DCs (* indicates p <0.05, ** indicates p <0.01;. (Paper III- Figure 6) [159].

Galactose grown planktonic and biofilm pneumococci have reduced growth rate and metabolism (Paper IV)

Studies have shown that glucose is not readily available in the human nasopharynx, whereas carbohydrates such as sialic acid, hyaluronic acid, N-acetyl glucosamine and galactose are available in the nasopharynx [160-162]. Galactose has been suggested to be an important carbon source during pneumococcal colonization and progression into infection[163, 164]. Therefore, we investigated the role of galactose during growth and its effect on the metabolism in pneumococci (both in planktonic and biofilm bacteria). First, we adapted multiple strains of pneumococci to glucose or galactose in chemically defined media (CDM) containing glucose or galactose as sole carbon sources. When monitoring growth over time, we observed that galactose-adapted bacteria grown in CDM-galactose grew slower than glucose-adapted bacteria grown in CDM-glucose and displayed and extended stationary phase. (**Figure 11**). Galactose-adapted bacteria formed biofilms with similar density compared to glucose-adapted bacteria but with less extracellular matrix. Further, we addressed the slow growth rate of galactose-adapted bacteria, both planktonically and in biofilms, by performing metabolic activity assays over time (such as oxidation assay, ATP assay and lactate/ hydrogen peroxide (H2O2) assays). Using an oxidation assay we observed

that, in both glucose-adapted bacteria and galactose-adapted bacteria oxidation of sugars did not vary over time in either planktonic or biofilm bacteria. While measuring energy production by ATP assays, we observed that galactose-adapted bacteria produced less ATP than glucose-adapted bacteria in both planktonic and biofilm forms. Further, we measured fermentation in the different bacterial populations by measuring lactate and H2O2, in the growth media. We found that a lower level of lactate was produced in the presence of galactose in both planktonic and biofilm bacteria grown compared with bacteria grown in glucose. On the other hand, a higher production of H2O2 was observed in both planktonic and biofilm bacteria grown in the presence of galactose, suggesting differences in fermentation pattern between bacteria grown in glucose and galactose. Finally, to understand the functionality of galactose-grown bacteria in biofilm form, we tested dispersal of bacteria after exposure to febrile temperature compared to biofilms from glucose-adapted bacteria. We observed that, galactose-adapted bacteria biofilms disperse less compared to biofilms formed by glucose-adapted bacteria. Overall, these results suggest that galactose plays an important role in bacterial metabolism and function, suggesting a potential role of metabolism for survival and colonization of bacteria in the nasopharynx.

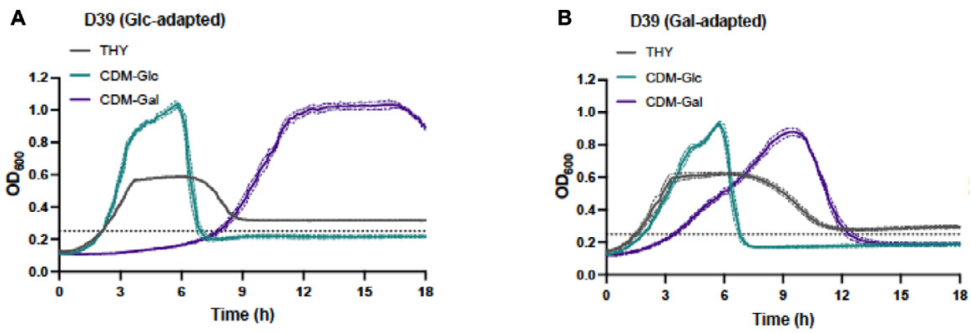

Figure 11. Bacterial growth in presence of either glucose (Glc) or galctose (Gal). (A) Glucose-adapted D39 bacteria were grown in either THY, CDM containing glucose or galactose. In the prescence of galactose (purple line) an extended stationary phase is observed compared to glucose-grown bacteria (green line) or THY-grown bacteira (black line). (B) Glactose-adapted bacteria (purple line) grown in galactose have an extended staionary phase compared to glucose-adapted bacteia (green line) grown in galactose (Paper IV-Figure 1).

Summary and future perspectives

In this thesis, to address the increasing problem with AMR in bacteria, we used HAMLET as a potential novel antimicrobial molecule against pneumococci. HAMLET exerts bactericidal activity against pneumococci. The overall goal of the thesis was to study HAMLET's antibacterial and immunomodulatory effects and as pneumococci colonizes the nasopharynx, we further attempted to study the metabolism and biofilm formation of bacteria grown in galactose present in the niche compared to glucose, which will give us a better understanding about pneumococcal colonization and disease progression as well as potentially identify novel therapeutic targets.

For the first time, to our knowledge, we show that HAMLET targets glycolysis in pneumococci. In bacteria, HAMLET binds to two central glycolytic enzymes and inhibits energy production leading to subsequent death **(paper I)**. However, in the future, studying HAMLET's interactions with glycolytic enzymes in HAMLET-sensitive vs resistant species like *Mycobacterium tuberculosis, Haemophilus influenzae* or *Moraxella catarrhalis, Staphylococcus aureus* [MRSA] and *Escherichia coli* would tell us whether HAMLET's activity is similar in all species or if it is specific to pneumococci. As HAMLET induces ion transport in HAMLET-resistant strains, this is an indication that HAMLET may have similar targets in bacteria and has conserved mechanistic pathways [43]. To understand the HAMLET-induced death mechanistically, we investigated whether the pneumococcal targets of HAMLET are activated sequentially or in parallel (**paper II**). We observed that the HAMLET-induced sodium and calcium transport is facilitated when energy production (ATP from glycolysis) is inhibited and partially associated with activation of serine/threonine kinase. On the other hand, inhibition of kinase activity did not influence the ion transport in the bacteria. These results from **paper II**, suggests that some targets, such as ion transport and ATP production, involved in HAMLET-induced bacterial death are associated and potentially linked. However, others, such as Ser/Thr kinase and ion transport are potentially activated independently in parallel. However, further studies are needed to understand the complex network of pathways initiated by HAMLET. Further, as the HAMLET targets are conserved among other bacterial species, the results from this study can lead to the identification of novel therapeutic targets in bacteria.

In **paper III** we show that HAMLET has immunomodulatory effects on monocyte-derived macrophages and dendritic cells. The HAMLET complex, compared to ALA or OA alone, induced morphological changes and

maturation of monocyte-derived dendritic cells as well as an M1-like surface phenotype of macrophages. HAMLET stimulation of macrophages and dendritic cells also induced release of many cell mediators and suggested the potential involvement and partial dependence on calcium-, NFκB- and p38-signalling pathways (from experiments using inhibitors). Functionally, HAMLET-stimulated cells were more efficient in phagocytosis of pneumococci and in inducing T cell proliferation compared to ALA-stimulated cells. The results from this paper propose a dual mechanism for HAMLET, i.e., a direct antibacterial activity and an indirect by activating immune cells. In the future we would like to validate the results acquired in this project *in vivo*.

In pneumococci, successful colonization and transition to infection is associated with niche/environmental factors such as host factors, other niche associated microbiota and nutrients. In **paper IV**, we studied the role of galactose in pneumococcal growth, biofilm phenotypes and the metabolic activity of pneumococci. The results from this paper show that in the presence of galactose, planktonic bacteria grow slower, biofilms formed are more resistant to antibiotics and have low metabolic activity compared to glucose-grown bacteria. Further, to assess the dispersal (spread) of bacteria from biofilms, by mimicking febrile temperature in the host (*in vitro*) we observed that galactose-grown biofilms are less prone to disperse than glucose grown bacterial biofilms, suggesting that galactose as a nutrient source is more suitable for colonization and establishment of biofilms, and that this can protect the bacteria from environmental factors. In future studies, incorporating other niche environmental factors such as viral co-infections, host factors (inflammation), normal flora and other available carbon sources would help in better understanding the colonizing environment and pneumococcal transition from colonization to infection.

Overall, the results from this thesis provided us with information about HAMLET-induced death pathways in bacteria and its immunomodulatory effects on human myeloid cells. Further, the results gave us a better understanding about pneumococcal growth and metabolism with sugar (galactose) readily available for bacteria in the nasopharynx compared to glucose that the bacteria encounter in the blood stream during invasive disease.

Conclusions

I. HAMLET's bactericidal activity in pneumococci is related to its ability to target and inhibit glycolytic enzymes.

II. The HAMLET-induced glycolysis inhibition and ion transport induction in bacteria are directly connected, whereas, serine/threonine kinase is partially activating ion transport resulting in depolarization of membrane and is activated by glycolysis inhibition.

III. HAMLET induces a pro-inflammatory phenotype in myeloid cells.

IV. Galactose influences pneumococcal metabolism, biofilm formation and biofilm dispersal.

Clinical significance

The results presented in this thesis provided a better understanding of HAMLET-induced targets and death pathways in bacteria. HAMLET could be good alternative treatment strategy because it has multiple targets in bacteria compared to traditional antibiotics which would potentially lead to less chance for resistant development. Due to its immunomodulatory effects it can serve dual proposes, i.e. both being directly antibacterial by killing bacteria and also act antibacterially by stimulating immune responses resulting in elimination of bacteria.

As it has no effect on healthy cells there is less chance to get side effects with HAMLET. Furthermore, it can sensitize bacteria to antibiotics, which would help in addressing resistance development in bacteria (not studied in this thesis).Additionally, in this thesis we also attempted to mimic and study the physiological environmental aspect of pneumococci comparing growth, metabolism and biofilm formation in glucose (present in the bloodstream) with galactose (present in the nasopharynx), to better understand pneumococcal pathogenesis, which in turn can provide information about pneumococcal infection progression and provide information for improved treatment strategies.

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Guru Brahma Guru Vishnu
Guru Devo Maheswara
Guru Saaksat Param Brahma
Tasmai Shri Guruve Namaha

These lines of shloka mean, Guru is Brahma (who plants the qualities of goodness), Guru is Vishnu (who nurtures and fosters the qualities of goodness), Guru is Maheswara (who weeds out the bad qualities) and last two lines means guru is like a creator/mentor/modulator. Please replace ´qualities or qualities of goodness with research` in above lines.

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