The adhesins of non-typeable Haemophilus influenzae

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Abstract

Introduction: Non–typeable *Haemophilus influenzae* (NTHi) is an opportunistic pathogen of the respiratory tract and the greatest contributor to invasive *Haemophilus* disease. Additionally, in children, NTHi is responsible for the majority of otitis media which can lead to chronic infection and hearing loss later in life. In adults, NTHi infection in the lung is responsible for the onset of acute exacerbations in chronic obstructive pulmonary disease (COPD). Unfortunately, there is currently no vaccine available to protect against NTHi infections.

Areas covered: NTHi uses an arsenal of adhesins to colonise the respiratory epithelium. The adhesins also have secondary roles that aid the virulence of NTHi, including mechanisms that avoid immune clearance, adjust pore size to avoid antimicrobial destruction, form micro-colonies and invoke phase variation for protein mediation. Bacterial adhesins are also ideal antigens for subunit vaccine design due to their surface exposure and immunogenic capabilities.

Expert commentary: The host-pathogen interactions of the NTHi adhesins are not fully investigated. The relationship between adhesins and the ECM play a part in the success of NTHi colonisation and virulence by immune evasion, migration and biofilm development. Further research into these immunogenic proteins would further our understanding and enable a basis for better combatting NTHi disease.

1.0 Introduction

H. influenzae is a Gram negative coccobacillus with six serotypes (a-f), determined by the composure of the polysaccharide capsule, and a non-encapsulated form known as non typeable *H. influenzae* (NTHi). Whilst encapsulated *Haemophilus* are relatively homologous regardless of their serotype, NTHi are genetically diverse [1, 2]. *H. influenzae* serotype b (Hib) was historically the greatest cause of invasive *Haemophilus* disease such as meningitis, pneumonia and epiglottitis [3]. In 2000 the World Health Organisation reported 386,000 deaths and 2-3 million cases of *Haemophilus* invasive disease globally of which Hib was identified as the causative organism for 90% [4]. Meningitis was the diagnosis in 50-65% of cases presented with the majority reported in children under the age of 5 years old [5]. The addition of the Hib vaccine to national immunisation programmes (NIP's) during the 1990's and early 2000's led to the drastic reduction of Hib in carriage and Hib related disease [6, 7, 8, 9]. Since the introduction of the vaccine, invasive *Haemophilus* disease has primarily been caused by the unencapsulated NTHi [10].

In addition to invasive disease, NTHi is also pivotal in the development of chronic obstructive pulmonary disease (COPD), a multi-faceted condition that causes accumulative and irreversible degradation of lung function and ranked the third largest cause of global morbidity [11]. Progression of the disease is brought about by episodes of worsening symptoms referred to as exacerbations which are associated with bacterial and viral infections of the COPD lung. NTHi is reported to be the largest bacterial cause of acute exacerbations in COPD [12, 13].

The carriage of NTHi in the nasopharynx of children has been reported from 11% - 57% [14, 15]. Bacterial carriage is known to be the precursor to disease but, to enable colonisation, microbes have to first successfully adhere to the epithelial layer despite the host's defenses. Mucins, defensive protein structures in the extracellular matrix (ECM), evasion of the immune system and penetration of the epithelial layer are all obstacles that need to be conquered for successful adherence and colonisation to occur. Like many other bacteria, NTHi have evolved to overcome these defensive mechanisms with adhesin proteins having an important role in doing so [16].

2.0 Adhesins

Figure 1 – Known secondary roles and receptor interactions of the NTHi adhesins - Hif, Omp1, Omp2, Omp4, Omp5, Protein E, Protein F, Hia, Hmw and Hap. Hif binds to mucins and displays phase variation. Omp5 also binds to mucins and has ICAM-1 and CEACAM-1 cell receptors. Omp1 binds to CEACAM-1 only and Protein E binds to ICAM-1, laminin, plasminogen, vitronectin and fibronectin. Protein expression of Hia and Hmw is mediated by phase variation. Omp4 binds to vitronectin and fibronectin. Hap also binds to laminin and is able to form microcolonies. Omp2 creates spontaneous point mutations and differing pore sizes.

2.1 The Trimeric Autotransporters

Trimeric autotransporters (TAA) consist of a c terminal anchor domain which embeds into the outer membrane of the bacterial cell and creates a pore through which a passenger domain mobilises to access the bacterial cell surface, facilitating interactions with host cells. NTHi produce adhesins of TAA structure, the heavy molecular weight proteins (Hmw1 and Hmw2), *H. influenzae* adhesion (Hia) and *Haemophilus* adhesion protein (Hap) [17, 18, 19, 20]. These proteins are expressed by *hmw*, *hia* and *hap* genes respectively [21, 22]. It is thought that the TAAs play an important part in the initial colonisation of the host for Gram negative bacteria [17].

Hmw and Hia are associated with highly adherent and invasive strains of NTHi suggesting an important role in virulence [21, 22, 23]. NTHi strains expressing *hia* and *hmw* have been identified as the causative organism in cases of infant meningitis with a report recording *hia* positive strains in five out of nine cases and *hmw* present in two out of nine [24].

The Hmw TAAs bind to a variety of cells with a high level of adherence and have been shown to outcompete *hmw* deficient strains in colonising rhesus macaques [23, 25, 26]. Despite these findings *hmw* positive strains of NTHi are more prevalently isolated from sites of non-invasive infection such as in the middle ear of otitis media cases than from throat and nasopharyngeal carriage [27, 28, 29]. A contradictory report did not observe an association between site of isolation and presence of *hmw* [30].

Despite the proposed role of Hmw in colonisation, Hmw along with Hia display high levels of immunogenicity [31] but both are able partially overcome this by mediating protein expression through phase variation (Figure 1) [32, 33, 34]. Hmw expression is reduced by the addition of 7bp tandem repeats inserted near the *hmw* promotor [33]. This results in a

stepped reduction of the protein with each increase of repeat, and is reversible, resulting in strains of NTHi that can switch between a spectrum of colonisation or immune evasion [34]. Similarly, a reduction in Hia is observed with an extension of a poly T tract again located near the promoter of the *hia* gene [32]. The size of the poly T tract correlates negatively with protein expression of Hia [32]. Hmw has been reported to have a supplementary role in auto immune disease [35]. Antibodies to hyperglucosylated Hmw1A have been found in the sera of multiple sclerosis patients, indicating Hmw as an exogenous agent that triggers an auto immune response in multiple sclerosis [35].

NTHi strains containing *hmw* are not ubiquitous with 45% – 80% of strains reportedly containing *hmw* [22, 29, 36]. The *hia* positive strains are less prevalent, reported in 8.3%-33% [22, 27, 36]. Strains containing both genes have been reported (3.1% -8.3%), as have strains negative for both [22, 36] However, ordinarily, NTHi containing *hmw* does not contain *hia* and vice versa but the majority (71%-95%) of strains are thought to contain one of the two. [22, 27, 37]. Hia and Hmw are both adherent proteins and those strains negative for both have been reported to be non–adherent highlighting the influence these genes and their resulting proteins may have on colonisation [22]. Hmw and Hia have been significantly associated with persistence and cross colonisation [36].

Hmw and Hia are present in serotypes a, e and f but not Hib [21, 27]. An interesting relationship exists between Hia and an adhesin found in Hib known as the Haemophilus surface fibril (Hsf) [38]. Whereas Hsf has a fimbril structure, Hia does not; however, genetically they are 81% similar and it has emerged that they share homology in areas of binding domains [38, 39]. Hsf contains three binding domains similar to that expressed by Hia [38, 39]. Only two out of these three facilitate adherence and contain the acidic binding pocket also present in Hia binding regions [39]. Adherence and internalisation of Hib strains is intensified by Hsf due to its ability to bind with vitronectin, a constituent of the ECM which conceals the bound Hib from the membrane attack complex evading immune clearance [40, 41]. There is the possibility therefore, that Hia may also interact with vitronectin in a comparable manner to Hsf due to their shared homology of essential binding regions. However, the adherent capacity of Hsf has been shown to alter with a single amino acid change in the binding regions [42]. Hia has displayed variance across NTHi strains which may affect the binding potential yet contrarily the need for both binding regions has been

demonstrated to be required for adherence [27, 42, 43]. The characteristics of Hia and its interactions with the ECM have yet to be investigated.

Hap is another TAA however, unlike Hmw and Hia it is found to be ubiquitous throughout NTHi [30]. Hap binds to collagen IV and laminin in addition to being the primary ligand to fibronectin within the ECM (Figure 1) [19]. Hap has the capability of creating microcolonies by bacterial aggregation (Figure 1) [44]. This is due to Hap_s, a serine protease which overcomes the repulsive forces of bacterial cells and self attracts [45, 46, 47]. Once the accumulation of Hap_s has reached an optimum concentration, it overcomes the inhibitive mechanisms of a host enzyme called secretory leucocyte protease inhibitor (SLPI) and disperses via auto proteolysis. Hap_s is cleaved from the bacterial cell as an extracellular protein, disrupting the epithelial layer and initiating migration, despite the dispersal of the microcolonies being counterintuitive to adherence [45, 48, 49]. It has been reported that Hap is not required for biofilm formation despite the understanding that microcolony formation is believed to be an important step in biofilm progression [44, 50]. Biofilms are speculated to have a pivotal role in pathogenesis and microbial survival on mucosal surfaces [51]. Hmw and Hia have been associated with biofilm formation with Hia positive strains resulting in denser biofilms than Hmw expressing strains [36]. The prevalence of *hmw* positive strains within OM suggests that Hmw proteins may possess some role within these associated biofilms [27, 28, 29, 51].

2.2 Surface Proteins

The outer membrane proteins (Omps 1,2, 4, 5 and 6) and Proteins E and F are important for adherence and have differing interactions with the epithelial layer and ECM (Figure 1). They are expressed by the genes *omp1*, *omp2*, *hel*, *ompA*, *omp6*, *pE* and *pF* respectively.

Omp5 fimbriae are universally found throughout NTHi and are important for binding to nasopharyngeal mucins which would otherwise facilitate clearance [52, 53]. In the event of co-infection of NTHi and respiratory syncytial virus, Omp5 is associated with a significant increase in attachment to epithelial cells although its interaction with receptors has been debated [53]. Initially it was thought that Omp5 was the primary ligand for the receptor carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1)[54]. This was

demonstrated on chinchilla epithelial cells however, further studies using human cells demonstrated residual adherence from mutant *omp5* strains determining a second ligand to the receptor must be available [54, 55]. Mutant strains of *omp1* were found to have no residual adherence to CEACAM-1 and therefore Omp1 was identified as the major ligand to the receptor [55]. Reduction of cell internalisation has been associated with *omp1* mutation indicating a joint role in adherence and internalisation as displayed by other adhesins such as Hsf. Similarly, Omp5 has displayed the ability to bind to and upregulate Intercellular adhesin molecule 1 (ICAM-1) which is found in the membrane of endothelial cells and leukocytes but this has been disputed by an alternate study [50, 56]. The capabilities of Omp5 appear to depend on the location of the epithelial cells. Adherence and internalisation of Omp5 to cells from the lower respiratory tract were seen to be more affected by strains of NTHi carrying a mutated ompA gene which expresses Omp5 than those cells from the upper respiratory tract [50]. Adherence of *ompA* positive strains were found not to be of high adherence capacity and sequence variation of *ompA* was reported to have no impact [23]. Conflicting reports for the importance of as a biofilm protein and as a requirement for growth have been published [50, 57]. Omp1, 2, 5 and 6 have all been determined as present in NTHi biofilms [57, 58]. Additionally, Omp5 is thought to play a part in immune resistance through affecting complement activation by binding to factor H which is a vital inhibitor of the alternative pathway and by decreasing the binding of IgG, a vital instigator of the classical pathway [59]. Like Hia, Hmw and Hap, Omp5 is a multi-purpose protein with roles in adhesion, biofilm, internalisation and immune evasion.

Protein E adheres to epithelial cells by binding and upregulating ICAM-1 and evades immune clearance by binding to vitronectin, similar to Hsf, and also plasminogen [60, 61]. Once bound to Protein E, plasminogen converts to plasmin, a serine protease which inhibits the complement pathway [62, 63]. Plasmin also enables cellular invasion and migration by degrading the ECM [64]. In addition to plasminogen, Protein E simultaneously binds to vitronectin and laminin and has been identified in 96.9% of NTHi [61, 65, 66].

Omp2 is a porin protein that encompasses approximately half of the cell membrane and enables adherence to nasopharyngeal mucins similar to Omp5 [52, 67]. The pore size varies in Omp2, mediated by sequence variation, and enables a decrease in antimicrobial susceptibility to broad spectrum treatments [68, 69, 70]. A potential method of immune

evasion has been identified by the presence of spontaneous point mutations [71]. Omp2 and Omp1 are also present in Hib. [72].

Fibronectin has been demonstrated as the main ligand for Omp4 in pharynx, alveolar and bronchial epithelial cells [73]. Omp4, however, also binds to laminin and vitronectin, mediating immune evasion and increased adherence. NTHi negative for Omp4 have been shown to result in reduced incidences of infection in the murine middle ear suggesting potential roles in immune evasion, colonisation and otitis media. [73].

The *omp6* gene is ubiquitous throughout NTHi but shows marked variation between strains [74, 75]. The majority of the Omp6 protein is located internally in the periplasmic space of the bacterial cell with only a small portion exposed to the surface [76]. It's interaction with peptidoglycan within the cell wall is thought to maintain cell integrity by affixing the cell wall to the outer membrane [77]. Omp6 triggers the activation of certain pro-inflammatory macrophage cytokines, inducing macrophage phagocytosis of NTHi [78]. NTHi are capable of survival after phagocytosis and this plays a large part of the pathogenesis of NTHi [78]. Omp6 is a constituent of NTHi biofilm but is also able to mediate expression by self-binding [57, 79, 80].

There is a paucity of data for Protein F although it has been discovered to interact with laminin and is thought ubiquitous throughout the NTHi subspecies [81, 82]. A reduction of 64% in adherence to bronchial epithelial cells has been observed in mutant Protein F strains [82].

2.3 Surface Protusions

The type IV secretion system (T4SS) is encoded by genes *pilA-D* and *comA-F* in NTHi with the major pili protein being expressed by *pilA*. High adherence to ICAM-1 on epithelial cells has been associated with the type IV pili [83]. Significant reduction in adherence to human bronchial epithelial cells has been reported in mutants of all *pil* and *com* genes, responsible for the expression of the T4SS, except *com*C [84]. Furthermore, the type IV pili has been demonstrated as important for formation and structural maintenance of NTHi biofilm [57, 79, 84, 85].

H. influenzae fimbriae (Hif) enable attachment to nasopharyngeal mucins and are expressed by genes *hif*A-E. [86, 87]. When comparing non fimbriated and fimbriated strains of NTHi, 95% reduction of attachment was observed in non –fimbrated strains [87]. Hif has been described in both Hib and NTHi but is more associated with non-invasive strains of the latter [88]. Phase variation plays a role in the mediation of Hif protein expression; a reduction resulting from the variation of 2bp TA repeats in the promoter region of the *hifA* and *hifB* genes [89]. This reduction could potentially be an immune avoidance mechanism or a tool to implement chronic colonisation of patients with conditions such as cystic fibrosis where secretions are not cleared [86, 87, 89, 90]. NTHi isolated from sputum exhibited a larger adhesion capability to mucins than those isolated from blood samples suggesting site of isolation may affect the adhesion efficiency and inferring the manifestation of an environmental pressure [91]. Conversely to *hmw* strains, NTHi containing *hifB and hifC* are significantly more present in throat isolates from healthy subjects compared to middle ear effusions from otitis media patients, supporting the hypothesis that Hif may be present in less pathogenic strains of NTHi [27, 88].

3.0 Immunogenicity and potential vaccine candidates

The majority of the adhesins discussed have been investigated for their ability to elicit an immune response and this has led to the focus of adhesin proteins for vaccine candidature. The adhesins are prime vaccine candidates due to their exposure on the cell surface.

Interest has been shown in the development of a vaccine composing of recombinant epitopes of PilA and Omp5. This chimeric vaccine has culminated in a strong immunogen and displayed the ability to significantly reduce biofilm production in the middle ear of a chinchilla model [92, 93]. Used without Omp5 but attached to an integration host factor, recombinant PilA administered to chinchillas resulted in degradation of biofilms from within the middle ear, eliminating both biofilm derived and planktonic populations of NTHi [92].

Hia and Hmw are highly immunogenic and are targets for opsonophagocytic activity [16, 31]. *hia* however is only observed in 8.3%-33% of strains and therefore may not be effective as a single target for immunisation purposes [22, 27, 36, 94]. A further complication arises with phase variation within *hia* and *hmw* mediating protein expression and resulting in immune

evasion, therefore potentially reducing effectiveness as vaccine candidates [32, 33, 34]. Anti-sera to Hmw, was unable to eradicate Hia positive strains and vice versa and although a small amount (5%) of NTHi are thought to carry neither proteins. The recommendation of a vaccine containing both could potentially present a vaccine sufficient to eradicate ~50% of strains [31].

Hap attached to a cholera toxin (CT-E29H) and intranasally administered to mice was able to reduce nasopharyngeal carriage providing a potential vaccine candidate in a protein ubiquitous throughout NTHi. [95].

Omp1, Omp2, Omp5 and Omp6 have been identified as important antigenic proteins via immunoprecipitation studies of intranasal immunisation with outer member vesicles (OMV) [96, 97]. As the most prevalent surface protein, Omp2 holds particular interest and has been shown to evoke an immune response in multiple studies; however, sequence variation resulting in strain specific immunity poses a drawback [98, 99, 100]. Using recombinant Omp2 has resulted in a more effective cross-reactive response and epitopes from the external loop structures have been identified as specific areas of focus [101, 102, 103]. More specifically, external loop structures 5 and 6 have demonstrated immunogenic capabilities and a conserved epitope from loop 6 found in a third of strains was observed to culminate in a multi strain response [71, 102].

Omp4 has been show to elicit an immune response and has been observed as ubiquitous within strains of NTHi [96, 104, 105, 106]. Recombinant Omp4 has been developed to remove enzymatic action to ensure suitability for vaccine [106]. Recombinant Omp4 attached to a cholera toxin has been shown to be successful in clearing intranasal carriage of NTHi from mice [73, 104].

Omp6 is able to elicit bactericidal antibodies despite the majority of the protein being internally positioned and non-antigenic [76, 107, 108, 109, 110, 111, 112]. A small percentage of the surface exposed section of Omp6 is immunogenic but is sufficient to upregulate cytokines IL-10, TNF-α and IL-8 [78]. Clearance has been observed of NTHi in murine sinuses, nasopharynx and middle ear after intranasal immunisation with recombinant Omp6 and cholera toxin or adamantylamide dipeptide. [108, 111, 112, 113]. Omp6 maternal antibodies are also reportedly passed to babies through breast feeding

increasing protection to NTHi infection; this has been demonstrated in maternal mice after intranasal administration with Omp6 [109, 114]. Evidence has shown that Omp6 shows sequence variation and is not conserved in all strains of NTHi with 4.9% - 5.6% displaying structural changes to Omp6 however this is a small percentage of strains [74, 75, 115].

Both Proteins E and F have shown promise as vaccine candidates. Protein E activates a pro-inflammatory IL-8 and ICAM response. Antibodies for Protein F are thought to be naturally present; identified in 26% of the healthy adult population. Furthermore, immunogenicity invoked by a protein F immunisation in mice has been shown to provide pulmonary clearance of NTHi [81]. Protein F and Protein E are conserved within NTHi and are found in 100% and 98.6% of the NTHi population respectively. [66, 82].

4.0 Summary

The first essential step for colonisation for NTHi is adhering to the respiratory epithelium. This is achieved by use of one or more different adhesins which are not all ubiquitous throughout NTHi [16, 22, 23, 37, 84, 94]. They are tools not only for adherence and colonisation but also demonstrate secondary roles in immune evasion and pathogenesis through biofilm development and migration deeper into the basement membrane due to the interactions with constituents of the ECM [27, 62, 64]. NTHi are able to form microcolonies and are present in biofilms of the middle ear and COPD, a critical survival tactic for NTHi in these environments. The majority of the adhesins have been shown to have some presence in the formation or development of biofilms [45, 49, 57]. Contradictory studies of genotypes responsible for proteins integral in biofilm formation reveal that the role of adhesins in NTHi biofilms is yet to be fully determined [36, 50, 57, 79, 84, 85].

A successful vaccine for NTHi has yet to be made available for public use to reduce the burden of disease of otitis media and devastating invasive disease, and lessen debilitating exacerbations in COPD. Complications arise from the complexities of the adhesins in NTHi in the form of the heterogeneity of the strains, strain replacement and mechanisms for evasion such as phase variation, point mutations and glycoprotein binding. Whole cell immunisation may not be viable for a species as heterogeneous as NTHi and conserved antigenic regions

could be more specific. However, with some conserved regions of interest only identified in a third of strains, strain replacement may follow.

Further investigation into the adhesins will allow a better understanding of their role in pathogenesis, establish those that are conserved and ubiquitous throughout the NTHi sub-population for vaccine candidature and further our understanding on how the presence of adhesins within NTHi enable them to infiltrate and colonise the respiratory tract and progress from commensal to pathogenic.

5.0 Expert summary

Recent findings have highlighted the many roles that adhesins play in the colonisation and virulence capabilities of NTHi. The intricate interactions between these surface expressed proteins and the host immune system are fascinating not least as they play a role in avoidance of phagocytosis. There remains much to be determined however, with Hia being a particularly interesting and currently unwritten story.

The homology between Hia in NTHi and Hif in the serotype b strains of *H. influenzae* indicates a shared evolutionary path between the capsulated and un-encapsulated strains and may give an insight into the ability of Hia to protect cells from immune clearance through binding to vitronectin. The fact that many other surface proteins enable binding to vitronectin suggests that this redundancy highlights a crucial mechanism for *H. influenzae* to avoid immune clearance. Hia is not ubiquitous throughout *H. influenzae* by any means, 33% is the largest proportion of *hia* positive strains reported in any one study, but its presence has been associated with higher adherence capacity and invasive disease. It has been noted that Hia is a highly immunogenic protein and reduces its protein expression by the mediation of phase variation and the extension of a poly T tract. This however may not be fully efficient in avoiding phagocytosis and give way to those strains without Hia and with more persistent capabilities, therefore resulting in a relatively small percentage of Hia positive strains.

Another possibility could be due to competitive binding with vitronectin, either with other strains of NTHi, or indeed other bacterial species, utilising adhesins with differing abilities to bind to vitronectin. C reactive protein (CRP) found in areas of inflammation also binds to

vitronectin, such as the COPD lung. Hia strains therefore, if present in cases of high inflammation may need to compete to bind to vitronectin. As it has not yet been revealed if and how Hia interacts with the ECM, it can only by hypothesised that the homology between Hia and Hsf in the binding regions also indicates that Hia may mirror the ability that Hsf has to bind to vitronectin. This may be a trade-off for this adhesin and therefore in certain sites of localisation or disease state be usurped by strains with more persistent capabilities.

Most adhesins have been investigated as vaccine candidates and are considered good targets as a consequence of their immunogenicity. However, the genetic diversity of NTHi and the varied distribution of adhesins suggests that identifying a perfect candidate may prove complicated. Indeed, genetic plasticity, varied antigen expression governed by phase variation and adhesin redundancy when coupled with vaccine pressure may result in rapid strain replacement of which the outcome cannot be predicted. Nevertheless, adhesins provide a group of interesting proteins which may further our understanding of host-pathogen interactions and provide avenues for future research into combatting NTHi disease.

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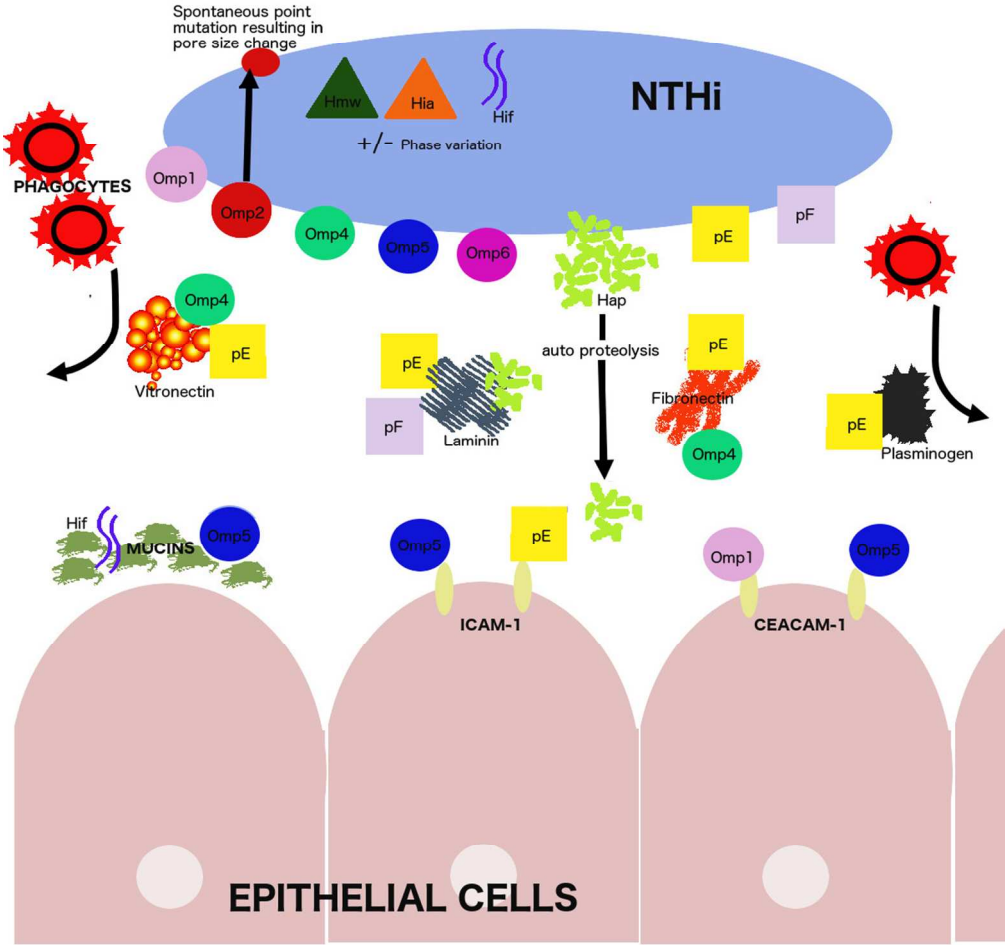

Figure 1 - Known secondary roles and receptor interactions of the NTHi adhesins - Hif, Omp1, Omp2, Omp4, Omp5, Protein E, Protein F, Hia, Hmw and Hap. Hif binds to mucins and displays phase variation. Omp5 also binds to mucins and has ICAM-1 and CEACAM-1 cell receptors. Omp1 binds to CEACAM-1 only and Protein E binds to ICAM-1, laminin, plasminogen, vitronectin and fibronectin. Protein expression of Hia and Hmw is mediated by phase variation. Omp4 binds to vitronectin and fibronectin. Hap also binds to laminin and is able to form microcolonies. Omp2 creates spontaneous point mutations and differing pore sizes.

101x101mm (300 x 300 DPI)