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Investigation of the role of the non-integrin laminin receptor in the pathogenesis of bacterial meningitis

by

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Declaration

I hereby declare that all the work described in this thesis is my own, unless otherwise stated, and that this thesis has not been submitted, neither in whole or in part, elsewhere for another degree.

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This Thesis is dedicated to my Mother

You have shown me the world.

You always stood by me in my best and worst times,

You have made me what I am,

Thanks for being such a wonderful mother

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PPI-Net
Protein-Protein Interactions Network

ABBREVIATIONS

A	**Alanine**	**CEACAM**	**Carcinoembryonic antigen-related cell-adhesion molecule**
ABC	ATP-binding cassette	**ChoP**	phosphorylcholine
ABM	Acute bacterial meningitis	**CNF1**	Cytotoxic necrotizing factor 1
ABTS	2, 2'-azino-bis; 3-ethylbenzthiazoline-6-sulphonic acid	**CNS**	Central nervous system
ATCC	American Type Culture Collection	**COAG**	Coagglutination
3ATM	3 ATM (Fc-FGFR2 IIIa TM; FGFR2 is a Fibroblast Growth Factor 2)	**COS7**	Fibroblast-like cell line derived from monkey kidney tissue
AP	Alkaline phosphatase	**CPS**	Capsular polysaccharide
AVV	Adeno-Associated Virus	***cps***	Capsular polysaccharide gene
***BamH*I**	Restriction endonuclease enzyme from *Bacillus amyloliquefaciens* H	**CRD**	Carbohydrate recognition domain
BBB	Blood-brain barrier	**CSF**	Cerebrospinal fluid
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium	**CTD**	C-terminal domain
BifC	Bimolecular fluorescence complementation	**Da/kDa**	Dalton/kilodalton
bLamR	Bacteria-produced laminin receptor	**DIG**	Digoxigenin-3-0-succinyl-ε-aminocaproic acid-N-hydroxy-succinimide ester Protein labeling kit
BM	Bacterial meningitis	**dH_2O**	Distilled water
BMECs	Brain microvascular endothelial cells	**DMEM**	Dulbecco's modified Eagle's Medium
bp	Base pair	**DMSO**	Dimethyl Sulfoxide
BSA	Bovine serum albumin	**DNA**	Deoxyribonucleic acid
CaPO4	Calcium phosphate	**ECM**	Extracellular matrix molecule
CBD	C-terminal choline-binding	**EDTA**	Ethylenediaminetetraacetic acid
CBM	Chronic bacterial meningitis	**EGCG**	(-)-epigallocatechin-3-gallate
CbpA	Choline-binding protein A	**eGFP**	Enhanced green fluorescent protein
cbpA	Choline-binding protein A gene	**eLamR**	Endogenous laminin receptor
CBPs	Choline-binding proteins	**ELISA**	Enzyme-linked ImmunoSorbent Assay

F	Phenylalanine	**LOS**	Lipooligosaccharide
FACS	fluorescence-activated cell sorting	**LPS**	Lipopolysaccharide
FBS	Fetal bovine serum	**LR**	The 67-kDa laminin receptor
Gal-3	Galectin-3	**LRP**	The 37-kDa laminin receptor
h	Hour	**LTA**	Lipoteichoic acid
Hap	Haemophilus adhesion and penetration protein	**LytA**	Autolysin
HBMECs	Human brain microvascular endothelial cells	**MAC**	Membrane attack complex
HEK293T	Human embryonic kidney cells	**MC58**	A meningococcal strain belonging to the serogroup B
Hia	*Haemophilus influenzae* adhesin	**mg**	Milligram
Hib	*Haemophilus influenzae* type b	**µg**	Microgram
Hif	*Haemophilus influenzae* fimbriae	**min**	Minute
hLamR	Human-produced laminin receptor	**ml**	Millilitre
HMW	High-molecular-weight proteins	**mM**	Milimolar
Hpd	Haemophilus protein D	**MST**	Medium-scale transfections
HRP	Horseradish peroxidase	***Nde1***	Restriction endonuclease enzyme from *Neisseria denitrificans*
HSPGs	Heparan sulphate proteoglycans	***Not*I**	Restriction endonuclease enzyme from *Nocardia otitidis-caviarum*
Hsf	*Haemophilus* surface fibrils	**NTHi**	Non-typeable *H. influenzae*
Ig	Immunoglobulin	**N-CAM**	Neural cell adhesion molecule
IgA	Immunoglobulin protease	**OapA**	Opacity-associated Protein A
IL	Interleukin	**OD**	Optical density
IM	Intracellular membrane	**Opa**	Opacity
IMAC	Immobilized metal ion affinity chromatography	**ORF**	Open reading frame
IPCR	Inverse polymerase chain reaction	**OS**	Oligosaccharide
IPTG	Isopropyl-1-thio-β-D-galactopyranoside	**OMP**	Outer membrane protein
L	Lysine	**OmpP5**	Outer-membrane protein P5
LamR	37/67-kDa non-integrin laminin receptor		
LB	Luria-Bertani	**PAF**	Platelet-activating factor

PAFr	Platelet activating factor receptor	**R**	Araginie
PFA	paraformaldehyde	**RIPA**	Radio immunoprecipitation assay buffer
PBS	Phosphate Buffered Saline	**rLRP**	Recombinant laminin receptor precursor protein
PBS-T	Phosphate buffered saline-Tween	**rpm**	Revolutions per minute
PCR	Polymerase chain reaction	**RNA**	Ribonucleic acid
PcpA	Pneumococcal choline-binding protein A	**SDS-PAGE**	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
pIgR	Polymeric immunoglobulin receptor	**T**	Tyrosine
PLY	Pneumolysin	**TA**	Teichoic acid
PPI	protein-protein interactions	**Tfp**	Type IV pili
PrP	Prion protein	**vYFLP**	Venus Full-length YFP fluorescence protein
PrP^{c}	Cellular prion protein	**vYFP**	Venus yellow fluorescent protein
PrP^{Sc}	Infectious prion protein called	**vYCL**	C-terminus of Venus yellow fluorescent protein
PS	Polysaccharide	**vYNL**	N-terminus of Venus yellow fluorescent protein
PspA	Pneumococcal surface antigen A	**VEE**	Venezuelan Equine Encephalitis Virus
PspC	pneumococcal surface protein C	**WBC**	White blood cell
pH	Unit of acidity/alkalinity	**WT LamR**	Wild type laminin receptor
PspC	pneumococcal surface protein C	**× *g***	The centrifugal force applied as multiples of the earth's gravitation
PorA	Porin A	**ºC**	Degrees Celsius
PorB	Porin B		
PBS	Phosphate buffered saline		

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ABSTRACT

The human non-integrin laminin receptor (LamR) is a multifunctional protein which is localised to a number of sub-cellular locations. LamR is a component of the ribosome and has a number of intracellular functions; it also acts as an extracellular receptor for laminin, growth factors, pathogenic microorganisms, prion proteins, toxins and the anticarcinogen epigallocatechin gallate (ECGC). Although LamR is present in most cellular compartments, its overexpression in many types of cancer cells suggests a vital role for LamR in tumor-cell migration and invasion. There are two isoforms of laminin receptor: the monomeric 37-kDa laminin receptor precursor (37LRP) and the mature 67-kDa laminin receptor (67LR). Although the precise molecular nature of 67LR is unclear, accumulating evidence strongly suggest that 37LRP can undergo homo- and/or hetero-dimerization with Galectin-3 (Gal-3) to form mature 67LR. A recent study demonstrated that both homo- and heterodimer LamR forms are present on the cell surface, where they form distinct populations.

Neisseria meningitidis, *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib) are major causes of bacterial meningitis. The contribution of LamR in traversal of the blood brain barrier (BBB) by neurotropic viruses is well established and interaction with LamR was recently found to be critical for initiation of bacterial contact with the blood brain barrier (BBB). These bacteria bind LamR via the surface protein adhesins: meningococcal PilQ and PorA; pneumococcal CbpA; and *H. influenzae* OmpP2.

Further investigations showed that the fourth and second extracellular loops of meningococcal PorA and OmpP2 of *H. influenzae*, respectively, are responsible for LamR binding.

The work presented here consists of two complementary projects in which a number of approaches were taken to characterise the ligand-binding domains of LamR. The first project aimed to identify sites on LamR that are critical for binding the ligands of bacterial meningeal pathogens. The second project aimed to identify residues that contribute to the stability of LamR homodimers and the heterodimer with Gal-3.

Several mutations were introduced into full-length human LamR, either by deletion mutations within the C-terminal domain (CTD) of LamR using inverse polymerase chain reaction (IPCR), or by single-amino acid substitution in the N-terminal domain (NTD) of LamR using site-directed mutagenesis. Protocols for large-scale expression of full-length and truncated LamR proteins in human cells were developed, as well as non-denaturing purification protocols.

We hypothesised that bacteria-binding domains could be located on both the NTD and CTD of LamR. *In vivo* examination using ELISA assays, in which the interaction of LamR and whole bacteria or purified recombinant PorA or PilQ were tested identified several novel sites on LamR that contributed significantly to binding of the bacterial ligands. These sites include amino acids 206-229 and 263-282, located within the CTD, and Tyrosine 156 in the NTD, each of which contributed to the binding of meningococcal PorA, and more specifically it's fourth extracellular loop. Furthermore, three sites on LamR comprising amino acids 206-229 and 263-282 within the CTD and Tyrosine 139 in the NTD were shown to contribute to binding pneumococcal CbpA, OmpP2 and Loop two of OmpP2 of *H. influenzae*. These results indicate that the three neuroinvasive bacteria share the same binding sites on LamR.

Bimolecular fluorescence complementation (BifC) coupled with flow cytometry and confocal microscopy revealed the vital contribution of amino acid residues Arginine 155, Tyrosine 156 and Lysine 166 (all within the NTD of LamR) for the homodimerization and heterodimerization of LamR with Gal-3. The dimerization of two meningococcal host receptors, LamR and Gal-3, may help to extend spectrum of their bacterial adhesins, which may act cooperatively or synergistically at different stages of infection. Information about the residues in LamR that contribute to the stabilization of LamR dimers has implications for the role of LamR dimers in the pathogenesis of bacterial meningitis. Identification of bacteria-binding domains on LamR is of particular interest for development of vaccines or therapeutic interventions that provide protection against the three major meningitis-causing bacteria.

The aim of the current work was firs to localise the domains of LamR responsible for binding with PorA and PilQ of *N. meningitidis*; CbpA and OmpP2 of *S. pneumonia*, and OmpP2 of *H. influenzae*. Also, previous studies have shown conspicuous homodimerisation of 37LRP and heterodimerisation with Gal-3. Our second aim was to identify of amino acid residues involved in 37LRP self-association and heterodimer formation with Gal-3.

In current work, several regions of LamR were hypothesised to constitute the binding site for the bacterial ligands; these predictions were based on previous studies on LamR binding domains and the crystal structure of laminin receptor. Also, to investigate both homo and heterodimerisation of LamR, the involvement of several putative amino acid residues within 37LRP in LamR dimerisation was was hypothesised.

CHAPTER ONE

General Introduction

CHAPTER ONE

1 GENERAL INTRODUCTION

1.1 Meningitis

Central nervous system (CNS) infections continue to be an important cause of mortality and morbidity (Tan et al., 2015). Meningitis, also termed leptomeningitis or arachnoiditis is an infection of the protective membranes (meninges) that surround the brain and spinal cord (Mace, 2008). This infection causes the meninges to become inflamed, which is characterised by the existence of polymorphonuclear cells in the cerebrospinal fluid (CSF) and can damage the nerves and brain in some cases (Harvey et al., 1999, Huang et al., 2000). Meningitis is caused by number of pathogenic microbes or less commonly by certain drugs. The pathogens that-cause meningitis include bacteria (bacterial or pyogenic meningitis), parasites, viruses and fungi. Pathogens capable of causing meningitis must have the ability to access the CNS (Ginsberg, 2004, Mace, 2008). Although several bacteria are able to attack the CNS and cause meningitis, meningitis infection can be fatal due to the inflammation's proximity to the brain and spinal cord; therefore, the condition is classified as a medical emergency (Saez-Llorens and McCracken, 2003, Tunkel et al., 2004).

1.2 Bacterial meningitis; epidemiology and clinical presentation

Bacterial meningitis (BM) was universally considered to be a life-threating disease from the time it was first reported by Vieusseux in 1806 in Geneva. Despite the extensive use of antibiotics and other advances in medical care, mortality and morbidity from bacterial meningitis still remain very high (Durand et al., 1993) accounting for an estimated 180, 000 deaths, per year, worldwide

(Durand et al., 1993, Jodar et al., 2002) with up to 50% of survivors developing long term neurological; neuropsychological sequelae and impairment (Saez-Llorens and McCracken, 2003, Tunkel et al., 2004, Hodgson et al., 2001, van de Beek et al., 2002, van de Beek and de Gans, 2004, Schmidt et al., 2006). Bacterial meningitis is now among the top 10 infectious causes of death worldwide (Grimwood et al., 2000) ; it usually presents as an acute illness, with a quick onset of symptoms, predominantly affects children and young adults (Boos et al., 2004) and rarely presents as a chronic infection in older adults. The most common cause of chronic bacterial meningitis (CBM) is *Mycobacterium tuberculosis* which accounts for 40–60% of cases (Anderson and Willoughby, 1987, Hildebrand and Aoun, 2003). Rarely, a few individual cases have been reported in the literature of CBM caused by *N. meningitidis* group B (Findler and Hoffman, 1981, Wynn et al., 1994). Acute bacterial meningitis (ABM) usually happens when bacteria get access into the bloodstream and migrate to the brain and spinal cord. In some cases, ABM occurs by direct bacterial inoculation of the meninges, as an outcome of a skull fracture or ear and sinus infection, or, rarely, after some surgeries. ABM is mainly caused by *Streptococcus pneumoniae* (pneumococcus); *Neisseria meningitidis* (meningococcus); *Haemophilus influenzae (*haemophilus), *Listeria monocytogenes* (listeria) and, in newborns, *Escherichia coli* K1 and Group B streptococcus (Harvey et al., 1999, Huang et al., 2000). Over 90% of all acute bacterial meningitis worldwide, outside of the neonatal period, is caused by the *S. pneumoniae*; *N. meningitidis* and *H. influenzae* (Luksic et al., 2013); these bacteria are all considered to be part of the normal flora in the nasopharynx and only occasionally cause disease (Steinbach and Shetty, 2001). They are transmitted via aerosolization or by contact with respiratory secretions of infected

persons (Roos, 2000, van de Beek et al., 2006, Schut et al., 2008, Mook-Kanamori et al., 2011).

The classical clinical presentation in the majority of patients with bacterial meningitis includes fever, neck stiffness and altered mental status; 80% of cases present with meningismus, and signs of cerebral dysfunction. Elderly patients and infants, however, may present with insidious disease manifested only by lethargy or variable signs of meningeal irritation without fever (Bamberger, 2010, Juganariu et al., 2012).

1.3 Diagnosis of bacterial meningitis

The clinical diagnosis of bacterial meningitis routinely requires the culturing of both blood and CFS (from lumbar puncture), beside the morphologic and chemical examination of CSF via Gram staining, cell count, glucose and protein levels. Wayson stain and Acridine orange are also used for microscopically examination to screen smears of CSF for bacteria (Daly et al., 1985). Cerebrospinal fluid glucose concentrations of <45 mg/dL and WBC count greater than 1000/µl, are indicative of bacterial meningitis (Mace, 2008). Isolation and culture from CSF is the gold standard to determine the causative organisms in bacterial meningitis. However, this infrequently achieved in many health institutions in developing countries, also around 50% of the CSF culture negative patients being pretreated with antibiotics (Caugant, 1998, Brouwer et al., 2010).

Several methods including coagglutination (COAG); latex agglutination (LA), EIA Enzyme immunoassays (EIAs) (Salih et al., 1989), and counter-immune electrophoresis (CIE), have been implicated widely in the clinical microbiology laboratories for a direct and rapid detection of having bacterial meningitis for the presence of the soluble bacterial antigens in the CSF of the patients (Naiman and

Albritton, 1980, Tilton et al., 1984, Salih et al., 1989, Gray and Fedorko, 1992, Carbonnelle, 2009).

Antigen-based bacterial identification is beneficial to detect *N. meningitidis* serogroups A, B, C, Y, and W135; *H. influenzae* type b, *S. pneumoniae* and *Streptococcus* group B which possess soluble, type-specific, capsular polysaccharide antigens which are released into surrounding body tissues and fluids as the bacteria proliferate, and also to identify group B *Streptococci* that possesses a soluble type-specific cell wall polysaccharide antigen (Coonrod, 1983).

Rarely, the Quellung capsular reaction can be also used to confirm the presence of organisms with a morphology typical of *N. meningitidis*; *H. influenzae* type b and *S. pneumoniae*, by using the antisera specific for the capsular polysaccharides of the bacteria mixed with the clinical specimens (Gray and Fedorko, 1992).

Also, in the CSF of patients with suspected bacterial meningitis, microbial DNA can be detected using the PCR using primers specific for *S. pneumoniae*, *N. meningitidis*, and *H. Influenzae* Type b (Hib)(Chiba et al., 2009, Kotilainen et al., 1998, Poppert et al., 2005).

1.4 Bacterial agents of Meningitis

The types of bacteria that cause bacterial meningitis vary depending on the infected individual's age group. Common bacteria that cause bacterial meningitis in each age group are listed in Table 1-1 (Thigpen et al., 2011).

Table 1-1 Meningitis-causing bacteria based on age group.

Age Group	Causes
Newborns	Group B Streptococcus, *E. coli*, *L. monocytogenes*
Infants and Children	*S. pneumoniae*, *N. meningitidis*, *H. influenzae* type b
Adolescents and Young Adults	*N. meningitidis*, *S. pneumoniae*
Older Adults	*S. pneumoniae*, *N. meningitidis*, *L. monocytogenes*

1.4.1 *S. pneumoniae*

S. pneumoniae is the leading cause of the bacterial pneumonia and meningitis. Pneumococci are pathogenic, encapsulated Gram-positive diplococci which usually grow in pairs, but may form short chains or grow singly. They are facultative anaerobes and grow best in 5% CO2. *S. pneumoniae* is α-haemolytic when grown on blood plates (Kadioglu et al., 2008, Andisi et al., 2012).

S. pneumoniae is a member of the normal flora of the human upper respiratory tract in up to10% of healthy adults; in children aged six years or younger, the carriage rates may reach 44%. Although carriage of the bacterium is usually asymptomatic, in some people symptomatic otitis media, pneumonia, bacteremia, or meningitis can be developed (Johnston, 1991).

S. pneumoniae is the leading cause of bacterial respiratory tract infections and is also a leading cause of septic infections and meningitis. This bacterium is also an occasional etiological agent of endocarditis (Uemura et al., 2001, Mook-Kanamori et al., 2011). Despite advances in medications, *S. pneumoniae* remains the leading cause of bacterial pneumonia, bacteremia, and otitis media in children below the age of five years. Due to emergence of antibiotic-resistant strains during the 1990s, *S. pneumoniae* has become the most common invasive pathogen in children and infants (Leowski, 1986, Hollingshead et al., 2000).

1.4.2 Pneumococci structure and virulence factors

The recognition and attachment of *S. pneumoniae* to human nasopharyngeal cells is the first and critical step in the interaction of the bacterium with human cells and this may lead to invasion of new sites within the body and/or contribute to the development of disease. *S. pneumoniae* possess several surface exposed proteins to mediate host-bacteria interactions as well as soluble toxins and structures protecting the pneumococcus from the host immune system. All of these structures significantly contribute to pathogenesis and might be involved in the disease process caused by these pathogens (Boulnois, 1992, Henrichsen, 1995, Jedrzejas, 2001). Pneumococcal virulence factors and their role in the pathogenesis are summarised in

Figure 1-1 and Table 1-2.

1.4.2.1 Capsule of *S. pneumoniae*

The capsular polysaccharide (CPS) is of great importance in the pathogenesis of pneumococcal infections. Encapsulated pneumococci are pathogenic for humans and may be isolated from clinical materials such as blood (Catterall, 1999, Martner et al., 2009), whereas the bacteria without capsular polysaccharides are unable to cause disease in animal models, illustrating the importance of capsule in pneumococcal virulence (Avery et al., 1944, Morona et al., 2002, Morona et al., 2006).

The capsule of the *S. pneumoniae* is encoded by a single genetic locus (*cps*) which has a cassette-like organization organised in a single transcriptional unit. Variation in the antigenic structure of the capsule allows more than 90 different serotypes of *S. pneumoniae* to be distinguished. The capsule is protects bacteria from phagocytosis and against complement-mediated phagocytosis and bacterial

killing by neutrophil extracellular traps (Garcia and Lopez, 1997, Hyams et al., 2010).

1.4.2.2 The pneumococcal cell wall

The two important structural components of the pneumococcal cell wall are teichoic acid (TA) and peptidoglycan, which found in almost equal proportions. Lipoteichoic acid (LTA) is attached to the cell membrane via a lipid moiety, and both teichoic and lipoteichoic acid contain phosphorylcholine (Fischer and Tomasz, 1985, Fischer et al., 1993). Two choline residues may exist on each carbohydrate repeat, these phosphocholine residues are vital for binding of a family of choline-binding receptors located on human cells and are also essential for the activity of autolysins (Tomasz, 1979). In contrast to capsular polysaccharides, cell wall polysaccharide (PS) can reproduce symptoms of typical pneumococcal diseases such as pneumonia, otitis media and meningitis in animal models by injection with cell wall components (Carlsen et al., 1992).

1.4.2.3 Pneumococcal pili

Pneumococcal pili were observed on the surface of pneumococci in 2006 and found to be significantly involved in pneumococcal virulence, and to mediate bacterial binding to host cells. The presence of pili on the pneumococcal cell surface enabled the bacteria to inhibit phagocytosis, promoted invasion, and stimulated proinflammatory cytokine production (Barocchi et al., 2006). Two types of pilus were found in *S. pneumoniae*. Pilus one is composed of three pilus subunits called RrgA, RrgB, and RrgC (Barocchi et al., 2006, Hilleringmann et al., 2009).

RrgA is located at the tip of the pilus, which make it a major pilus-associated adhesin, while RrgB forms the backbone of the pilus structure, and the RrgC is an addition proteins which positioned at the internal ends of the pili. In the second pneumococcal pilus type, the structural protein is called PitB, which is involved in adherence of pneumococci to epithelial cells (Nelson et al., 2007).

A study by Bagnoli and co-workers revealed that pilus one covers most of pneumococcal surface, while pilus two is found in single copies that extend from the bacterial cell (Bagnoli et al., 2008). Furthermore, several studies results have shown that pilus one was expressed in less than 30% of clinical isolates, while pilus two is expressed in less than 17% of isolates, suggesting that pilus genes are not present in all pneumococcal strains and clinical isolates (Adamou et al., 2001, Basset et al., 2007, Moschioni et al., 2008).

1.4.2.4 Pneumococcal surface proteins (PSPs)

Cell-surface proteins have attracted considerable attention due to their important roles in the pathogenesis of *S. pneumoniae*. There are therefore significant efforts to develop a vaccine using cell-surface protein antigens as they have the potential to stimulate production of opsonic antibodies. PSPs may be divided into three major groups: (i) choline-binding proteins, (ii) lipoproteins, and (iii) LPXTG-containing proteins, in which X denotes any amino acid (Jedrzejas, 2001, Bergmann and Hammerschmidt, 2006).

1.4.2.4.1 Choline-binding proteins (CBPs)

Pneumococcus expresses several, surface-exposed, choline-binding proteins (CBPs) which function as specific adhesins for glycoconjugates on eukaryotic host cells (Rosenow et al., 1997, Gosink et al., 2000). Genomic data demonstrated

that all CBPs share similar C-terminal choline-binding domains, which are responsible for non-covalently binding the phosphorylcholine moiety of the pneumococcal cell wall, while their N-termini are distinct, reflecting different functions of CBPs. In the context of pathogenicity, the CBPs family comprises several proteins which are implicated in pneumococcal adhesion and virulence. For instance, pneumococcal surface protein A (PspA) acts as a protective antigen; Choline binding protein A (CbpA) and Pneumococcal choline-binding protein A (PcpA) act as adhesins; and LytA, B, and C are autolysins. Each group of CBPs are implicated as significant determinants of virulence in *S. pneumoniae* (Rosenow et al., 1997, Gosink et al., 2000, Perez-Dorado et al., 2012).

1.4.2.4.1.1 Pneumococcal surface protein A (PspA)

Pneumococcal surface protein A is a choline-binding protein and the gene encoding this protein is present in all pneumococci (Crain et al., 1990) It seems to be necessary for complete virulence. On the pneumococcal surface, PspA was found to inhibit spontaneous classical complement activation (Weiser et al., 1994, Weiser, 1998) and killing by the bactericidal peptides of lactoferrin (Smillie, 1938, Weiser et al., 1996).

McDaniel reported that inactivation of the PspA in murine infection models resulted in reduction of pneumococcal virulence (Mcdaniel et al., 1987). Several studies have suggested that immunization with PspA can confer protection against pneumococcal infection (Briles et al., 1989, Mcdaniel et al., 1987, Briles et al., 1996, McDaniel et al., 1998, Briles et al., 2000).

1.4.2.4.1.2 Pneumococcal choline-binding protein A (PcpA)

Pneumococcal choline-binding protein A (PcpA) is a surface protein which is expressed in the lungs and blood throughout invasive disease. Selva and

colleagues found that PcpA was expressed in more than 90% of tested pneumococcal isolates (Selva et al., 2012). The protein consist of two domains: the C-terminal choline-binding domain, which is responsible for attachment of PcpA with the bacterial surface; and the N-terminal domain, which contains several leucine-rich repeats (LRR) (Sanchez-Beato et al., 1998) that may play a role in adhesion (Seepersaud et al., 2005).

1.4.2.4.1.3 Choline binding protein A (CbpA)

The Choline binding protein A (CbpA), also referred to pneumococcal surface protein C (PspC) and secretory pneumococcal surface protein A (SpsA)(Hammerschmidt et al., 1997, Iannelli et al., 2002), is a multifunctional, major member of CBPs family. It acts as an adhesin, binding to the polymeric immunoglobulin receptor (Hammerschmidt et al., 1997, Zhang et al., 2000). CbpA has been shown to be important in pneumococcal disease and plays a significant role in protecting the pneumococcus from the host immune system by binding to the complement regulatory protein factor H and thus confers resistance to complement (Dave et al., 2001). Its ability to bind lactoferrin may aid in the colonization of the host mucosae (Hakansson et al., 2001). PspC is known to cross-react with PspA, which may in part be the reason antibodies elicited by PspC are protective against infection with pneumococci (Brooks-Walter et al., 1999, Daniels et al., 2010).

Eleven different CbpA subtypes have been identified to date; they are classified into two subgroups depending on their anchorage in the bacterial cell envelop. The classical CbpA proteins (subtypes 1-6, members of subgroup 1) belong to the choline-binding proteins (Iannelli et al., 2002) and CbpA-like proteins (subtypes 7-11) are placed in subgroup 2. Both subgroups of CbpA proteins contain a leader

peptide and an N-terminal region with a high similarity in sequence and structure, followed by one or two single repeated domains (called R1 and R2), and a proline-rich sequence. Like other members in CBP family, the C-terminal choline-binding domain (CBD) is conserved and enables non-covalent attachment to the cell wall through an interaction with the phosphorylcholine of lipoteichoic and teichoic acids (Hammerschmidt et al., 1997, Iannelli et al., 2002, Luo et al., 2005). CbpA is also known as secretory pneumococcal surface protein A (SpsA) reflecting its function in binding protein including secretory IgA or polymeric immunoglobulin receptor (pIgR), which is responsible for transports of IgA (Hammerschmidt et al., 1997, Zhang et al., 2000, Elm et al., 2004).

CbpA was found to be involved in human nasopharyngeal adherence and colonization as knockout of the *cbpA* resulted in significant attenuation in the ability of the bacteria to colonize the nasopharynx and to bind epithelial cells (Rosenow et al., 1997, Cheng et al., 2000, Dave et al., 2001). Also, several studies demonstrated a role for CbpA in human epithelial cell invasion as it promotes pneumococcal adherence and invasion of these cells via interaction with the secretory component of pIgR (Elm et al., 2004, Hammerschmidt et al., 1997, Zhang et al., 2000). More recently, CbpA was found to bind a receptor on human brain microvascular endothelial cells (HBMECs), called laminin receptor (LamR) to initiate bacterial contact with the blood brain barrier (BBB)(Orihuela et al., 2009).

1.4.2.4.1.4 Pneumococcal hydrolytic enzymes

The family of choline-binding protein also includes the hydrolytic enzymes LytA (autolysin), LytB, LytC, CbpA and CbpE which are implicated in nasopharyngeal colonization and adhesion to host tissues. Among these enzymes, LytA is

considered to be major pneumococcal autolysin which is characterized by binding to the phosphorylcholine moiety of the cell wall via its choline binding domain (Garcia et al., 1986). LytA was also found to contribute to pathogenesis by allowing release of the intracellular toxin pneumolysin (Canvin et al., 1995) and cell wall degradation components that can provoke inflammatory responses of the host.

Two other cell wall hydrolases, LytB and LytC, have recently been described, but their roles in virulence have not been assessed. LytB plays a role in pneumococcal daughter cell separation (Garcia et al., 1999).

1.4.2.4.2 Pneumococcal Lipoprotein

S. pneumoniae is endowed with a variety of lipoproteins that have been demonstrated to contribute to virulence. Pneumococcal surface antigen A (PsaA) is a conserved antigen which is expressed in all *S. pneumoniae* strains and functions to transport manganese. When PsaA was identified, it was thought to be an adhesin due to the similarity of its sequence with putative adhesins from other streptococci (Sampson et al., 1994).

PsaA is a lipoprotein member of an ATP-binding cassette (ABC) transporter complex. The two pneumococcal ABC transporters, PiuA and PiaA have been identified as possible lipoproteins localised to the cytoplasmic membrane of *S. pneumoniae* and responsible on iron uptake. In animal models, PiuA and PiaA were found to be required for full virulence (Brown et al., 2001).

1.4.2.4.3 LPXTG-anchored surface proteins

LPXGT-anchored surface proteins are a group that comprises several proteins that are anchored via G+ LPXTG attachment motif and are pneumococcal virulence

factors (Bergmann and Hammerschmidt, 2006, Kadioglu et al., 2008). The most prominent enzymes in this group are: hyaluronidase, neuraminidase (NanA), and the serine protease PrtA which all characterised by their location on the surface of the pneumococcus.

NanA was found to be important in colonization and development of otitis media in animal models due to its ability to cleave N-acetylneuraminic acid or sialic acid from glycolipids, glycoproteins, lipoproteins and oligosaccharides in the mucosal cell surfaces. Thus, it unmasks potential receptors in the surface of the human epithelial cells and enhances pneumococcal colonization (Tong et al., 2000, Tong et al., 2001). Furthermore, NanA enhances invasion by modifying the function of sialidated proteins of the host which play role in clearance of bacteria (Kadioglu et al., 2008, Mitchell and Mitchell, 2010).

PrtA is another enzyme in group of LPXTG-anchored surface proteins which exhibit proteases activity and is considered to be a pneumococcal virulence factor due to its ability to cleave host proteins such as complement compounds, immunoglobulins and proteins of the extracellular matrix (Bethe et al., 2001, Bergmann and Hammerschmidt, 2006, Frolet et al., 2010).

1.4.2.4.4 Pneumolysin

Pneumolysin (PLY) is a 53 kDa protein belonging to the family of pore-forming toxins and is a key virulence factor which has several roles in the pneumococcal infection. PLY was originally described as a haemolysin and is present in all pneumococci (Nabors et al., 2000). Several *in vivo* studies results have been suggested that PLY have no role in the inflammation associated with meningitis (Shapiro et al., 1991, Ralph et al., 1994). However, this toxin is implicated in deafness associated with pneumococcal meningitis (Rosenow et al., 1997), and in

bacteraemia and pneumonia (Rosenow et al., 1997, Nuorti et al., 2010). It is also involved in activation of complement. Using animal models and a PLY-deficient mutant of *S. pneumoniae*, PLY has been shown to play a significant role in lung cell recruitment and redistribution of T-lymphocytes and B-lymphocytes in and around inflamed bronchioles in response to respiratory tract infections (Mitchell and Mitchell, 2010, Harvey et al., 2014). Lack of PLY also resulted in a significant reduction in the numbers of the bacteria in the nasopharynx, trachea, and lungs in an intranasal infection model (Kadioglu et al., 2002). Braun and co-workers also reported the role of PLY in brain damage during pneumococcal meningitis in the rabbit model of infection (Braun et al., 2002).

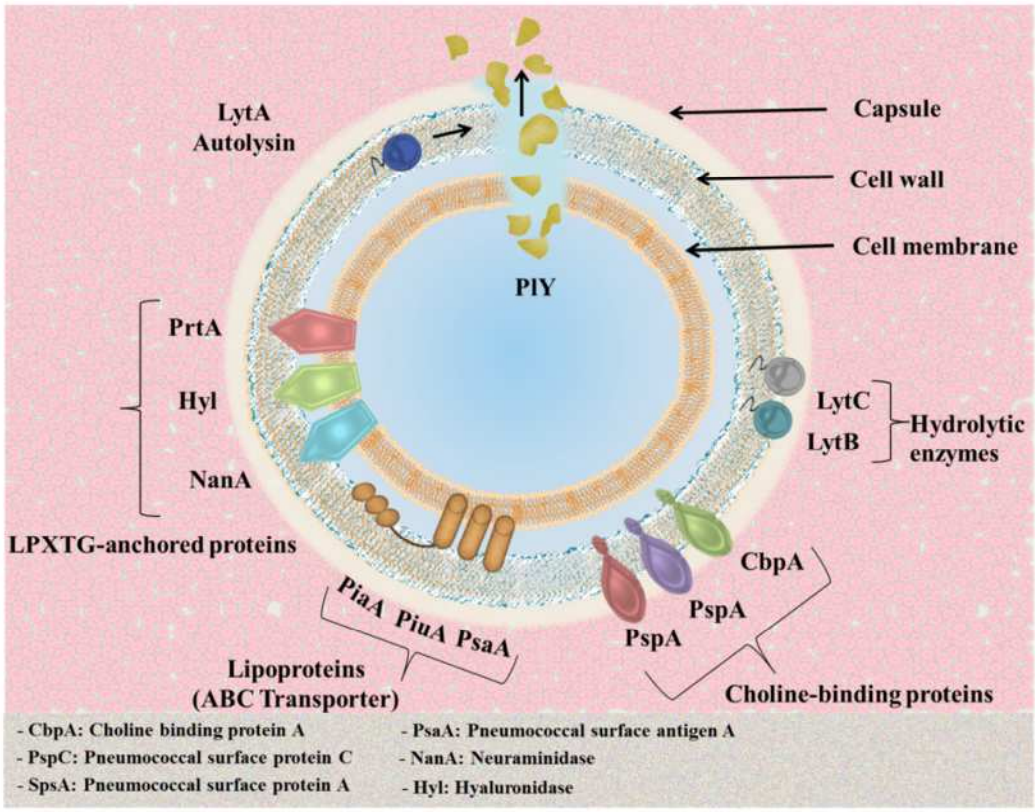


Figure 1-1 Schematic diagram for pneumococcal main virulence factors.

Table 1-2 The role of each virulence factors in the pathogenesis of *S. pneumoniae*.

Virulence factors	Role in pathogenesis	Description
Capsule	Colonization	. Bacterial escape from mucosal secretions to the epithelial cells . Antiphagocytic
Type IV pili	Adhesion/Invasion	. Adherence to respiratory epithelium
PspA	Adhesion/Invasion	. Prevent binding of the complement components on the surface of pneumococci . Inhibit the bactericidal activities of the lactoferrin
PcpA	Adhesion/Invasion	. Expected to play role in cellular Adherence
CbpA	Adhesion/Colonization and Invasion	. Binds to human secretory IgA or polymeric immunoglobulin receptor (pIgR) during the first stage of translocation across the epitheliums . Binds a receptor on HBMECs, called laminin receptor (LamR) to initiate bacterial contact with the blood brain barrier (BBB)
LytA	Colonization/Invasion	. Digests the cell wall, which results in the release of Ply
Hyl	Adhesion/Colonization	. Breaks down hyaluronan-containing extracellular matrix components.
NanA	Colonization	. Reveal receptors for adherence, modifying the function of host clearance glycoproteins
Ply	Colonization	. Cytolytic toxin that also activates Complement

1.4.3 *H. influenzae*

H. influenzae is a pleomorphic, Gram-negative cocco-bacillus that is human-specific pathogen and exists as a commensal within the upper airways. This bacterium is classified into two categories according to the presence of a polysaccharide capsule. Encapsulated strains are characterized by expression of one of six different capsular polysaccharides, designated types a-f, where type b (Hib) is the most virulent. Non-encapsulated strains are characterised by their inability to react with antisera against the recognized *H. influenzae* polysaccharide capsules and are considered nontypable (NTHi). In healthy carriers of *H. influenzae*, the isolated bacterium is often NTHi, and is commonly associated with middle ear infection and sinusitis. By contrast, Hib is often usually responsible for invasive disease and mostly affects infants and children, causing potentially life-threatening conditions such as severe sepsis, epiglottitis and meningitis (Kuklinska and Kilian, 1984, Howard et al., 1988, Murphy et al., 2009, Watt et al., 2009).

1.4.3.1 Structure and virulence factors in *H. influenzae*

H. influenzae is expresses a number of surface structures that act as virulence factors. Some of these factors are present in both encapsulated and non-encapsulated strains, while others are strain-specific. These virulence determinants are related to host defence, transmission, adherence, colonization, and invasion (Read et al., 1991, Wilson et al., 1992, Vitovski et al., 2002, Hallstrom and Riesbeck, 2010). The virulence factors of *H. influenzae* and their role in the pathogenesis are summarised in Figure 1 2 and Table 1 3.

1.4.3.1.1 Polysaccharide capsules of *H. influenzae*

Serotype b (Hib) is the most clinically important group that commonly associated with human disease (Zhou et al., 2007, Agrawal and Murphy, 2011, King, 2012, Su et al., 2014). The presence of the polysaccharide capsule is an important virulence feature of encapsulated *H. influenzae* strains; it appears to be essential for bacteremia and meningitis caused by Hib and allows the bacteria to avoid phagocytosis and opsonisation processes (Ulanova and Tsang, 2009, King and Sharma, 2015).

1.4.3.1.2 Adhesins of *H. influenzae*

Adherence to the epithelial surface is a critical step in the process of host colonization. Several bacterial surface elements influence the course of bacterial adherence with the host cells.

High molecular weight (Hmw) proteins 1 and 2, type IV pilin protein (PilA), outer-membrane protein P5 (OmpP5), Haemophilus protein D (Hpd) and Haemophilus adhesive protein (Hap) are surface proteins involved in the adherence of non-typeable *H. influenzae* (Vuong et al., 2013).

1.4.3.1.2.1 Types of Pili in *H. influenzae*

Some isolates of type b and nontypable *H. influenzae* (NTHi) are capable of expressing adhesive pili. Two types of pili have been recognized in *H. influenzae* strains type IV pili (Tfp) and hemagglutinating pili. The former type are primarily associated with NTHi strains and likely contributes in adherence of the bacteria to human respiratory epithelial cells (Guerina et al., 1985, Clemans et al., 2001, Bardy et al., 2003). Hemagglutinating pili are mainly found in Hib strains but are also found in approximately 15% of NTHi strains, mediate the adherence of the bacteria to respiratory epithelial cells, hence it plays a role in colonization of the

upper respiratory tract by *H. influenzae*. Due to the differences in their antigenic structures especially in NTHi strains, Hemagglutinating pili may not be a potential candidate for vaccine development (Gilsdorf et al., 1997, Rao et al., 1999, St Geme, 2000).

1.4.3.1.2.2 HMW1 and HMW2 adhesins in *H. influenzae*

Two proteins, HMW1 and HMW2 with high-molecular weight (125 kDa for HMW1 and 120 kDa for HMW2) are adhesins present in *H. influenzae* (Grass and St Geme, 2000) which play crucial role in adherence to human epithelial cells. These two proteins are members of the autotransporter family of proteins and are expressed abundantly by almost 80% of NTHi strains (Giufre et al., 2006, Yeo et al., 2007) and are commonly associated with strains that cause otitis media and nasopharyngeal infection; these proteins are expressed rarely in encapsulated strains (Remaut and Waksman, 2004). In NTHi strains which lack HMW1 and HMW2, *H. influenzae* was found to express either Hia protein or heamagglutinating adhesins. Despite the presence of some conserved homologous regions in HMW1 and HMW2, which could be utilized for the development of vaccines against non-typeable NTHi. HMW proteins were found to be mainly heterologous among different stains (Remaut and Waksman, 2004, Yeo et al., 2007).

1.4.3.1.2.3 Hia and Hsf adhesins in *H. influenzae*

Adherence of *H. influenzae* that lack HMW1/HMW2 adhesins to human epithelial cells is mediate by two adhesins, *H. influenzae* adhesin (Hia) and Haemophilus surface fibrils (Hsf) which are expressed by NTHi and Hib strains of *H. influenzae*, respectively. Hia is a trimeric autotransporter protein, expressed in approximately 25% of the clinical NTHi isolates (Rodriguez et al., 2003) and

enhances bacterial adherence to respiratory epithelial cells through two homologous binding domains, named HiaBD1 and HiaBD2 (Yeo et al., 2004). By using Hia mutant NTHi strains, Barenkamp & St Geme observed a significant reduction in bacterial attachment to various human epithelial cell types (Barenkamp and St Geme, 1996). Hsf is structurally similar to Hia, however, it is expressed universally by Hib strains and exists on the cell surface as short fiber-like structures that are considered to be one of the main virulence factors of Hib strains and plays an essential role in respiratory tract colonization (St Geme et al., 1996, Rodriguez et al., 2003).

Interestingly, Hia and Hsf are conserved at their N-terminal and C-terminal ends and there is 72% amino acid sequence identity and 81% similarity between the two proteins, Recent evidence indicates that these two proteins confer identical adhesive specificities by binding to the same receptors, suggesting that *hia* and *hsf* are alleles of the same locus (St Geme et al., 1996).

1.4.3.1.2.4 The Hap adhesin of *H. influenzae*

Studies of mutant strains lacking HMW1/2 or Hia led to the identification of an additional adhesin, Haemophilus adhesion and penetration protein (Hap) which also belongs to the autotransporter family and shares homology with IgA protease (St Geme et al., 1994, St Geme, 2002). The presence of the hap gene in all encapsulated *H. influenzae* type suggests that Hap plays a critical role in pathogenesis and it is known to contribute to bacterial binding to human epithelial cells (Rodriguez et al., 2003) as well as extracellular matrix proteins such as fibronectin, laminin, and collagen IV (Hendrixson and St Geme, 1998, Fink et al., 2002).

1.4.3.1.3 Opacity-associated Protein A (OapA) in *H. influenzae*

OapA is a surface-associated protein found in all *H. influenzae* strains and is responsible for opaque colony morphology of *H. influenzae* on a transparent growth medium. Several studies revealed that OapA is required for efficient nasopharyngeal colonization in the infant rat model (Weiser et al., 1995, Prasadarao et al., 1999a). Prasadarao et al. showed that mutation of OapA in Chang epithelial cells resulted in a significant decrease in adherence compared with the isogenic parental strains (Prasadarao et al., 1999a). In the context of vaccine design, OapA was found to be a potentially useful antigen for vaccine design (Fu et al., 2013).

1.4.3.1.4 Immunoglobulin protease (IgA) in *H. influenzae*

IgA is a predominant immunoglobulin found in mucosal tissues and it is known to be involved in host defence by inactivation of bacterial toxins and by inhibiting the microbial adherence and invasion (Bakema and van Egmond, 2011, Pan et al., 2014). Indeed, the IgA1 subclass in the respiratory tract comprises 90% of all IgA present (Brandtzaeg, 1992). Therefore, to overcome the protective effects of IgA, more than 97% of NTHi strains that colonise the human respiratory mucosa, possess IgA protease that cleaves the hinge region of both the serum and the secreted forms of IgA1. Hence, it inactivates human immunoglobulin by splitting the antigen-binding Fab domains from the Fc portion of the antibody (Kilian et al., 1996, Fernaays et al., 2006). Recently, it has been found that some strains of NTHi possess a second IgA protease called IgAB which is a potential virulence factor in nontypeable *H. influenzae* (Fernaays et al., 2006).

1.4.3.1.5 Lipopolysaccharide in *H. influenzae*

Like several respiratory pathogens, *H. influenzae* lipopolysaccharide (also called lipooligosaccharide or LOS) lacks the long polysaccharide side chains that are characteristic of many other Gram-negative bacteria and instead has just one oligosaccharide unit (Kilian et al., 1996, Fernaays et al., 2006). *H. influenzae*, LOS contains a conserved lipid A core region which confers a net negative charge to the bacterial surface. The lipid A is connected to highly variable oligosaccharides which consist of various sugars, including glucose, galactose, N-acetylglucosamine, and sialic acid (Wong et al., 2011).

In NTHi, LOS mediates endotoxin activities, attracts opsonization and phagocytosis, also it constitutes a first line of defense against the innate immune response which is encountered in the blood and at mucosal surfaces (Andersson et al., 1994, Narkio-Makela et al., 2001, Marc et al., 2004, Van Zele et al., 2009). The structure and antigenic similarity of *H. influenzae* LOS with the human glycosphingolipids confers protection to these bacteria. NTHi can attach to host cells by binding to platelet-activating factor receptor (PAFr) via LOS glycoforms that contain phosphorylcholine (ChoP)(Fink et al., 2003, Forsgren et al., 2008). *H. influenzae* LOS has also been investigated as a potential antigen for NTHi vaccine development (Choi et al., 2014).

1.4.3.1.6 Outer membrane proteins (OMPs) in *H. influenzae*

The major outer membrane proteins of *H. influenzae* are P1-P6, of which P2 is the most abundant (Poolman et al., 2000). Two OMPs, OmpP2 and OmpP5, are porin proteins and are characterised by their capacity to bind mucin. It is believed that these two proteins facilitate the establishment of infection caused by *H. influenzae* (Reddy et al., 1996).

OmpP2, is highly immunogenic, but also highly antigenically variable, which allows bacteria to evade protective antibodies (Forbes et al., 1992, Hansen et al., 1988). OmpP2 was found to be expressed by all *H. influenzae* strains and its molecular weight ranges from *ca* 38 to *ca* 40 kDa. OmpP2 is usually present as trimer with porin activity (Coulton et al., 1992). It has 16 predicted transmembrane regions which are highly conserved among strains, and 8 surface-exposed loops that exhibit a considerable heterogeneity (Arbing et al., 2001, Galdiero et al., 2006). Antigenic drift *OmpP*2 enables the bacteria to escape clearance by potentially protective antibodies, thus it is involved in the development of chronic infection (Smith-Vaughan et al., 1997). Interestingly, in animal models and human infected by NTHi, the antibodies produced against OmpP2 protein have bactericidal activity, and were able to confer protection against *H. influenzae* (Winn et al., 2006).

The P6 protein is a 16.6-kDa peptidoglycan-associated lipoprotein that constitutes 1-5% of all OMPs, and it is expressed in both typeable and nontypeable *H. influenzae* strains. In animal models of infection it has been shown to elicit bactericidal antibodies (Winn et al., 2006). In contrast to OmpP2, OmpP6 is a conserved protein; the amino acid analysis of the OmpP6 showed a high homology (97%) among NTHi strains (Winn et al., 2006) making OmpP6 a promising candidate vaccine against NHTi strains.

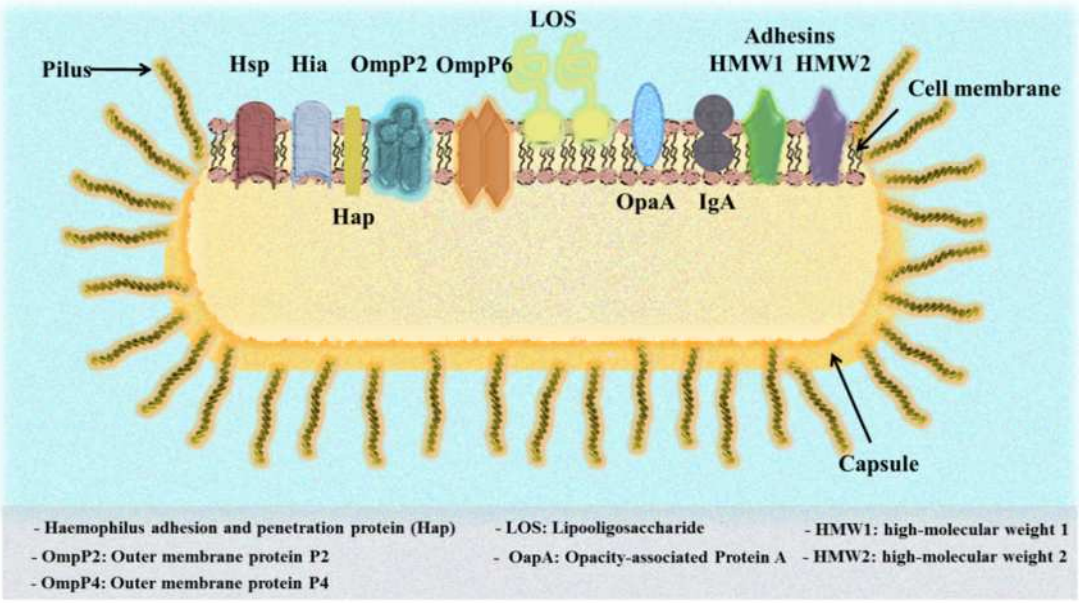


Figure 1-2 Schematic diagram for the main virulence factors in *H. influenzae*.

Table 1-3 The role of each virulence factors in the pathogenesis of *H. influenzae*.

Virulence factors	Role in pathogenesis	Description
Capsule	Colonization	. Resist phagocytosis and . Opsonisation Antiadherent
Pili **Type IV pili/ Hemagglutinating pili**	Adhesion	. Mediates haemagglutination and adherence to human respiratory epithelial cells
HMW1/HMW2	Adhesion	. Mediate bacterial adherence to human epithelial cells
Hia and Hsf Adhesins	Adhesion	. Mediate bacterial adherence to human epithelial cells in strains lack HMW1/HMW2
Hap Adhesin	Adhesion	. Adhesion to human epithelial cells and extra cellular matrix proteins such as fibronectin, laminin, and collagen IV
OapA	Adhesion/ Colonization	. Mediates adherence to epithelial cells in culture and is required for pharyngeal colonization.
IgA Protease	Adhesion/Invasion	. Inactivates the human immunoglubolin A1
LPS	Endotoxin	. Poses endotoxin activities . Attracts opsonization and phagocytosisgen
OmpP2	Colonization	. Enable bacteria to escape clearance by protective antibodies . Binds to LamR on HBMECs to initiate bacterial contact with the blood brain barrier (BBB)s
OmpP6	Colonization	. Induce production of bactericidal antibodies . Induce P6-specific humoral and cell mediated immunitys

1.4.4 *N. meningitidis*

N. meningitidis, or the meningococcus, is a strict human commensal, aerobic or facultatively anaerobic, Gram-negative diplococcus, commonly occurring as pairs of cells with adjacent flattened sides. This bacterium belongs to the family *Neisseriaceae*. Of the 15 human originated Neisseria species, only two are frequently pathogenic: *N. gonorrhoeae* and *N. meningitidis*. The remaining Neisseria species, such as *N. lactamica* and *N. polysaccharea*, are commensal bacteria that may rarely cause disease in immunocompromized patients (Catlin, 1973, Lee et al., 2010).

N. meningitidis is relatively fastidious and grows on blood agar, chocolate agar, modified New York City medium, and Mueller Hinton agar. *N. meningitidis* grows optimally at 35-37ºC at pH 7.0-7.4 in a humid atmosphere containing 5-10% CO_2 (Ala'Aldeen and Turner, 2006).

1.4.5 Structure and virulence factors in *N. meningitidis*

The meningococcus can evade host defense mechanisms and establish infection by expressing numerous surface components and secretion of various molecules. The main virulence factors of *N. meningitidis* and their role in the pathogenesis are summarised in Figure 1-4 and Table 1-4.

1.4.5.1 Meningococcal Polysaccharide

A polysaccharide capsule covers the outer membrane of meningococci (Prather et al., 2004) and is considered to be a major virulence factor that plays vital roles in invasive meningococcal disease (Schneider et al., 2007) and in the organism's survival during invasion of the bloodstream or CSF by its antiphagocytic, antibactericidal and antiadherent properties (Manchanda et al., 2006). The

antigenic and chemical differences among pathogenic meningococcal polysaccharide structures are used to classify meningococci to thirteen serogroups: A, B, C, D, E, H, I, K, L, W, X, Y and Z (Morley and Pollard, 2001). Serogroups: A, B, C, W and Y were found to be responsible for more than 90% of invasive disease worldwide (Tzeng et al., 2005, Lo Passo et al., 2007). However, around 50% of the strains isolated from carriers were unencapsulated and therefore non-serogroupable (Claus et al., 2002).

Several separate clinical and epidemiologic studies support a role for capsule in meningococcal pathogenesis. In studies conducted by Schwartz et al., 1989 and Stephens et al., 2007, they revealed that the majority of clinical isolates of *N. meningitidis* from patients with invasive disease were encapsulated strains belonging to serogroups A, B, C, Y and W-135 (Schwartz et al., 1989, Stephens, 2007). While Ala'Aldeen et al.; and Cartwright et al. showed that the majority of *N. meningitidis* isolates recovered from the nasopharynx lack a polysaccharide capsule (Cartwright et al., 1987, Ala'Aldeen et al., 2000). Indeed, meningococcal capsular polysaccharides for most invasive groups (A, B, C, Y and W) are composed of sialic acid derivatives (N-acetylneuraminic acid, NANA), with the exception of serogroup A, which, is formed of N-acetyl mannosamine residues (Liu et al., 1971). However the composition of the sialic acid in capsules differs among serogroups (Ala'Aldeen and Turner, 2006).

Sialylation of meningococcal capsules is thought to protect the bacteria by reducing the ability of the human immune system to recognize these encapsulated bacteria (Hill et al., 2010). Interestingly, the (α2→8)-linked polysialic acid capsule of serogroup B meningococci is structurally mimics human neural cell adhesion molecule, N-CAM. This similarity, which constitutes a form of

molecular mimicry, is likely to contribute to the adaptation and protection of the organism within the host; this property explains why meningococcal serogroup B capsule is poorly immunogenic: a property which has been a major obstacle to vaccine development against serogroup B strains (Diaz Romero and Outschoorn, 1994, Stephens, 2009).

1.4.5.2 Meningococcal type IV pili (Tfp)

Meningococcal pili are the first adhesin to contact human cells, particularly in the presence of the bacterial capsule. In *N. meningitidis*, pili have been shown to contribute significantly to meningococcal pathogenesis as they are required for the interaction of bacteria with both human epithelial and endothelial cells leading to the bloodstream invasion from the nasopharynx, and the crossing the blood-brain barrier (BBB) (Taha et al., 1998, Nassif et al., 1999, Mairey et al., 2006). In addition to adhesion, several other functions of meningococcal pili have been reported. For example, their association with the ability of bacteria to mediate transformation (horizontal genetic exchange)(Jodar et al., 2002), modulate target-cell specificity (Jonson et al., 2005); biofilm formation (Lappann et al., 2006) and twitching motility (Mattick, 2002).

Pili are adhesive hair-like projections (also known as fimbriae), that extend beyond the capsule and are assembled from protein subunits called pilins (Strom and Lory, 1993, Pizarro-Cerda and Cossart, 2006). Based on a systematic genetic analysis, 15 proteins, designated pilC-pilX and ComP, have been identified to be important in pilus biogenesis, assembly and disassembly of pili (Virji, 2009, Brissac et al., 2012). Meningococcal pili belong to the Type IV family of pili, and a single meningococcus has the ability to produce several antigenically different pili subunits (Nassif et al., 1997, Nassif et al., 1999). In addition to *N.*

meningitidis, type IV pili were also identified in other Gram-negative bacterial pathogens including *Moraxella bovis*, *Pseudomonas aeruginosa*, and *Vibrio cholera* (Virji et al., 1989, Eriksson et al., 2015).

Type IV pili are homopolymers composed of thousands copies of the single-chain pilin protein encoded by the *pilE* gene (Craig et al., 2006) and an adhesive subunit at the tip encoded by the *pilC* gene (Pizarro-Cerda and Cossart, 2006). To build the Neisserial pilus fibre, several pilin subunits (~15-17) are assembled and arranged in a helical configuration (Craig et al., 2006).

In *N. meningitidis*, PilE, PilC, PilF and PilQ are considered to be key protein subunits as they are necessity for biogenesis and maturation of pilus as well as bacterial adherence (Carbonnelle et al., 2005).

The PilE pilin, is the major protein subunit that constitutes the pilus shaft and is initially localised to the inner membrane (IM), it also anchored to the membrane by a hydrophobic N-terminal sequence. PilE is essential for pilus biogenesis and indirectly for host adhesion by promoting functional display of the PilC adhesin in the context of the pilus fiber (Winther-Larsen et al., 2001).

Meningococcal pili also contain another important pilus protein termed PilC, which is located at the extreme tip of the pilus (Rudel et al., 1995) and appears to be essential for bacterial adherence to both epithelial and endothelial cells (Rudel et al., 1995, Kirchner et al., 2005). Adhesion of meningococcal cells to endothelial cells of the blood-CSF barrier in the clinical samples has been shown to be related to expression of PilC (Pron et al., 1997). In addition to adhesion function, PilC was also found to play a crucial role in pilus expression on the bacterial surface and transformation competence (Nassif et al., 1994). Meningococci *pilC* genes contain a poly-G tract within the coding sequence causing phase variation. There

are two highly homologous PilC variants in meningococci, designated PilC1 and PilC2 (Jonsson et al., 1991). It was demonstrated by Nassif et al. (1994) that mutation of PilC1 in *N. meningitidis* resulted in a non-adhesive, piliated, transformation-competent strain (Nassif et al., 1994) suggesting that although PilC1 and PilC2 are necessary for pilus biogenesis, only PilC1 is mediates adherence to human cells (Nassif et al., 1994, Ryll et al., 1997, Rahman et al., 1997). These authors suggested that the interaction was mediated via the membrane cofactor protein MCP/CD46 (Kallstrom et al., 1997). More recently, Morand and co-workers demonstrated that meningococcal PilC2 also has ability to mediate adhesion; however pili containing these two variants of PilC had differing cell specificities and mentioned diverse effects on the host cells (Morand et al., 2009).

Another important component of the pilus of both *N. meningitidis* and *N. gonorrhoea*, is the PilT protein. PilT protein is a hexameric ATPase located in the inner membrane; it mediates pilus retraction, which is required for twitching motility that enables the bacteria to move across the host cell surface prior to intimate attachment (Pujol et al., 1999, Merz and So, 2000). PilT is implicated in force-induced elongation allowing bacteria to control interactions with surfaces by modulating the tension on their pili (Maier et al., 2004). Twitching motility mediated by PilT also appears to be essential for invasion by pathogenic meningococcus whereby the pilus rapidly extends and retracts to move the bacterium (Skerker and Berg, 2001), and is generally associated with bacteria expressing type IV pili (Whitchurch et al., 1991). It was observed by Wolfgang et al., 1998 that twitching motility of *N. meningitidis* was abolished by mutation *pilT*; this mutant also lacks competence for sequence-specific uptake of DNA

facilitating natural transformation (Wolfgang et al., 1998). Subsequent to the primary attachment of bacteria to host tissue, PilT causes the pilus to retract, bringing the bacterium into closer contact with the cell surface and thus facilitating the progression from localised to diffuse adhesion (Pujol et al., 1999) suggesting a role for meningococcal PilT in microcolony formation (Merz et al., 1999).

PilQ is the other significant meningococcal pilus protein involved in type IV pilus biogenesis; other pilus-related functions, such as adhesion, DNA uptake, and twitching motility also require the expression of PilQ (Bille et al., 2005, Frye et al., 2006). PilQ is a member of a large family of proteins called secretins. Meningococcal secretin PilQ forms a pore in the outer membrane allowing the extruded pilus to emerge (Collins et al., 2001, Collins et al., 2005). PilQ is also required for phage extrusion of a filamentous phage that is encoded in the genome of many meningococci, especially strains belonging to hypervirulent lineages (Bille et al., 2005). PilQ has also been shown to be an adhesin with specificity for the human laminin receptor (Orihuela et al., 2009).

PilG, which is present in most meningococcal strains is required for piliation, and contributes to protein export and competence (Tonjum et al., 1995). The lipoprotein PilP was also shown to be involved in pilus assembly through its interaction with the cap region of the PilQ complex (Carbonnelle et al., 2006, Balasingham et al., 2007). Another meningococcal pilus component, a PilV, a PilE-like pilin was also shown to be essential for pilus biogenesis, and another pilin-like protein, PilX is co-localized with the pilus fiber; it is not necessary for pilus assembly but appears to be important for aggregate formation and adhesion to host cells (Helaine et al., 2007).

Moreover, several studies demonstrated the role of PilP in assembly of meningococcal PilQ outer ring (Jain et al., 2011, Berry et al., 2012). PilG and PilT are not required for pilin biogenesis, however, they are necessary for pilus retraction. PilF is a cytoplasmic ATPase which has been shown to contribute to in pilus elongation (Tonjum et al., 1995, Wolfgang et al., 2000).

During the biogenesis of Tfp and after synthesising of the pilin as a precursor (prepilin) in the cytoplasm, the individual pilin subunits are inserted into the inner membrane by the Sec machinery (Peek and Taylor, 1992). For pilus assembly, each of the individual pilin subunits (PilE) is assembled at the inner membrane-periplasm interface after inserting the PilE into the cytoplasmic membrane by PilF ATPase and PilG (Carbonnelle et al., 2006). Indeed, PilE has a hydrophobic N-terminal, which is attributed in the polymerization of the pilin subunit to form Tfp fibre. In this stage, the inner membrane prepilin peptidase (PilD) play a role in cleaving of the N-terminal leader sequence (first seven amino acids), and methylate it at its N-terminal Phenylalanine in order to generate the mature pilin (Strom and Lory, 1993). Then, the processed PilE assembled into a helical filament from a platform complex comprising PilM, PilN, PilO and PilP at the inner membrane (Carbonnelle et al., 2006). After translocation across the inner membrane and assembling of mature pilin, the Tfp subsequently extruded across the outer membrane through a secretin pore, PilQ by the cytoplasmic ATPase PilF (Bayan et al., 2006, Carbonnelle et al., 2006). Two inner membrane ATPases, PilF and PilT, were found to promote pilus extension (assembly) and pilus retraction (disassembly), respectively (Wolfgang et al., 1998). In addition to mentioned proteins, the other pili proteins, PilW, PilX, PilV and ComP were also contributed in pilus biogenesis after pilus assembly, for instance, PilW was found

to play a role in fibre stability and function after pilus assembly (Deghmane et al., 2002, Carbonnelle et al., 2006).

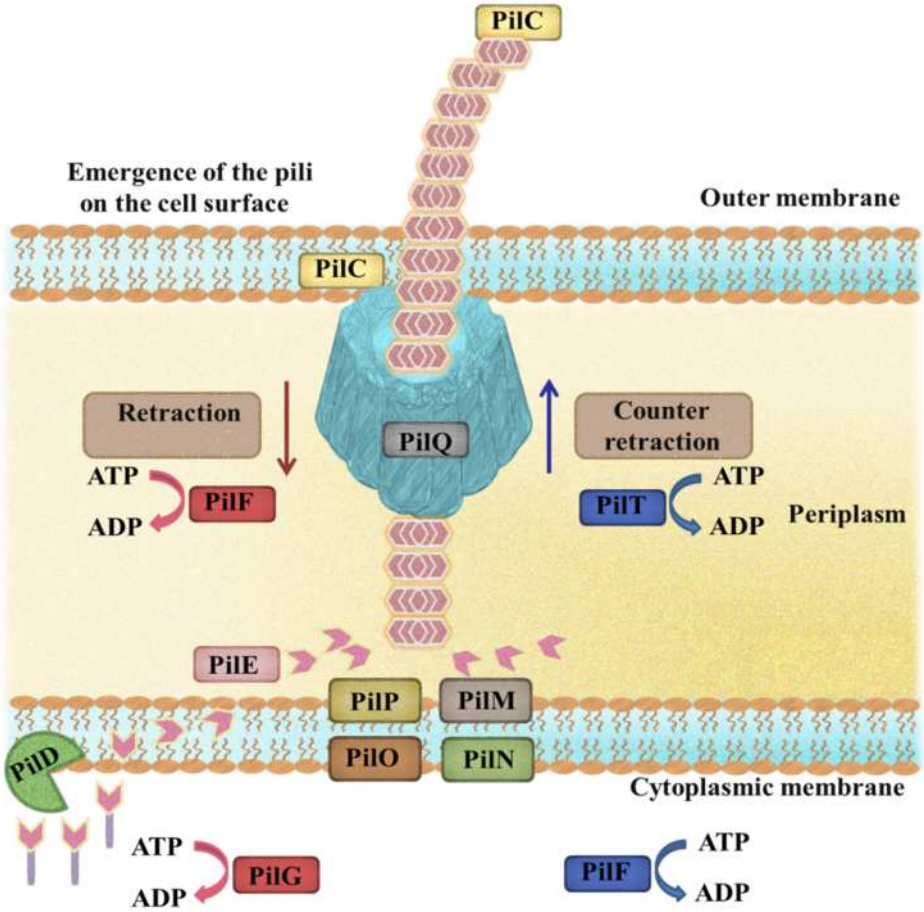


Figure 1-3 Various components of meningococcal TFP pili involved in pilus assembly.

In the cytoplasmic membrane, the pilin subunits called PilE is inserted by help PilF ATPase and PilG and assembled. For Pilus maturation, the inner membrane prepilin peptidase PilD cleaves the N-terminal leader sequence and methylates it at its N-terminal Phenylalanine. Then, processed PilE as well as PilM, PilN, PilO and PilP form a platform complex. Subsequently, the mature pilus extruded across the outer membrane through PilQ with help of cytoplasmic ATPase PilF. The pilus extension and retraction mediate by two inner membrane ATPases, PilF and PilT. While, the other pili proteins, PilW, PilX, PilV and ComP were also participated in pilus biogenesis after pilus assembly. Adapted and modified from (Carbonnelle et al., 2009).

1.4.5.3 Meningococcal porin proteins

Neisseria spp., including the human pathogens *N. meningitidis* and *N. gonorrhoeae* possess porins which are β-barrel proteins that assemble into trimer forming pores in the bacterial outer membrane and comprise up to 60% of the proteins present in the outer membrane (Tommassen et al., 1990, van der Ley et al., 1991). Unlike other Neisseria spp. which express a single porin, *N. meningitidis* possess both class 1 (PorA, 45 kDa), and either class 2 or class 3 (PorB, 33 kDa)(van der Ley et al., 1991). Neisserial porins function as pores and are essential for bacterial survival as they modulate the exchange of ions between the bacteria and the surrounding environment. Having both PorA and PorB proteins in the OMPs aids selective passage of cations and anions across the cell membrane respectively (Tommassen et al., 1990). Topology models of Neisserial porins predict PorA to be composed of 16 transmembrane segments that form a β-sheet structure with eight extramembranous surface-exposed loops designated I–VIII; VR1 (loop I) and VR2 (loop IV) contain the most amino acid variation among different meningococcal strains (van der Ley et al., 1991). It has been found that the amino acid sequences of these variable regions are stable within an isolate but vary between unrelated isolates, and thus they have been used for differentiation of meningococci in a subtyping system. For this reason, PorA is an important epidemiological marker in meningococcal disease and has been used as an immunogenic antigen in several nonpolysaccharide-based serogroup B meningococcal vaccine trials (Claassen et al., 1996, Jessouroun et al., 2004, Sexton et al., 2004, Devoy et al., 2005).

1.4.5.4 Meningococcal Opacity proteins

The class 5 major outer membrane proteins of in Neisseria Spp. are known as the opacity proteins which include Opacity-associated (Opa) and Opc proteins. Opa and Opc proteins are so named due to their association with opaque agar-grown colony phenotype (Virji, 2009, Hill et al., 2010). Although these two proteins belong to the same class of OMPs and have similar molecular weights (27-31 kDa), they share only limited sequence homology. Opa is found in both meningococci and gonococci, while Opc is present only in meningococci (Aho et al., 1991, Olyhoek et al., 1991, Zhu et al., 1999). Both, Opa and Opc play key roles in adhesion and invasion of meningococci to host epithelial and endothelial cells, but only in the absence of a polysaccharide capsule (Virji et al., 1992, Virji et al., 1995, de Jonge et al., 2003, Hauck and Meyer, 2003).

Meningococci express 3-4 Opa proteins (Aho et al., 1991), that has eight transmembrane domains arranged in β-parallel strands and four extracellular loops. Three of these loops are highly variable within and among different *N. meningitidis* strains (Malorny et al., 1998, Prince et al., 2002).

Meningococcal Opa proteins were found to bind heparan sulphate proteoglycans (HSPGs)(Virji et al., 1995, Unkmeir et al., 2002, Sadarangani et al., 2011) and recognize members of the carcinoembryonic antigen-related cell-adhesion molecule (CEACAM) family that harbor the CD66 epitope (Chen and Gotschlich, 1996, Virji et al., 1996).

Opc is one of the most important meningococcal surface adhesions and is encoded by a single *opcA* gene, which is phase variable through transcriptional regulation (Chen and Gotschlich, 1996, Virji et al., 1996). Structurally, Opc is a β barrel-containing- protein that consists of 10 transmembrane domains producing

five surface-exposed loops (Zhu et al., 1999). Neisserial Opc bind fibronectin, vitronectin, and endothelial cell integrins (Sa et al., 2010). Although Opa and Opc proteins have capability to elicit a strong bactericidal response, their high phase and antigenic variabilities limited their utility in vaccine development (Virji et al., 1995, Unkmeir et al., 2002).

1.4.5.5 Meningococcal lipooligosaccharide (LOS)

Meningococcal lipopolysaccharide (LPS) which also called lipooligosaccharide (LOS) as it lacks repeating polysaccharide O-antigens, is the major constituent of the outer leaflet of the outer membrane of pathogenic Neisseria (Kahler and Stephens, 1998). Due to having immunomodulatory activities, like LOS is also referred to as consider endotoxin; it is a major contributor to meningococcal septic shock, multiple organ failure and death (Brandtzaeg et al., 1989).

Meningococcal LOS molecule consists of an oligosaccharide (OS) portion attached to the toxic lipid A moiety. An oligosaccharide (OS) portion comprises the conserved inner core region, which is composed of diheptose sugars (HepI and HepII), Lipid A moiety composed of two glucosamine residues connected to fatty acids. Each carbohydrate unit normally contains a phosphate group. The heterogeneous mixture of variable number of sugars which present in meningococcal LOS has been used as the basis for meningococcal classification into 13 immunotypes, designated L1–L13 (Mandrell and Zollinger, 1977, Verheul et al., 1993, Scholten et al., 1994, Raetz et al., 2009). Both, inner and outer core of Meningococcal LOS lipid A and the inner core are embedded in the cell membrane; while the variable oligosaccharide chains are surface exposed. Meningococcal LOS is one of the important virulence factors in the pathogenesis of *N. meningitidis* for its different biological activities. Due to the presence of

sialic acid residues which act as molecular mimics for human antigens, Meningococcal LOS confers defensive properties on the meningococcus that protects the bacteria by facilitating its evasion from the immune system, such as antibody and complement. Furthermore, meningococcal LOS is subject to phase variation, enabling the organism to switch between immunotypes (Mandrell et al., 1991, Virji, 2009, Berrington et al., 2002).

Besides the above roles that are shared with the capsule, the lipid A component of LOS has been shown responsible for initiating the intense inflammatory response observed during sepsis (Brandtzaeg et al., 2001). Additionally, LOS exert high levels of toxicity toward endothelial cells *in vitro* (Virji, 2009). Interestingly, the presence of pili has been shown to synergistically enhance this toxicity, indicating the cooperation between the pili and LOS in signalling to endothelial cells (Dunn et al., 1995). Van der Ley and Steeghs have also demonstrated that Meningococcal LOS can facilitate bacterial adhesion and invasion independently as mutation of LOS abolished the bacterial adhesion and invasion (van der Ley and Steeghs, 2003).

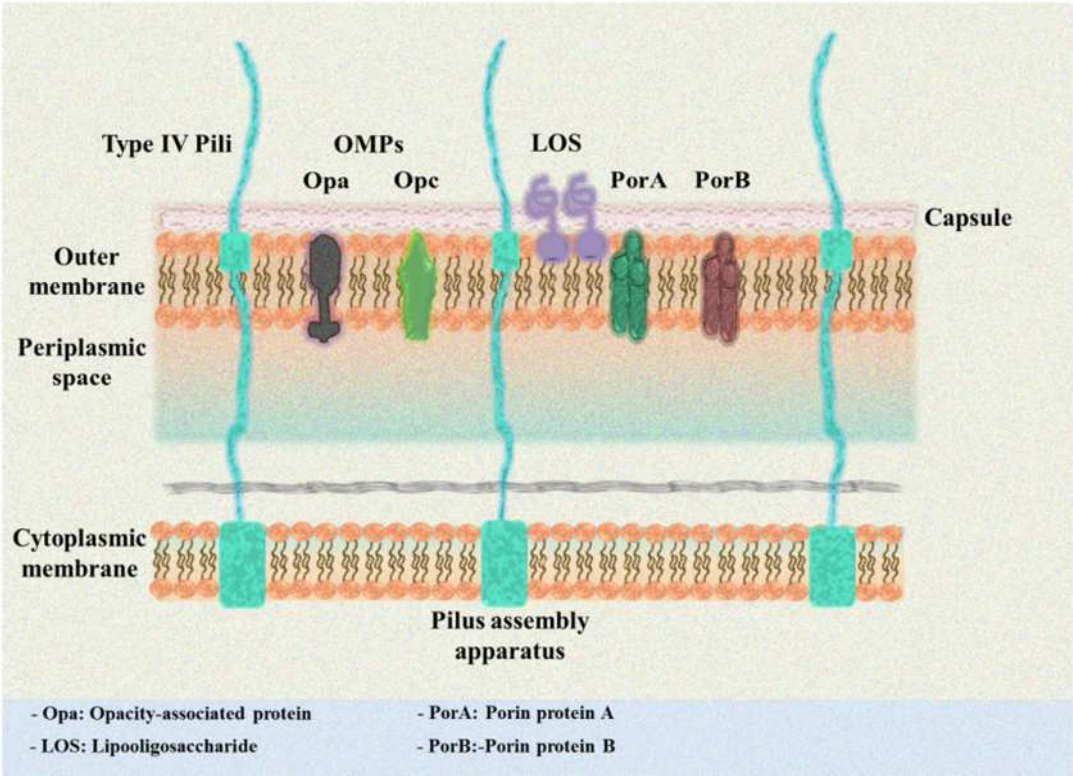


Figure 1-1 Schematic diagram for meningococcal main virulence factors.

Table 1-4 The role of each virulence factors in the pathogenesis of *N. meningitidis*.

Virulence factors	Role in pathogenesis	Description
Capsule	Colonization	. Bacterial survival in the bloodstream . Bacterial survival in the CSF . Antiphagocytic, Antibactericidal and Antiadherent
Type IV pili	Adhesion/Invasion	. First contact with the human epithelial and endothelial cells
PorA **PorB**	Porin/Adhesion	. Bacterial survival by modulating the exchange of ions between the bacteria and the surrounding environment. . Help in selective passage of cation and anion across the cell membrane . PorA binds human endothelial cells
Opa	Adhesion/Invasion	. Binds human epithelial/endothelial cells and fibronectins
Opc	Adhesion/Invasion	. Binds human epithelial/endothelial cells, fibronectins and vitronectin
LOS	Endotoxin	. Meningococcal septic shock, multiple organ failure and death

1.5 Bacterial Meningitis

Despite advances in antimicrobial therapy, bacterial meningitis remains a significant cause of mortality and morbidity. The high prevalence of this condition is in part due to an incomplete understanding of its pathogenesis (van de Beek, 2012, Tan et al., 2015). Indeed, bacterial meningitis usually develops through several stages of bacteria-host interactions; a key factor for all these steps is the involvement of numerous bacterial and human receptors to mediate specific interactions that result in meningitis. Significant progress has been made in understanding the molecular interaction between the human cells and meningeal pathogens. (Ribet and Cossart, 2015). Prior to initiation of meningitis, bacteria initially colonize mucosal cells, usually of the upper respiratory or gastrointestinal tract (Stephens, 1999), following this they may invade the intravascular space. At this stage, bacteria may survive and multiply in the bloodstream resulting in

bacteraemia. From the blood stream they may further transverse the BBB (Mereghetti et al., 2008, Nakamura et al., 2011). Multiplication of bacteria in the subarachnoid space elicits an inflammatory responses resulting in pathophysiological alterations such as pleocytosis and BBB disruption (Pfister et al., 1992).

In the context of CNS infections, the brain is usually protected from the infection by an intact blood-brain barrier (BBB), which is composed mainly of specialized brain microvascular endothelial cells (BMVEC). This barrier maintains the neural microenvironment in the central nervous system (CNS) by regulating the passage of molecules and ions into and out of the brain. Despite the highly restrictive nature of the BBB, certain bacterial pathogens are able to gain access into the CNS by crossing the BBB to invade CNS resulting in serious disease including bacterial meningitis (Zhang and Tuomanen, 1999, Hawkins and Davis, 2005, Kim, 2008, Cardoso et al., 2010). Globally, *Neisseria meningitidis* (meningococcus), *Haemophilus influenzae* type B (Hib) and *Streptococcus pneumoniae* are the most common causes of meningitis in infants and adults, while *Escherichia coli* K1 and Group B streptococci are most commonly isolated in newborns (Eskola et al., 1987, Peltola, 2000, Maruvada et al., 2008, Thigpen et al., 2011).

1.6 Mechanisms of bacterial entry into BBB

Meningitis-causing bacteria have the ability to enter the brain via several complex mechanisms that enable them to cross and disrupt the BBB. Although the exact mechanisms of entry are not clear, several studies suggest that the pathogens may disrupt the BBB and traverse into the CNS via transcellular penetration,

paracellular entry, and/or by means of so-called Trojan Horse carriage across the BBB within infected immune cells (Kim, 2008).

In transcellular passage, the pathogen crosses the microvascular endothelial cell layer without altering barrier permeability using pinocytosis or receptor-mediated mechanisms (Kim, 2008). This mode of invasion has been observed for many bacterial pathogens, such as *Neisseria meningitidis* (Pujol et al., 1997), *Streptococcus pneumoniae* (Ring et al., 1998), and *Escherichia coli* (Prasadarao et al., 1999b). The pathogen penetrates through barrier cells with and/or without evidence of tight-junction disruption. Paracellular penetration of the BBB has also been suggested for the Hib (Chang et al., 2004). On the other hand, some pathogens which have ability to survive within the phagocytes can be carried across the BB by infected immune cells, such as monocytes; this mechanism is sometimes called the Trojan Horse pathway and, has also been suggested for some viral pathogens, such as simian immunodeficiency virus (Erlander, 1995).

1.7 Bacterial attachment and entry into HBMECs

The mechanisms of BBB presentation by specific bacteria are summarized below.

1.7.1 *N. meningitidis*

A first step in the course of meningococcal infection is initiated by colonization of nasopharynx. The type IV pili mediate the attachment with human nasopharyngeal mucosal cells, which is a critical step for establishing the infection *in vivo*. Then the bacteria use other virulence factors to bind more intimately with these host cells. For instance, the opacity (Opa) proteins recognize and bind to carcinoembryonic antigen cell adhesion molecules (CEACAMs,

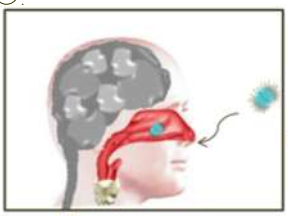
formerly CD66), a cell-surface molecule which is located on nasopharyngeal epithelial cells (Kirchner et al., 2005, Sanders et al., 2011).

Following the initial pilus-mediated contact, the bacteria invade the mucous and meningococcal capsular polysaccharides bind to collectins, and can inactivate IgA1 via the autotransporter protease (Vidarsson et al., 2005). Then, *N. meningitidis* cross the mucosa by transcytosis to invade the bloodstream (Pujol et al., 1997, Unkmeir et al., 2002). Meningococcal can survive and multiply in the bloodstream due to the presence of a number of virulence factors including lipo-oligosaccharide (LOS) and capsular polysaccharide, which can lead to a high-degree of bacteremia. Interaction of LOS with immune cells and other cells lead to the production of proinflammatory cytokines and chemokines, including interleukin 1 (IL-1), IL-6, and tumor necrosis factor (TNF)(Brandtzaeg, 1992, Zughaier et al., 2004). The central role of LOS at this stage has been demonstrated as LOS-deficient meningococcal mutants were observed to have remarkable reductions in bacterial adherence (Albiger et al., 2003) and induction of serum cytokines (van der Ley and Steeghs, 2003). The antiphagocytic properties of capsular polysaccharide has also been shown to be fundamental for bacterial growth in the bloodstream, a prerequisite for septicaemia and meningitis (Spinosa et al., 2007).

After multiplication in the bloodstream, meningococcal can invade HBMECs. Attachment to these cells is mediated via type IV pili which were thought to recognize the CD46 molecule (Kallstrom et al., 1997), although interaction of pili and CD46could not be verified in further studies and it is possible that the type IV pilus attaches to host cells independently of CD46 (Kirchner and Meyer, 2005).

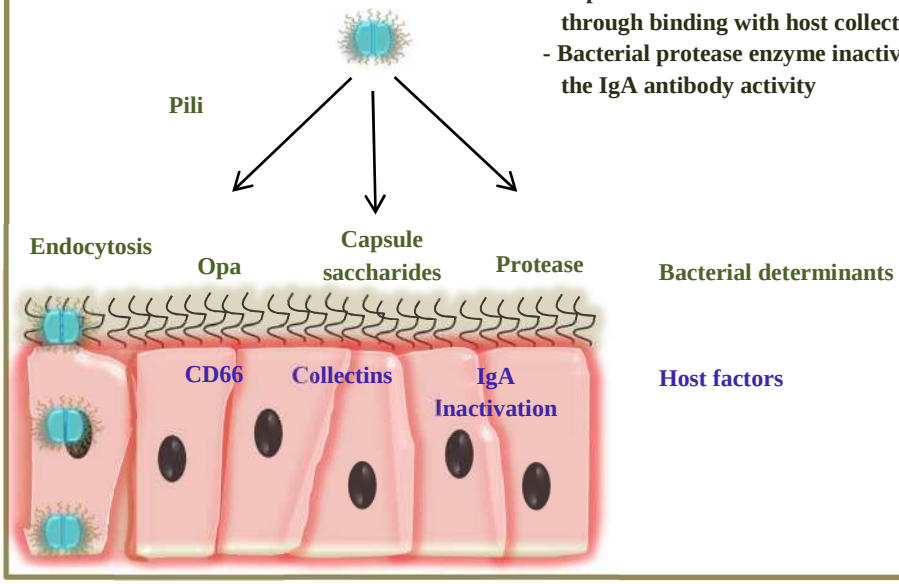
Furthermore, PorA and PilQ have been recently shown to bind to the laminin receptor (LamR) on HBMECs. After primary anchorage on the epithelium by way of pili, Opc was also found to mediate a direct contact between the pathogen and host cells by binding with fibronectin(Meyer, 1999). Attachments of these bacterial adhesins contribute to traversal of the BBB by endoctyosis or transcytosis after disorganizing the cell polarity via binding to type IV pili. Meningococcal invasion of the meninges and replication in the CNS triggers the production of cytokines and chemokines by cells of the immune system leading to increased BBB permeability and pleocytosis; this in turn enhances the local inflammatory response with subsequent increased intracranial pressure and edema, ultimately resulting in neuronal injury (Pfister et al., 1992, Sanders et al., 2011). See also Figure 1-5 that surmises the main steps in the bacterial meningitis caused by *N. meningitidis* including host and bacterial-receptors involved in these steps.

1 Colonization of nasopharynx



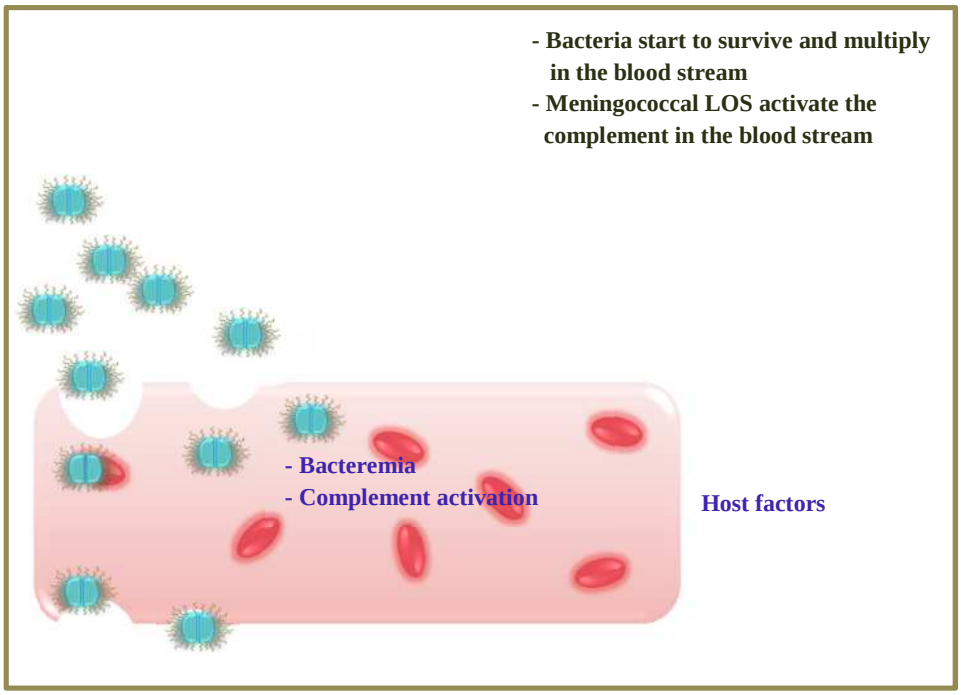
2 Nasopharyngeal mucosal cells adherence and invasion

- Pili mediate initial attachment
- Opa recognize host receptor, CD66
- Capsule enhance bacterial adherence through binding with host collectins
- Bacterial protease enzyme inactivate the IgA antibody activity



3 Meningococcal survival and multiplication in the bloodstrem

- Bacteria start to survive and multiply in the blood stream
- Meningococcal LOS activate the complement in the blood stream



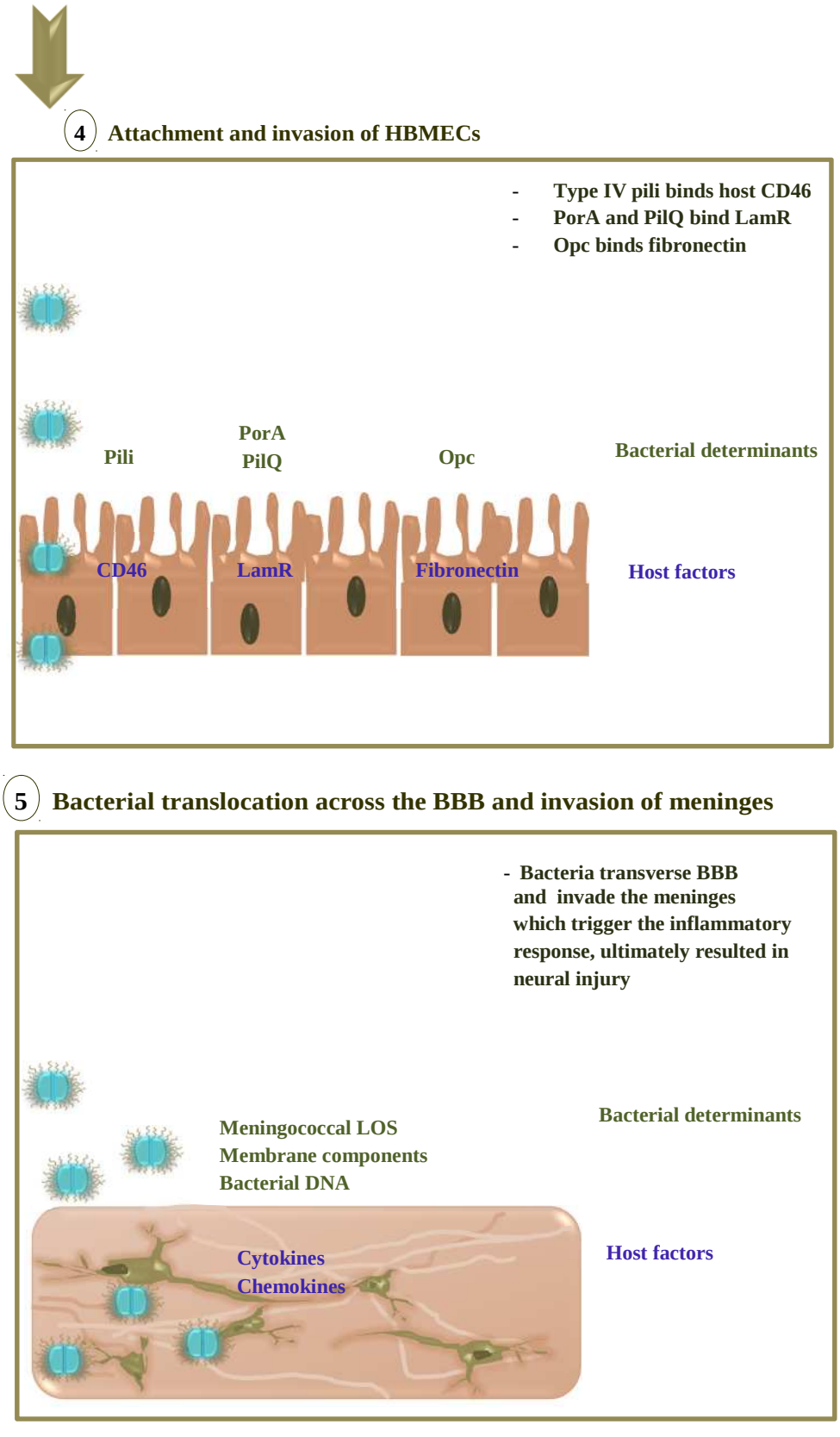
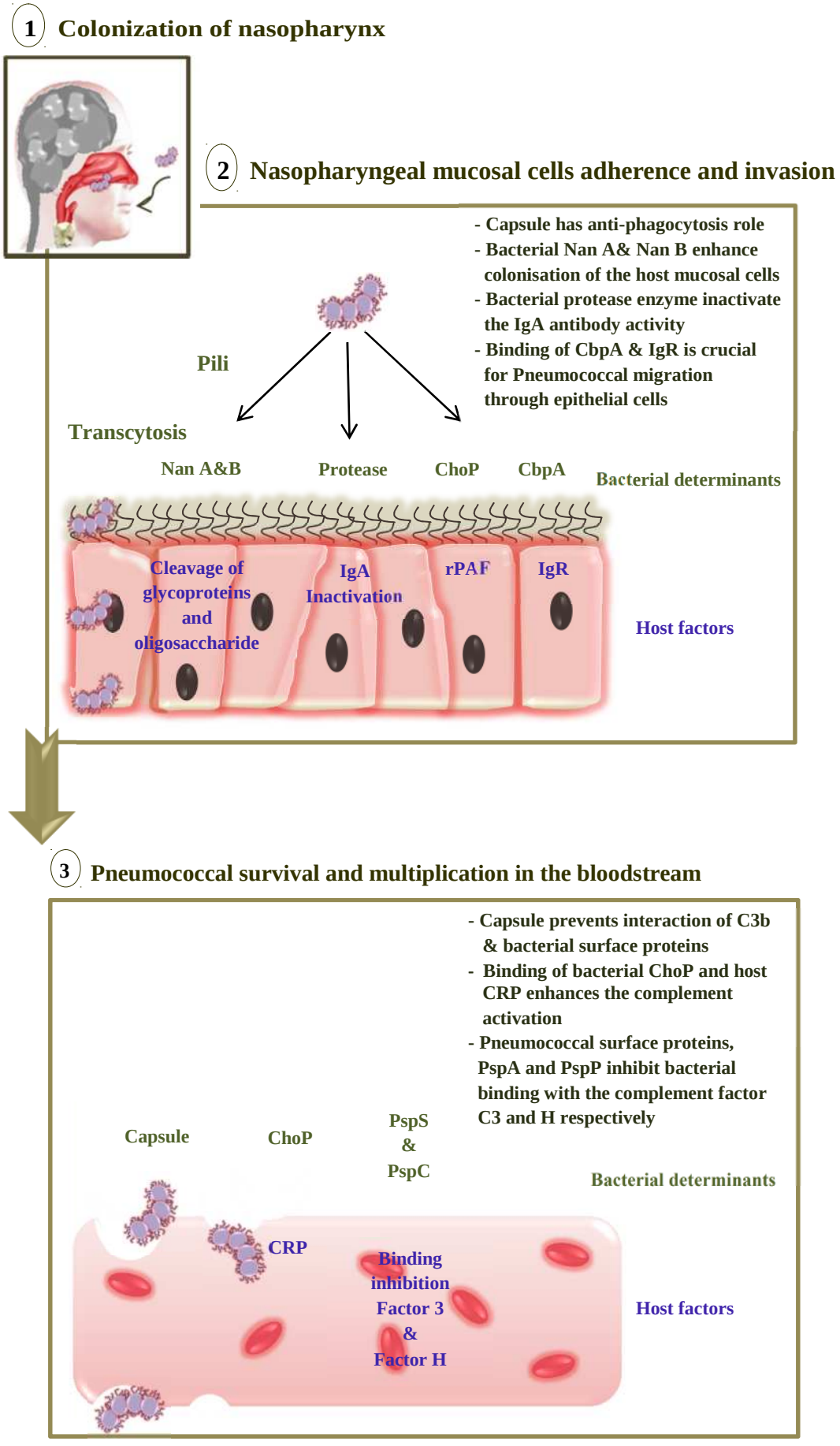


Figure 1-5 Main steps in the bacterial meningitis caused by *N. meningitidis* including host and bacterial-receptors involved in these steps. Adapted and modified from (Sanders et al., 2011).

1.7.2 *S. pneumoniae*

S. pneumoniae express a range of surface proteins that mediate adherence and colonisation of the mucosal epithelium of the nasopharynx. The pneumococcal polysaccharide capsule prevents entrapment in the mucus and inhibits phagocytosis (Sanders et al., 2011). Neuraminidases (NanA and B) cleave glycoproteins and oligosaccharides in mucus, thus it enhances bacterial colonization by decreasing the viscosity of the mucus (Tong et al., 2000). Pneumococcal IgA1 protease further facilitates colonization by degrading local secretory IgA, which may protect the bacteria from type-specific antibody during colonisation (Poulsen et al., 1998). Also, Phosphorylcholine (ChoP) binds to the receptor for platelet activating factor (PAF) and activates host cell signalling (Thornton et al., 2010). After adherence and colonisation, mucosal invasion is a pre-requisite for bacteremic spread. For that, Pneumococci upregulate choline-binding protein CbpA); this protein recognises and binds to epithelial polymeric immunoglobulin receptor (IgR), which is critical for the adherence and migration of pneumococci via epithelial cells Then, the bacteria traverses mucosa by transcytosis (Zhang et al., 2000). Pneumococcal ChoP binds to C-reactive protein (CRP) and enhances complement activation. In addition, two pneumococcal surface proteins named PspA and PspC were found to inhibit complement activation by interfering with binding of the bacteria to complement factor C3 and H respectively (Kadioglu et al., 2008, Sanders et al., 2011). At this stage, polysaccharide inhibits the interaction between any C3b bound to surface proteins and receptors on phagocytic cells (Hyams et al., 2010, Melin et al., 2010). Prior to invasion of the meninges, the bacteria must adhere to the human endothelial cells that line the vessels. Two pneumococcal proteins, CbpA and ChoP bind to LamR

and PAFr respectively; leading to invasion of the meninges and bacterial replication in the CNS (Kadioglu et al., 2008, Orihuela et al., 2009). Bacterial autolysis via the action of the autolysin LytA has been implicated in releasing bacterial sub-capsular components, which subsequently trigger the inflammatory response, cytokine and chemokine production leading to an inflammatory response and increased BBB permeability, pleocytosis, BBB disruption, cerebral oedema and increased intracranial pressure, ultimately resulting in neuronal injury (Mitchell and Mitchell, 2010, Sanders et al., 2011). See also Figure 1-7 that surmises the main steps in the bacterial meningitis caused by *S. pneumoniae* including host and bacterial-receptors involved in these steps.


1 Colonization of nasopharynx
2 Nasopharyngeal mucosal cells adherence and invasion
- Capsule has anti-phagocytosis role
- Bacterial Nan A& Nan B enhance colonisation of the host mucosal cells
- Bacterial protease enzyme inactivate the IgA antibody activity
- Binding of CbpA & IgR is crucial for Pneumococcal migration through epithelial cells
Pili
Transcytosis
Nan A&B
Protease
ChoP
CbpA
Bacterial determinants
Cleavage of glycoproteins and oligosaccharide
IgA Inactivation
rPAF
IgR
Host factors
3 Pneumococcal survival and multiplication in the bloodstream
- Capsule prevents interaction of C3b & bacterial surface proteins
- Binding of bacterial ChoP and host CRP enhances the complement activation
- Pneumococcal surface proteins, PspA and PspP inhibit bacterial binding with the complement factor C3 and H respectively
Capsule
ChoP
PspS & PspC
Bacterial determinants
CRP
Binding inhibition Factor 3 & Factor H
Host factors

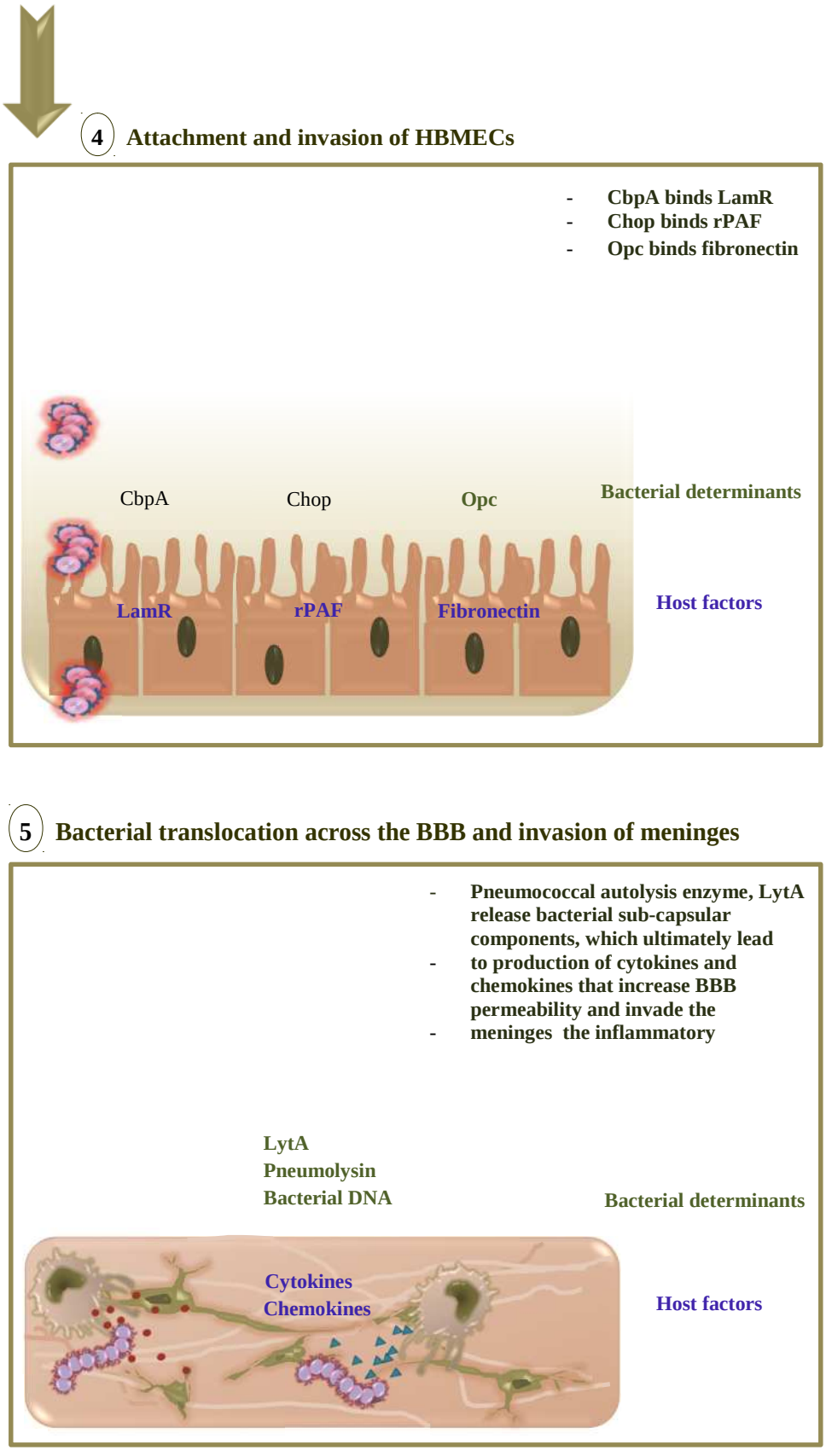


Figure 1-2 Main steps in the bacterial meningitis caused by *S. pneumoniae* including host and bacterial-receptors involved in these steps. Adapted and modified from (Sanders et al., 2011).

1.7.3 *H. influenzae*

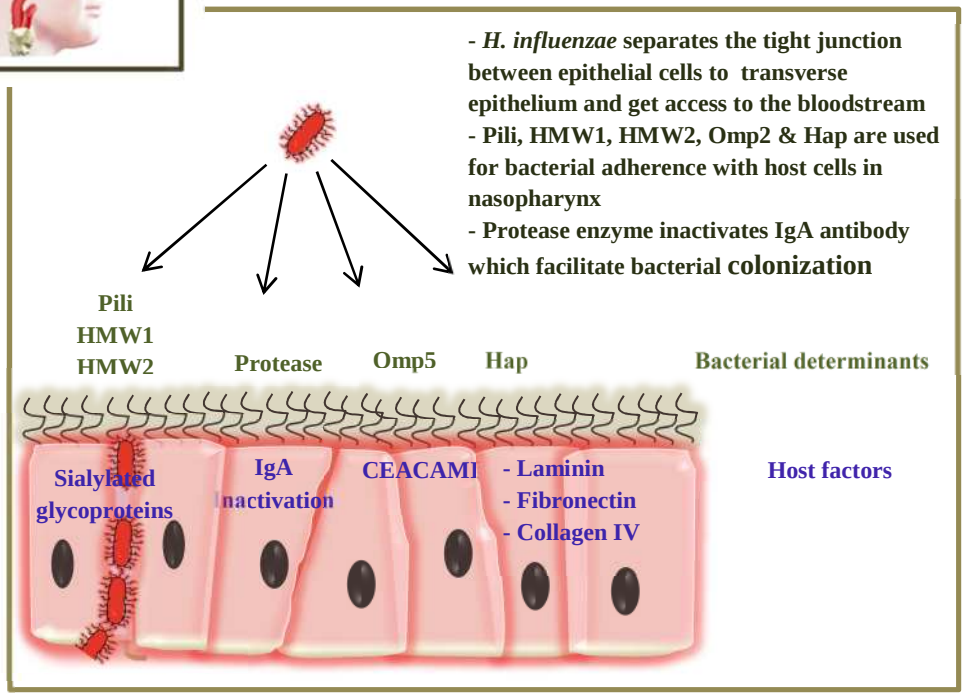
H. influenzae gain access into the host by colonizing the nasopharyngeal mucosal epithelium. Pilus-mediated adhesion is the initial step in a series of events that may lead to infection (van Alphen et al., 1987). Haemophilus pili interact with human oropharyngeal epithelial cells by recognition of sialylated lactosylceramide derivatives (van Alphen et al., 1991). They also promote bacterial adherence to respiratory mucus (Kubiet et al., 2000), and heparin-binding extracellular matrix proteins, such as the heparin binding domain of fibronectin and mouse heparin-binding growth-associated molecule (Virkola et al., 2000). In addition to adhesins associated with pili or fimbriae, significant levels of attachment to mammalian cells have been demonstrated in non-piliated *H. influenzae* that is mediated by highmolecular-weight adhesins HMW1 and HMW2 which bind mammalian surface structures containing glycoproteins related to heparan sulfate (Foxwell et al., 1998). Nontypeable *H. influenzae* has been shown to adhere to human nasopharyngeal mucin (Reddy et al., 1996) and human CEACAM 1 by *H. influenzae* outer membrane proteins P5 (Omp5) (Reddy et al., 1996, Hill et al., 2001). Two proteins, Hi autotransporter adhesin (Hia) and Haemophilus adherence and penetration protien (Hap), which are members of autotransporter family, also have adherent properties to respiratory epithelial cells. Two homologous binding domains, named HiaBD1 and HiaBD2 were found to be responsible for binding of Hia to host cells. While Hap binds to laminin, fibronectin and collagen IV (St Geme et al., 1994, Hendrixson and St Geme, 1998, Fink et al., 2002, Yeo et al., 2007). Bacterial colonization of the upper respiratory tract is also facilitated by IgAl proteases that cleave and inactivate human IgAl (Plaut, 1983, Kilian et al., 1996).

Once in the blood stream, *H. influenzae* can survive due to its polysaccharide capsule which enhances its survival in the blood by interfering with the binding of opsonising antibodies and complement to the bacterial surface (Marrs et al., 2001). Like meningococci and pneumococci. *H. influenzae* binds to the LamR receptor on HBMECs, in this case via its porin protein OmpP2 (Orihuela et al., 2009, Abouseada et al., 2012). Prior to invasion, the bacteria interact with the host endothelial cells by binding to the host platelet-activating factor receptor (PAFr) via LOS glycoforms containing ChoP (Swords et al., 2001). Bacteria crossing the BBB and invading the meninges, will trigger a host inflammatory cascade, which subsequently leads to abnormalities in the cerebral blood flow and increases in the permeability of the BBB leading to neuronal damage (van Sorge and Doran, 2012). See also Figure 1-7 that surmises the main steps in the bacterial meningitis caused by *H. influenzae* including host and bacterial-receptors involved in these steps.

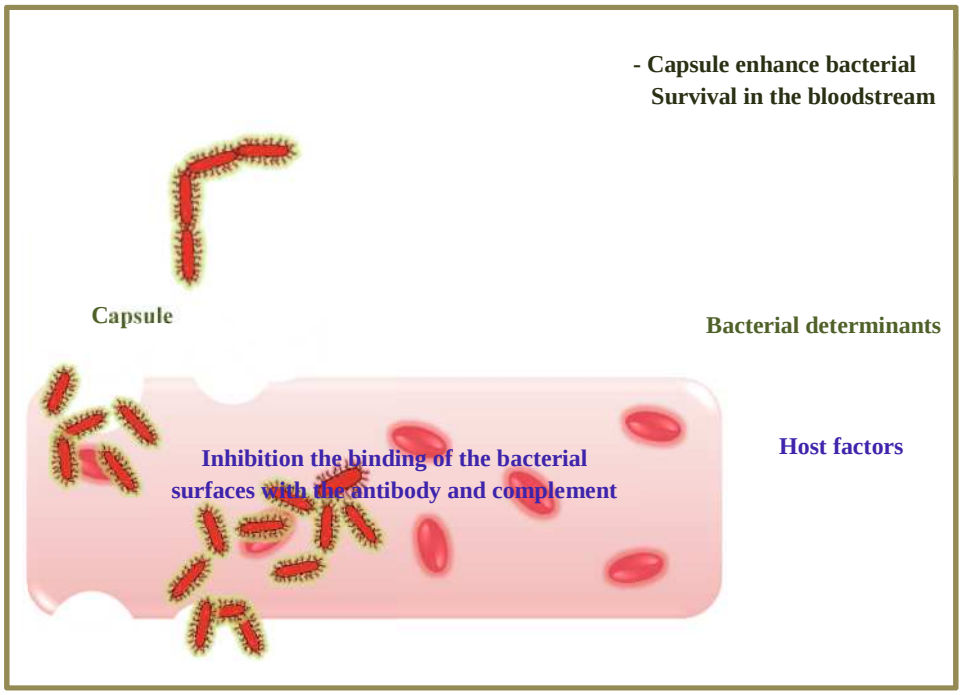
1 Colonization of nasopharynx



2 Nasopharyngeal mucosal cells adherence and invasion



3 Surviving and multiplication of *H. influenzae* in the bloodstream



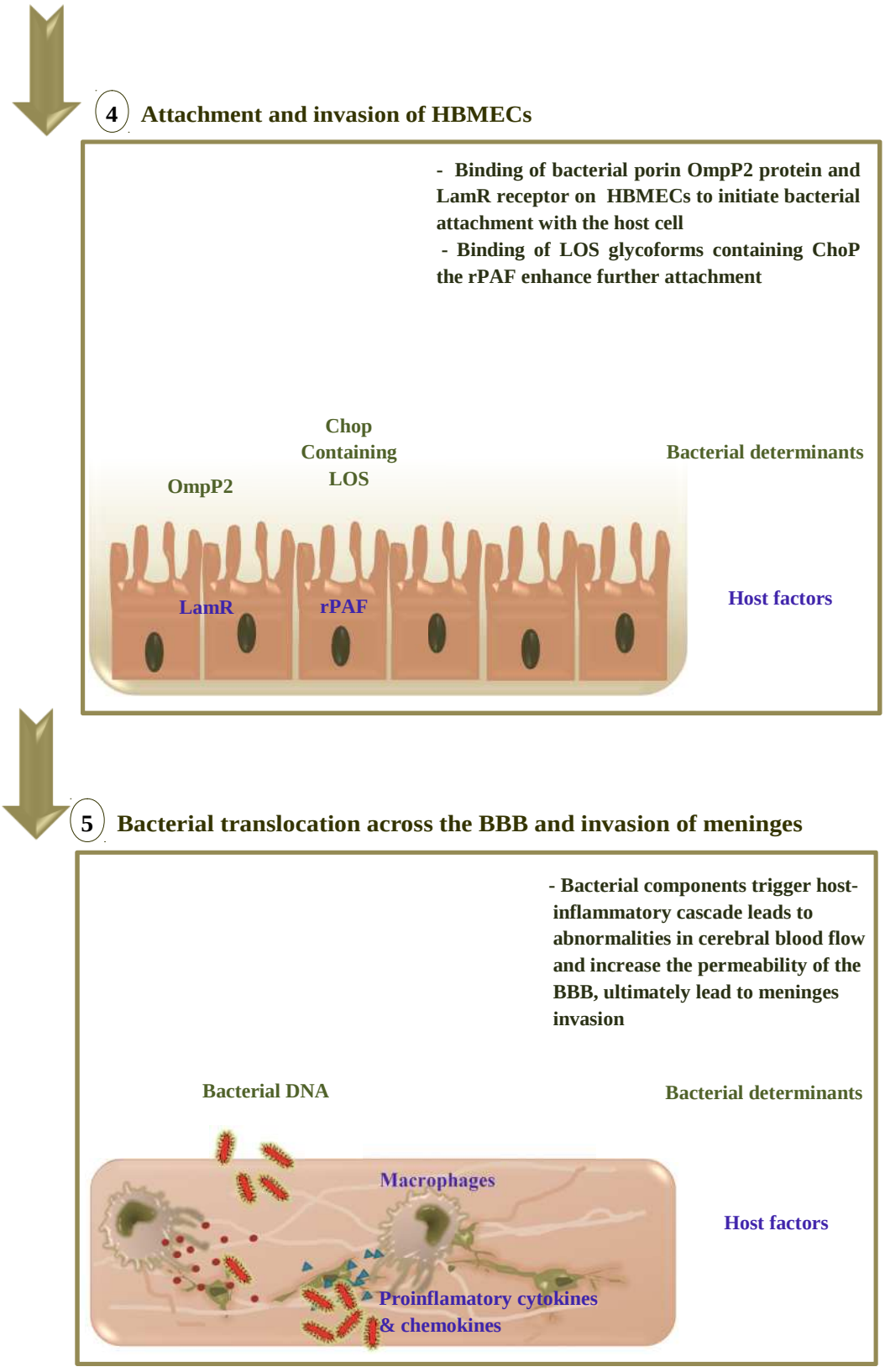


Figure 1-7 Main steps in the bacterial meningitis caused by *H. influenzae* including host and bacterial-receptors involved in these steps.

1.8 Development of vaccines against bacterial meningitis

Significant progress has been made in developing vaccines against *H. influenzae*, *S. pneumoniae* and *N. meningitidis* in the last century to prevent bacterial meningitis. Many of the most important vaccines to be developed are described below.

1.8.1 Vaccines against *H. influenzae*

Numerous vaccine strategies have been attempted in the battle to control bacterial meningitis caused by *H. influenzae*. These efforts have concentrated on the use of lipopolysaccharides (LPS), outer membrane proteins, or capsular polysaccharides. The Hib capsular polysaccharide was the first licensed for use in humans against Hib in 1985. This vaccine was composed of polyribosyl ribitol phosphate (PRP)(Morris et al., 2008). Although PRP vaccine conferred adequate protection in children above two years, it failed to provide a full protection in infants younger than 18 months (Peltola et al., 1977).

To overcome the T-cell independent characteristics of PRP and to address the issue prevention of bacterial meningitis in children in younger age groups, researchers redirected their efforts to find vaccine based on PRP conjugated to a carrier protein, such as diphtheria toxoid conjugate (PRP-D), tetanus-toxoid protein (PRP-T) or meningococcal outer membrane protein conjugate (PRP-OMP) (Trotter et al., 2008, Pollard et al., 2009, Theodoratou et al., 2010). These proved to be highly immunogenic vaccines that provided T-cell-dependent memory as well as induction of immunological memory B cells that could not be achieved with a capsular polysaccharide-antigen alone (McVernon et al., 2003). Although Hib conjugate vaccines were effective in protection against *H. influenzae*, failures were reported in several regions of the world (Garpenholt et al., 2000, McVernon

et al., 2003, Rijkers et al., 2003). Also, Hib vaccines did not preventing disease caused by non-type b *H. influenzae* (Trollfors et al., 1992). After developing a Hib-vaccine, researchers have made significant advances with regard to the development of vaccine against NTHi (non-capsulated strains). However, developments of these vaccines and their implementation have faced major challenges in terms of absence of capsule as well as the high heterogeneity among these strains. Currently, there is no licensed vaccine against NTHi commercially available. Numerous NTHi antigens have been investigated in *in vivo* models to develop effective vaccines and some have yielded promising results with regard to their ability to trigger the desired immune protection. These include LOS (Hong et al., 2010) , Omp26 (Riedmann et al., 2011) , P4 (Hotomi et al., 2005) and P5+PilA (Novotny et al., 2013). However, translation of these findings to human vaccine requires further investigations prior to licensure.

A highly conserved lipoprotein named protein D (PD) which is present in both NTHi and Hib strains (Janson et al., 1993) and enhances bacterial adhesion and invasion showed promising results as a vaccine candidate in preclinical trials of an 11-valent pneumococcal conjugate vaccine containing PD (Forsgren et al., 2008). In addition a surface-exposed protein of NTHi, Protein F (PF), has been investigated as vaccine candidate. This protein with host cell adhesive properties is highly conserved and may be a promising vaccine candidate against NHTi (Jalalvand et al., 2014).

1.8.2 Vaccines against *S. pneumoniae*

To prevent pneumococcal meningitis, several vaccines have been developed against different serotypes of the pneumococcus. In the 1980's the first generation vaccine was licensed named 23-valent polysaccharide vaccine

(PPSV23 or Pneumovax® 23 Merck). This vaccine contains polysaccharide antigens from 23 most prevalent invasive pneumococcal serotypes which are responsible for 90% of the most serious pneumococcal disease in developed countries (Herva et al., 1980). The 23-valent vaccine was shown to have no efficacy in children less than 2 years age or in immunocompromised patients; these groups have the highest rate of invasive pneumococcal disease and pneumococcal meningitis (Black et al., 2000, McClure et al., 2006). For that reason, in many industrialized countries a second generations of pneumococcal vaccines have been developed by conjugation of the capsular polysaccharides with carrier proteins from a nontoxic variant of diphtheria toxin (CRM197), tetanus toxoid, or a meningococcal outer membrane protein complex, leading to dramatic declines in pneumococcal meningitis in infants and young children, these vaccines called pneumococcal conjugate vaccine (PCVs). By conjugating polysaccharide vaccine antigens to a protein carrier the antigen is converted from a T cell-independent one to a T cell-dependent one (Black et al., 2000, Dennehy, 2001).

In 2000, a 7-valent pneumococcal conjugate vaccine (PCV-7) was the first PCV to be licensed. This vaccine contains polysaccharides from several serotypes (6A, 6B, 9V, 14, 19F, and 23F), which are conjugated to an inactivated nontoxic diphtheria toxin CRM197(Ghaffar et al., 2004). Although the effectiveness of PCV-7 vaccine has been very good, there has been a reported increase in invasive disease due to non-vaccine serotypes in countries where PCV7 has been introduced into the routine infant immunization schedule (Kaplan et al., 2004, Byington et al., 2005). To address this issue, several pneumococcal conjugated vaccines have been integrated in the vaccination program including: 9-valent, 11-

valent and 13-valent conjugate vaccines which cover additional pneumococcal serotypes (Klugman et al., 2003, Grando et al., 2015, Hirose et al., 2015).

Due to serotype-specificity of pneumococcal conjugated vaccines, current researches are focused on bacterial surface proteins as pneumococcal vaccines candidates for use in developing countries. Currently, there are three candidate vaccines: pneumococcal surface protein A, pneumococcal surface adhesion A and pneumolysoid (a mutant pneumolysin-like molecule). Common protein vaccines are less expensive to manufacture than the polyvalent vaccines (which use the capsular polysaccharide as the immunizing antigen) since they can be produced in large amounts using inexpensive recombinant technology (Briles et al., 1996, Yamamoto et al., 1997, Feldman, 2014).

1.8.3 Vaccines against *N. meningitidis*

There have been extensive global efforts to develop efficacious vaccines against the five major pathogenic meningococcal serogroups: A, B, C, W, and Y. In the 1960s, the first major success towards vaccination against meningococcal disease was achieved by Gotschlich and co-workers, who successfully demonstrated the importance of anti-polysaccharide antibodies in the human immune response against serogroup A and C disease (Morley and Pollard, 2001).

Capsule based vaccines against *N. meningitidis* serogroups A, C, W and Y have been available and used since the 1970s. These include a bivalent vaccine (serogroups A and C), a trivalent vaccine (A, C, Y), and a tetravalent vaccine (A, C, W, and Y). Although, several investigations revealed that these vaccines have the ability to activate high enough titres of bactericidal antibodies to all four polysaccharide antigens in adults (18 years and above), these plain polysaccharide vaccines have several drawbacks including their inability to confer the protection

in children less than 2 years and their inability to provide long-term protection to young children, protection typically lasting just for one year (Lepow et al., 1986, Hankins et al., 1982, Bilukha et al., 2005), since they generate only T-cell-independent responses (Reingold et al., 1985). Also, these vaccines ineffective in providing protection against group B meningococcal disease.

As a result of the success of conjugate vaccines to protect against invasive disease caused by *H. influenzae* type b and *S. pneumoniae*, conjugate vaccines against specific serogroups of *N. meningitidis* were also developed. These vaccines contain meningococcal polysaccharide conjugated to a protein such as tetanus toxoid, diphtheria toxoid, or CRM197 (Burrage et al., 2002). They are immunogenic and induce immunological memory in young children (Jodar et al., 2002). Additionally, conjugate vaccines are characterized by their efficiency in children below two years, their ability to reduce nasopharyngeal carriage, and hence their ability to produce herd immunity (Jodar et al., 2002, Snape and Pollard, 2005).

The carrier proteins that have been employed in conjugate vaccines include toxoids of diphtheria toxin (for example CRM 197) or tetanus toxoids (Burrage et al., 2002). There is also variation among the polysaccharides employed in conjugate vaccines regarding their structures. Additionally, the polysaccharide component can be attached to the carrier protein either directly or through a chemical linker (Broker and Fantoni, 2007).

The first serogroup C meningococcal conjugate vaccine (MenC) was introduced into the routine childhood immunisation schedule in 1999 (Ramsay et al., 2001). This had a huge impact in reducing serogroup C carriage and disease (Ramsay et al., 2003, Maiden et al., 2008). Later on, in January 2005, a quadrivalent

meningococcal conjugate vaccine (MCV4) was developed and licensed (Harrison, 2006). Meningococcal conjugate vaccines have been proven to be safe and able to trigger an efficient immune response in infants (Andersson et al., 1994, Richmond et al., 1999, Campagne et al., 2000). Besides, these vaccines are able to prime immunologic memory, and lead to a booster response to subsequent doses (Twumasi et al., 1995, Borrow et al., 2000).

In addition to serogroup C, the conjugate vaccines have been also introduced against serogroup A in Africa (MenAfriVac) beside the quardivalent conjugate vaccine (A/C/ Y/ W) that confer protection in individuals aged 11-55 years (Keyserling et al., 2006, Kristiansen et al., 2011). Indeed, it was believed that developing a vaccine against serogroup B using carrier protein conjugation may not be safe as it may lead to autoimmune reactions due to the structural homology between serogroup B polysaccharides and host molecules (Finne et al., 1987, Nedelec et al., 1990, Trotter and Ramsay, 2007).

With declining group C disease following, the introduction of multivalent conjugate vaccines for infants, Group B disease remains the major public health challenge. Despite the major successes with other serogroups, serogroup B meningococci (MenB) vaccine development has been impeded by the similarity of the group B capsule to host polysialic acid in glycoproteins from human neural tissue rendering the conjugate vaccines (Hung and Christodoulides, 2013, Vesikari et al., 2015). Although, extensive attempts have been made to solve this problem by replacing the N-acetyl groups of polysialic acid residues to yield a conjugate vaccine that was safe in phase I clinical trials, these vaccines did not prove to be efficious in bactericidal assays, opsonophagocytic tests (Holst, 2007). Thus other approaches have been adopted to develop vaccines using non-capsular

antigens either as outer membrane vesicles (OMVs) or as individual antigens by identifying conserved antigen variants, or by compiling a cocktail of immunogenic antigens to provide broad coverage (Zollinger et al., 2011, Moran et al., 2012). Of the non-capsular vaccine candidates, the most complex are outer membrane vesicle (OMV)-based vaccines that contain a mixture of antigens including lipo-oligosaccharide (LOS) and outer membrane proteins (Vesikari et al., 2015). In several different countries, OMV-based vaccines have been utilized as a basis for several outer-membrane-vesicle-based vaccines to protect against MenB disease. For instance, MeNZB (Norwegian Institute of Public Health and Novartis), a tailor-made outer-membrane-vesicle vaccine, stimulated the immune responses directed against PorA and proved to be effective in controlling epidemic meningitis infection in New Zealand. Unfortunately, antigenic variation of PorA among geographic isolates has proven to be a major limitation in the use of OMV-based vaccines more widely (Zollinger et al., 2011).

A new strategy called 'reverse vaccinology' has been adopted more recently (Rappuoli, 2000). By this technique, in which protective antigens were identified in a multi-level screen starting with bioinformatics prediction of conserved surface-exposed proteins followed by expression, purification and screening for the ability to elicit protective antibodies in *in vivo* models, a multicomponent vaccine was developed which combined specific surface proteins with OMV; the resulting vaccine is known commercially as Bexsero (4CMenB) and was produced by Novartis (now Glaxo Smithcline) and was licensed for use in Europe (Vesikari et al., 2015). Another vaccine has been developed and is now being marketed by Pfizer; it is directed against two variants of fHbp (Fletcher et al., 2004, Tan et al., 2010, McNeil et al., 2013).

Using same technique, six neisserial antigens: factor H–binding protein (GNA1870), GNA2091, *N. meningitidis* adhesin A (NadA, or GNA1994), Neisserial heparin-binding antigen (NHBA; also known as GNA 2132), and GNA 1030 (fused with NHBA) as well as the later added outer membrane porin protein PorA-containing OMV vaccine, were combined in a vaccine formulation named rMenB (Zollinger et al., 2011, Serruto et al., 2012, Costa et al., 2014, Brendish and Read, 2015, Oviedo-Orta et al., 2015).

1.9 Human Laminin receptor (LamR)

The 37/67 kDa human laminin receptor (p40, RPSA, 37LRP, 67LR, LamR) is a multifunctional protein, with a number of intercellular activities, which also acts as an extracellular receptor. In 1983, the 67 kDa laminin receptor (LamR) was first discovered by three independent laboratories by affinity chromatography on laminin sepharose and identified as a non-integrin cell surface receptor for the extracellular matrix molecule (ECM) laminin (Lesot et al., 1983, Malinoff and Wicha, 1983, Rao et al., 1983).

1.9.1 Molecular nature of human LamR

The precise molecular nature of LamR and the relationship between the 37kDa laminin receptor protein (LRP) and the 67 laminin receptor (LR) is unclear. Although 37kDa LRP has been suggested to be the precursor of the 67kDa high-affinity laminin receptor (LR), however, the exact mechanism by which the 37kDa precursor forms the mature 67kDa isoform has not been demonstrated, although several conflicting hypotheses have been proposed to account for their relationship. These include homodimerisation, heterodimerisation with another molecule such as Galectin-3 (Gal-3) or fatty acylation (Rao et al., 1989, Buto et

al., 1998). Data from yeast two-hybrid analysis showed that LRP failed to interact with itself (Hundt et al., 2001); an argument against hypothesis of a direct homodimerisation. Analysis of the membrane-bound 67kDa LR indicated that acylation of LRP is involved in the processing of the receptor (Landowski et al., 1995a). Additional studies suggested that the 67kDa LR is a heterodimer stabilized by fatty acid-mediated interactions (Buto et al., 1998).

1.9.2 The human LamR gene

Laminin receptor (LamR) is encoded in humans by the ribosomal protein SA gene (*RPSA*, NCBI Gene ID: 3921, Accession No.: AC-000135). The gene is a 5,833-bp stretch along chromosome location 3p22.2 (3p21.3 by *in situ* hybridization) consisting of seven exons and six introns (Jackers et al., 1996).

Multiple copies *RPSA* gene have been found in mammalian genomes. For instance, the human genome contains 26 copies, while the mouse genome has only 6 (Jackers et al., 1996).

The human LamR sequence displays 99% homology with the rat sequence and 98.3% with the mouse and bovine sequences (Menard et al., 1997).

RPSA has homologues in widely divergent organisms including *Saccharomyces ccrevisiae, Arabidopsis thaliana, Drosophila mclanogaster, Chlorohydra zjiridissima* and the archaebacterium *Haloarcula masmortzri* (Ardini et al., 1998). The homologue present in the Archaea is 40% identical to *RPSA,* which places the human gene as a member of the S2 family of ribosomal proteins (Scheiman et al., 2010a).

1.9.3 The human LamR protein

The laminin receptor is likely to have originated ancestrally as a ribosomal component and the human LamR precursor protein and the p40 ribosomal protein are encoded by the same gene (37LRP/p40)(Ardini et al., 1998). The gene encodes a protein of 295 residues with a calculated molecular weight of 37 kDa (Jamieson et al., 2008). The amino acid sequence of LamR is 98% identical in all mammals. LamR is not glycosylated but is acylated in position Ser2 by fatty acids (Zidane et al., 2013). The acylation contributes to its conversion to the 67 LR form and the residue Tyr139 is phosphorylated *in vivo*. LamR is comprised of two main domains: an N-terminal (a.a. 1-209) and a C-terminal (210-295) domain (Ould-Abeih et al., 2012). The N-terminal domain (NTD) is involved in the ribosomal functions and is characterised by homology with the prokaryotic ribosomal proteins S2. It is thus likely that the function of the 37LRP/p40 as a ribosomal protein has been maintained during evolution from prokaryotes to eukaryotes (Ould-Abeih et al., 2012). Scheiman and co-workers revealed that the N-terminal domain of LamR is sufficient for ribosomal binding, as truncated LamR protein (1-220) is incorporated into ribosomes and can rescue translational defects caused by LamR siRNA knockdown (Scheiman et al., 2010a).

The C-terminal extension or domain (CTD) is involved in the extracellular functions and is highly conserved among vertebrates. It binds laminin, its laminin-binding capability constituting an additional function gained during evolution (Ardini et al., 1998, Ould-Abeih et al., 2012).

Homologues to 37LRP/67LR can be found in all three domains of life and all five traditionally recognised kingdoms (archae, bacteria, fungi, plants and animals) and are likely to be present in all cellular organisms. Phylogenetic analysis of the

sequence suggested that acquisition of laminin-binding capability by 37LRP is linked to the evolution of the C-terminus, and more specifically to the appearance of the palindromic sequence LMWWML (Ardini et al., 1998). This sequence appeared during evolution concomitantly with laminin and the basement membrane, the key foundation structure for multicellular tissue formation (Ardini et al., 1998, Nelson et al., 2008). Since only multicellular animals have laminin-containing basement membranes, it is clear that N-terminal homologies with proteins from unicellular organisms indicated alternative or additional functions in organisms other than metazoans. Interestingly, some pathogenic organisms have cell-membrane-associated receptors for laminin, presumably as an adaptation to aid in attachment to the basement membrane during invasion of the host. For example, *Leishmania donovani* (a unicellular haemoflagellate parasite, which is the causative agent of leishmaniasis) expresses a laminin receptor on its cell membrane. However, on the basis of partial sequence analysis and western blotting, this protein appears not to be homologous with LamR, although it does have functions in common (Ghosh et al., 1996, Bandyopadhyay et al., 2003).

1.9.4 Crystal structure of LamR

The crystal structure of human LamR protein likely to provide insights into its functions and to facilitate the design of novel therapeutics that target this receptor. Kazmin and co-workers performed the first comparative modelling of the N-terminal domain of 37 LRP using the crystal structures for the ribosomal protein S2 from *Thermus thermophilus* (PDB code 1J5E) as a template in order to study the ribosomal functions of LamR (Kazmin et al., 2003). This 37LRP/P40 model was generated on the basis of proteins from organisms evolutionarily distant from

humans and as it lacked a significant portion of the rRNA-binding domain and they were unable to model the laminin-binding C-terminal domain.

Jamieson and colleagues were the first to produce a high-resolution crystal structure of the majority of human LamR, which comprised residues 1-220, abbreviated $LamR^{220}$) (PDB code 3BCH) (Badger et al., 2005, Jamieson et al., 2008). However, the C-terminal region, and thus the bulk of the putative laminin-binding region was not contained with the $LamR^{220}$ structure. Nevertheless, $LamR^{220}$ bound laminin with similar affinity to full-length LamR and inhibited Sindbis virus infection *in vitro* (Ardini et al., 1998). By contrast, the *A. fulgidus* S2p did not exhibit any laminin-binding activity; these two results support the hypothesis that LamR acquired ECM functions during evolution (Jamieson et al., 2008, Jamieson et al., 2011).

1.9.5 Insights into LamR functions

The fact that most of the LamR sequence and structure is highly conserved suggests that LamR involved in several processes in the cell. Notably, yeast homologs of LamR were found to be important cell viability (Demianova et al., 1996), processing of 20S rRNA to 18S rRNA, maturation of the 40S ribosomal subunit, and 80S monosome assembly (Ford et al., 1999). Figure 1-9 and Figure 1-9 shows different LamR functions and distributions respectively.

1.9.5.1 Binding of LamR to laminin

LamR was first identified for its direct binding with laminin-1 (Lesot et al., 1983, Malinoff and Wicha, 1983, Rao et al., 1983); laminin-binding properties presages the importance of LamR in numerous cellular functions, such as cell adhesion to the basement membrane and in signalling transduction following Laminin-LamR

binding (DiGiacomo and Meruelo, 2015). Both 37LRP and 67LR have been shown to bind laminin with high affinity (Jamieson et al., 2008, Fatehullah et al., 2010).

1.9.5.2 Ribosomal function of LamR

The first evidence for a ribosomal role for LamR was provided by Auth & Brawerman, who observed the association of cytosolic p40 with ribosomal particles in mice (Auth and Brawerman, 1992). Later, several studies confirmed the association of LamR with the 40S small ribosomal subunit and demonstrated that the N-terminal domain (NTD) was sufficient for ribosomal binding (Tohgo et al., 1994, Scheiman et al., 2010a, Scheiman et al., 2010b, Anger et al., 2013).

LamR contributes in the maturation and assembly of the ribosome through its associations with 18s rRNA and the S21 ribosomal protein, and it is implicated in the translational processes through its association with 40S ribosomal subunit (Sato et al., 1999, Scheiman et al., 2010a, Scheiman et al., 2010b).

1.9.5.3 Nuclear function of LamR

LamR has also been shown to localize to the nucleus and (DiGiacomo and Meruelo, 2015) bind to histones H2A, H2B and H4, which seems to be controlled in a cell cycle dependent manner (Sato et al., 1996, Kinoshita et al., 1998, DiGiacomo and Meruelo, 2015). Association of 37LRP/p40 with both the nuclear envelope and chromatin during interphase, but only with chromatin during mitosis has been observed by Kaneda and co-workers (Kaneda et al., 1993). The same group later suggested that LamR may serve as a ligand connecting chromatin to the nuclear envelope (Kaneda et al., 1998). LamR was also found to be involved

in cell signalling pathways that are important for cell survival (Givant-Horwitz et al., 2004).

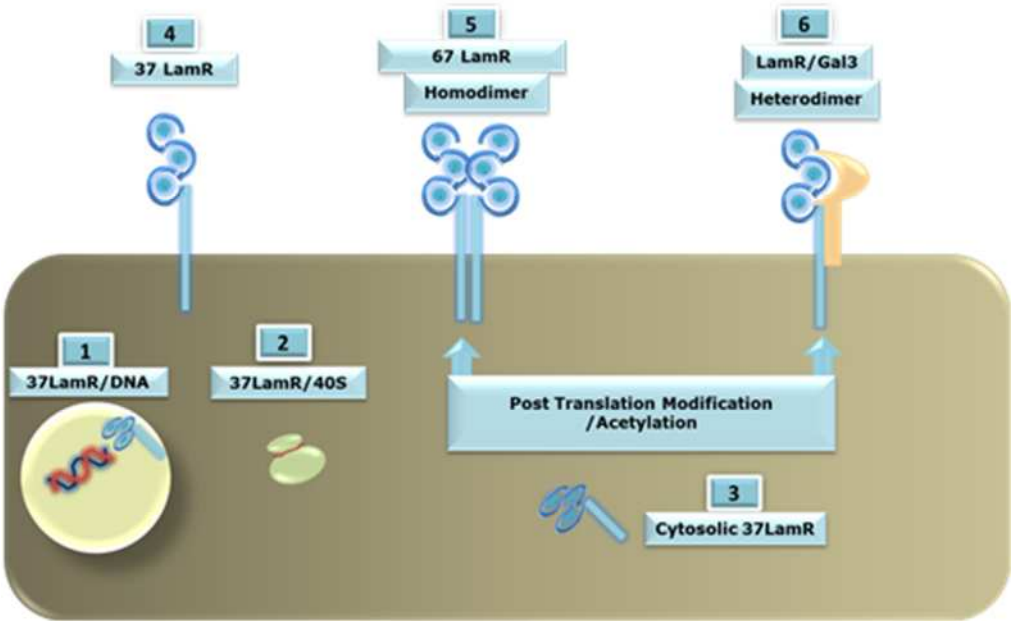


Figure 1-8 Schematic showing the multiple functions and localization of LamR.
(1) LamR located in the nucleus binds DNA via the histones, (2) LamR in the cytoplasm associated with the 40s ribosomal subunit, (3) 37 kDa LamR may have additional function/associations in the cytosol; Post-translational modifications such as fatty acid acylation enable 37 kDa LamR to form: (4) The 67 kDa homodimer LamR (by interacting of 37kDa LamR with itself and form 67 kDa homodimer LamR and/or (5) The 67 kDa heterodimer LamR (by interacting of 37kDa LamR with Galactin 3 to form 67 kDa heterodimer); 67 kDa LamR localize to the cell surface (6) 37kDa on the cell surface.

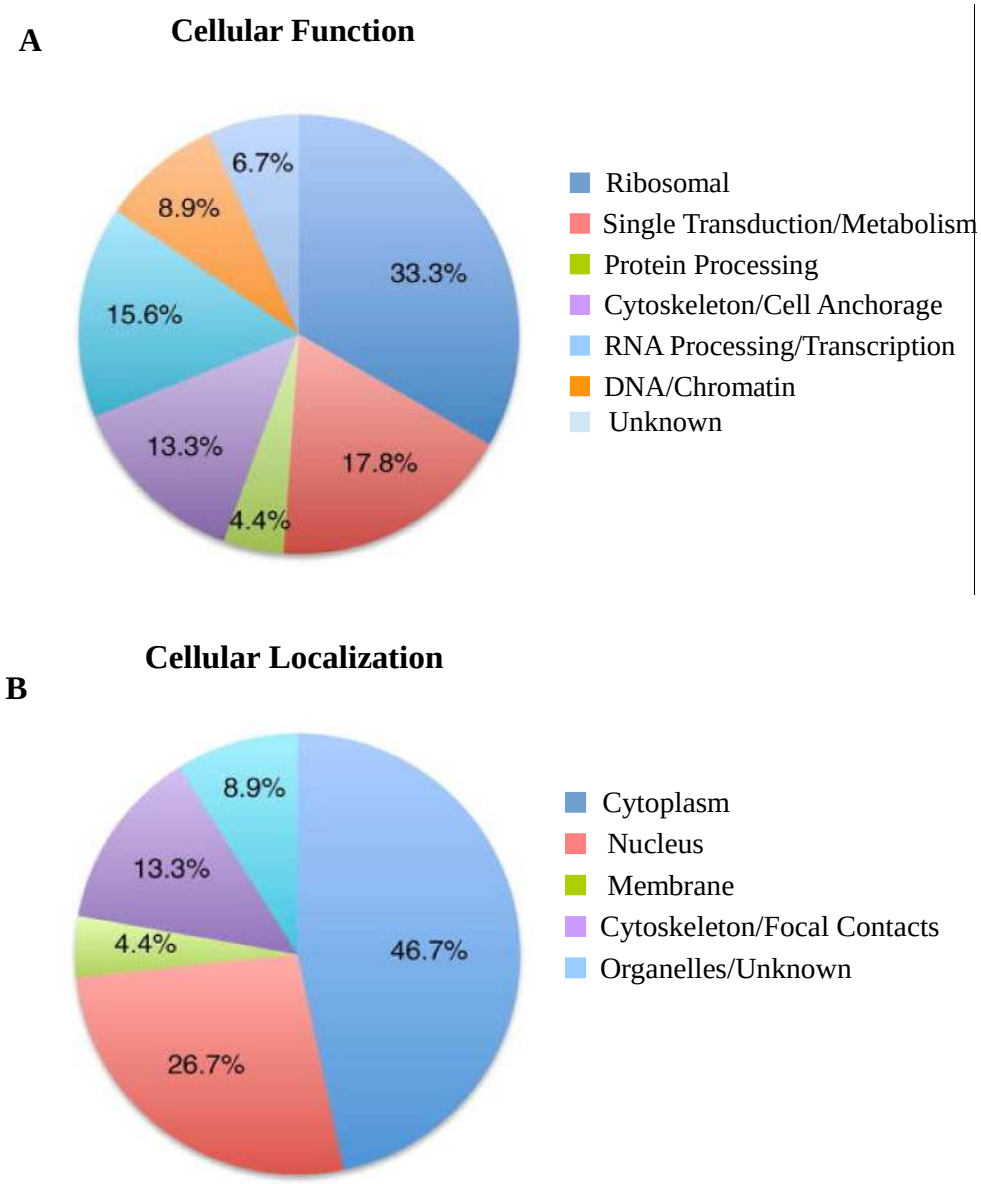


Figure 1-9 Distribution of LamR.

(A) Distribution of LamR based on predicted function. (B) Distribution of LamR by theoretical cellular localization (Venticinque and Meruelo, 2012).

1.9.5.4 Pathological functions of LamR

1.9.5.4.1 Role of LamR in cancer

LamR overexpression has been associated with many types of cancers including breast (Martignone et al., 1993), gastric (de Manzoni et al., 1998), lung (Satoh et al., 1992), colon (Cioce et al., 1991, Sanjuan et al., 1996), ovarian (Liebman et al.,

1993, vandenBrule et al., 1996), liver (Ozaki et al., 1998), thyroid (Montuori et al., 1999) , and melanoma (Vacca et al., 1993, Taraboletti et al., 1993). However, it is not known whether 37 LRP and 67 LR have separate roles in tumor development. It has been found that the high-affinity interactions between LamR and laminin mediate changes in the cell environment (Zuber et al., 2008c). The overexpression of LamR also enhances the capability of circulating cancer cells to attach to and migrate on laminin substrates. All these changes lead to increase the metastatic capability of the tumor cells attachment to the basement membrane, and form vasculature more effectively (Narumi et al., 1999). These features render LamR a useful prognostic marker for determining the severity of tumors of human cancer patients (DiGiacomo and Meruelo, 2015).

The effect of siLamR on cell morphology *in vivo* is much more profound, indicating that the ribosomal functions of LamR may be more critical for tumor-cell survival in a more physiological environment. The ability of LamR to regulate translation, proliferation, and survival as a ribosomal protein may therefore be as important, if not more, than its extracellular functions in tumor development (Tohgo et al., 1994, Scheiman et al., 2010a, Scheiman et al., 2010b, Anger et al., 2013).

1.9.5.4.2 Role of LamR in Prion diseases (PDs)

Prion diseases, also called transmissible spongiform encephalopathies (TSEs), are a group of rare, fatal neurodegenerative diseases, that affect humans and animals (Belay, 1999). A protein known as cellular prion protein (PrP^c) is a glycolipid anchored protein which is highly conserved among mammalian species. PrP^c is normally expressed in several cell types, mainly neuronal cells without causing disease (Kretzschmar et al., 1986, Wopfner et al., 1999). Several lines of evidence

suggested the physiological activities of PrP^{C} including its role in cellular uptake or binding of copper ions, protection against apoptotic and oxidative stress, transmembrane signaling, and adhesion to the extracellular matrix. However, the evidences are not conclusive for any them (Isaacs et al., 2006, Westergard et al., 2007). Prion disease develops when endogenous PrP^{c} is converted to an abnormal form, the infectious prion protein called PrP^{Sc}, which accumulates in the brains of affected animals and humans (Prusiner, 1998).

Interestingly, both LamR monomer and dimer forms were found to act as receptors for cellular and infectious prion protein isoforms (Gauczynski et al., 2001). Both PrP^{c} and PrP^{Sc} were found to use LamR as part of a mechanism of cellular internalization and propagation, they also bound heparin sulphate proteoglycans (HSPGs) as co-receptors, to facilitate their interaction with LamR (Hundt et al., 2001). Furthermore, the prion protein was also found to be related to Alzheimer's Disease (AD), and LamR thus also have indirect role in AD. The identification of the LamR as prion receptor has opened a new direction for the development of a prion disease therapy such as anti-prion therapy (Vana et al., 2009).

1.9.5.4.3 LamR binding to pathogens and exogenous ligands

A role for LamR in the development of bacterial meningitis development was first reported by Kim and co-workers, who showed that LamR was required for internalization of *E. coli* K1 into brain endothelial cells (Kim et al., 2005). More specifically, a toxin secreted by these bacteria, called cytotoxic necrotizing factor 1 (CNF1) was found to bind LamR and initiate cytoskeletal rearrangement which ultimately lead to the uptake of the pathogen (Chung et al., 2003). More recently, three neurotropic bacteria which are the major bacterial agents of meningitis,

Neisseria meningitidis, *Haemophilus influenzae* and *Streptococcus pneumoniae*, were reported to employ a similar mechanism to initiate bacterial interaction with the BBB by binding with LamR on HBMECs (Orihuela et al., 2009). These bacteria mediate attachment to LamR via the surface protein adhesins: meningococcal PilQ and PorA, *H. influenzae* OmpP2 and pneumococcal CbpA. More specifically, the fourth and second surface-exposed loops of meningococcal PorA and *H. influenzae* OmpP2 respectively, and the surface-exposed loop of the R2 domain of pneumococcal CbpA were shown to act as ligands for LamR. Using synthetic peptides corresponding to loop4/PorA and loop2/OmpP2 LamR-binding to the bacterial ligands was blocked, confirming their role as the LamR ligands (Orihuela et al., 2009, Abouseada et al., 2012).

In addition to bacteria, numerous of neurotropic viruses have been shown to utilize LamR as a receptor to bind host microvascular endothelial cells. These viruses include: Adeno-associated virus (AAV; serotypes 2,3,8 and 9) (Akache et al., 2006), Dengue virus; serotypes 1,2 and 3, Dengue virus (serotypes 1,2 and 3) (Tio et al., 2005), Sindbis virus (Wang et al., 1992), Venezuelan equine encephalitis virus (VEE) (Ludwig et al., 1996), tick-borne encephalitis (TBE)(Protopopova et al., 1999) and the West Nile Virus (Bogachek et al., 2008).

LamR was also shown to bind (-)-epigallocatechin-3-gallate (EGCG)(Tachibana et al., 2004), the major active polyphenolic compound in green tea with reported anti-cancer effects inducing promotion of apoptosis of tumorigenic cells. Interestingly, EGCG has been shown to inhibit tumor cell growth *in vitro* and *in vivo* (Umeda et al., 2008).

1.9.6 Binding domains of LamR

1.9.6.1 Laminin, heparin and ribosome binding domains on human LamR

Three regions of the C-terminal domain of LamR are believed to be implicated in laminin binding: 53 amino acids on C-terminal containing four acidic TEDWS repeats (Landowski et al., 1995b), also the putative helical stretch between amino acids 206–229, and the heparin-sulfate-dependent laminin-binding region from 161–180 (peptide G) using utilized synthetic peptide segments of LamR (Graf et al., 1987, Castronovo et al., 1991b, Landowski et al., 1995b).

Recently, the laminin binding sites on human LamR were mapped by Jamieson and colleagues by substitution of amino acid residues that were most highly conserved among mammalian species. Residues Phe32, Glu35, and Arg155 were identified as primary ligands for LamR as mutations of these residues resulted in significant reduction in laminin binding activity as well as migration in cellular assays (Jamieson et al., 2011).

Both N-terminal and C-terminal portions of LamR can independently bind laminin-1 (Jamieson et al., 2008, Jamieson et al., 2011, Zidane et al., 2013). In addition to Laminin, LamR is also binds directly to the sulfated polysaccharide heparin through peptide G domain (161−180). Heparin and laminin compete for binding its peptide G; this may represent a further level of regulation of the interaction between 67LR and the basement membrane. Heparin and laminin are also both bound by a second domain: 206-229 on LamR (Nelson et al., 2008, Malygin et al., 2009).

1.9.6.2 Prion protein binding domains on human LamR

Prion protein binding domains have been identified on both NTD and CTD of human LamR using yeast two-hybrid assays and cell-binding studies on neuronal and non-neuronal cells (Tubulekas et al., 1997).

Binding of LamR to PrP is mediated either directly or indirectly depending on the presence of heparin sulfate proteoglycan (HSPG) on the cell surface (Hundt et al., 2001). On the NTD, the region between amino acid residues 161 and 180 was identified as a direct cellular PrP binding region using a yeast two-hybrid screen. A second PrP-binding site was identified between amino acids 205 and 229 using a mutant LamR protein lacking the direct PrP-binding domain; the site required the presence of HSPG (Kazmin et al., 2000). Two comparatively short PrP-binding sites on LamR, amino acids 263-278 and 187-210, were identified using polyclonal and monoclonal antibodies that interact with these sites and inhibit binding of PrP (Malygin et al., 2009).

Two LamR-binding domains of PrP, comprising amino acid residues 53-93 called PrPLRbd1, and 144-179 called PrPLRbd2 that mediate HSPG and direct binding with LamR respectively, were identified using a yeast two-hybrid screen (Hundt et al., 2001).

1.9.6.3 LamR-binding domains for bacteria, virus and EGCG

Despite the critical role of LamR in bacterial meningitis, only two studies have focused on the identification of bacteria-binding domains on LamR. In one of these studies, peptide G (residues 161-180) was identified as a binding site for the *E coli* K1 toxin, cytotoxic necrotizing factor 1(CNF1). Preliminary results suggested that residues between 263-282 in the C-terminal portion of LamR acted as a binding-domain for *N. meningitidis* (Orihuela et al., 2009).

Kim and co-workers identified amino acids 263-282 of LamR as binding domain for Venzuelan equine encephalitis virus (Kim et al., 2005) and Malygin and colleagues identified two regions corresponding to amino acids 263-278 and 187-210 of LamR as binding regions for the E2 protein of this virus, as well as tick-borne encephalitis virus using monoclonal antibodies (Malygin et al., 2009).

The functional domains of LamR that bind EGCG and are thus responsible for mediating its anti-cancer activity were identified within the CTN of LamR using a peptide containing the 10-amino acid residues, IPCNNKGAHS, corresponding to residues 161-170 as well as deletion/replacement of this region. This region as well as the basic amino acids, K166 and H169 were shown to interact with EGCG and to inhibit cancer cell growth (Fujimura et al., 2012).

Table 1-1 Reported differences in the laminin receptor-mediated neurotropism for various microbial pathogens from viruses to bacteria. Adapted from (Huang and Jong, 2009).

Virulence factors	Role in pathogenesis	Description	*In vitro* model	*In vivo* model
N. meningitidis	Meningitis	PilQ and PorA (adhesion)	BMECs	BBB
S. pneumoniae	Meningitis	CbpA (adhesion)	BMECs	BBB
H. influenzae	Neonatal meningitis	OmpP2 (adhesion)	BMECs	BBB
E. coli K1	Adhesion/Invasion	CNF1 (toxin)	BMECs	No
Sindbis virus	Sindbis fever	?	Hamster cells	No
Dengue virus	Dengue fever	GP E Domain II	HepG2	No
West Nile virus	Meningitis/encephalitis	GP E Domain II	Vero/PEK cells	No
Adeno-associated virus	?	Capsid protein	HeLa cells	No
TBE virus	Encephalitis	GP E Domain II	RH cells	No
VEE virus	Encephalitis	GP E	C6/36 (mosquito cell line)	No
Prion	Prion diseases	Prion protein	Neuronal cultures	No

?: Data unknown.

BBB: Blood–brain barrier; BMEC: Brain microvascular endothelial cell; CNF: Cytotoxic necrotizing factor;PEK: Pig embryo kidney; RH: Human embryonic kidney cell; TBE: Tick-borne encephalitis; VEE: Venezuelan equine encephalitis; Vero: African green monkey kidney epithelial cell

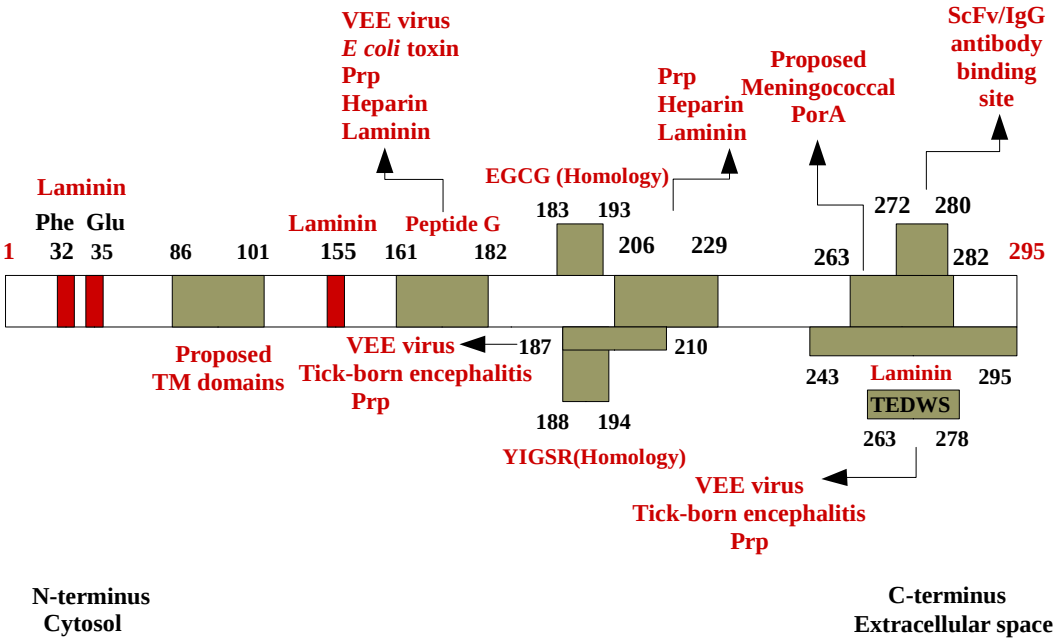


Figure 1-3 Schematic of proposed LamR Binding regions shows different binding sites.

The map shows either LamR binding regions (gray) and single-amino acid residue (red). Residues 86-101 constitute a proposed transmembrane domain; residues 32 ,35 and 155 constitute laminin-binding domain, 161-182 (also called peptide G) binds multiple ligands including laminin, heparin, prion protein, *E coli* toxin CNFI and -Venezuelan equine encephalitis virus (VEE); residues 183-193 represent a domain responsible for binding the green tea polyphenol epigallocatechin-3-gallate (EGCG); residues 188-194 (also called- YIGSR homology domain) constitute the binding site for the minimal ligand sequence of laminin required for interaction with LamR; residues 206-229 represent the binding site for laminin, heparin and prion protein: residues 243-295 constitute an additional binding sites for laminin.

1.9.7 LamR targeting for therapeutic purposes

LamR has garnered much attention as a potential target for anti-cancer therapeutic intervention. Several studies have examined possible strategies to block the interaction of LamR and ECM, either by silencing or using antibodies to block the interaction of LamR and ECM component. Several studies have confirmed the effectiveness of these strategies in reduction of cellular invasion and loss of cell attachment to laminin (Leucht et al., 2004, Zuber et al., 2008a, Zuber et al., 2008b).

Immunization of mice with recombinant LamR loaded on liposomes was not toxic and was shown to be efficient in reducing tumor metastasis. Indeed, rLamR has been included in a human cancer vaccine trial for breast cancer patients (Barsoum et al., 2009).

Furthermore, due to its ability to bind EGCG, LamR has been utilized to uptake the EGCG-containing medicine via receptor-mediated endocytosis, enabling them to exert their therapeutic effects. This strategy was applied to deliver Ruthenium Nanoparticles (RuBB-Loaded EGCG-RuNPs) antiliver cancer therapy. Notably, same medicine and strategy have been used to control Alzheimer's disease (Zhou et al., 2015).

The implications of LamR as therapy have been utilized in a variety of other diseases, including prion disease. LamR was used to reduce peripheral PrP^{Sc} propagation in the spleen (Ludewigs et al., 2007, Zuber et al., 2008a, Zuber et al., 2008b) as well as by viral delivery to reduce PrP^{sc} propagation in the brain (Zuber et al., 2008d).

In addition, truncated LamR that only expresses residues 102-295 is secreted from cells to serve as a decoy in binding and inhibiting PrP^{Sc} propagation (Vana and

Weiss, 2006). This truncated LAMR construct has therapeutic effects *in vivo* (Pflanz et al., 2009).

1.10 Galectins

1.10.1 The Galectin family

Galectins are a group of β-galactoside-binding lectins which play vital roles in cell trafficking, signalling and inflammation. Members of galectin family are present across a variety of tissues from diverse species ranging from lower invertebrates such as sponges and nematodes to mammals, including humans (Hirabayashi and Kasai, 1993). They are highly conserved in their carbohydrate recognition domain (CRD)(Barondes et al., 1994a, Dumic et al., 2006)

Currently, fifteen mammalian galectins have been described in the literature, eleven of which are expressed in humans (Cooper, 2002). These lectins are implicated in a wide range of biological processes, including tumour cell adhesion, inflammation, wound healing, and immunity (Dumic et al., 2006). Based on the structure of the CRD, galectins have been classified by Hirabayashi and Kasai into three subtypes: (i) Proto-type galectins; (ii) Tanden-repeat galectins; and (iii) Chimera-type galectins, Proto-type galectins include: galectins 1, 2, 5, 7, 10, 11, 13, 14 and 15; they each contain a single CRD. Tandem-repeat galectins includes: galectins 4, 6, 8, 9 and 12; they contain two CRDs that are joined by a linker peptide. Chimera-type galectins includes only galectin 3, which has only one CRD connected by a collagenase-sensitive domain to the N-terminus (Hirabayashi and Kasai, 1993).

1.10.2 Galectin-3 (Gal-3): structure and cell localization

Galectin-3 (Gal-3) is a 31-kDa chimera-type lectin which was initially identified as a Mac-2 antigen on the surface of murine macrophage (Ho and Springer, 1982). Based on its function, localization and biochemical properties, Gal-3 has been assigned several names including CBP-35 (Roff and Wang, 1983); IgE-binding protein (Liu and Orida, 1984); RL-29 (Cerra et al., 1985); L-34 (Raz et al., 1987) and HL-29 (Sparrow et al., 1987). After establishing the nomenclature of galectins, all these names were re-designated Galectin-3 (Gal-3)(Barondes et al., 1994a).

Among vertebrate galectins, Gal-3 seems to be unique in its structure (Houzelstein et al., 2004). It is made up of three distinct structural domains: (i) an atypical short N-terminal domain (NTD), which lacks glycan binding activity and consists of 110-130 amino acids (Barondes et al., 1994a), in which its first twelve amino acids were reported to be important for Gal-3 secretion and nuclear localization (Menon and Hughes, 1999) (ii) a repetitive collagen-like sequence; and (iii) a C-terminal carbohydrate-recognition domain that contains only one CRD and is composed of approximately 130 amino acids. The CTD of Gal-3 is responsible for the glycan binding activity due to containing glycan binding sites (Barondes et al., 1994b).

Although, Gal-3 is largely monomeric in solution, recent studies suggested that it may self-associate to form homodimers via its C-terminus. In the presence of a ligand, Gal-3 polymerizes into pentamers employing its N-terminal domain (Nieminen et al., 2007, Kavanaugh et al., 2013).

Galectin-3 is reported to be localized in the cytosol, the nucleus and the cell surface. In addition, it can be excreted extracellularly by an unknown mechanism

(Dagher et al., 1995, Hughes, 1999). Gal-3 has ability to bind various cytosolic proteins enabling it to contribute to a number of intracellular events (Dumic et al., 2006). Its ability to bind the apoptosis repressor protein, Bcl-2 conveys Gal-3 with anti-apoptotic activity (Yang et al., 1996).

Interaction of intracellular Gal-3 with host proteins is mainly via glycan independent protein-protein interaction. On the cell surface it is implicated in cell-cell adhesion as well as mediating the interaction with ECMs via its binding to cell surface glycoproteins (Ochieng et al., 2004, Dumic et al., 2006).

Unlike nuclear Gal-3, extracellular Gal-3 interacts with several glycosylated molecules on the cell surface in a glycan dependent pattern (Dumic et al., 2006).

In contrast to nuclear Gal-3, extracellular Gal-3 has pro-apoptotic activity in activated T cells (Fukumori et al., 2003). In addition, extracellular Gal-3 is implicated in cell signalling as well as migration of monocytes and macrophages (Sano et al., 2000, Ahmad et al., 2004).

1.10.3 Interaction of Gal-3 with different pathogenes

Gal-3 has been shown to recognize some pathogens and their results indicated that these interactions are mediated via the host-like glycan components in the pathogens. *Leishmania major*; *Candida albicans*; *Schistosoma mansoni*; *Trypanosoma cruzi* and *Neisseria gonorrhoeae* have all been reported to bind Gal-3 (John et al., 2002, Pelletier and Sato, 2002, Kleshchenko et al., 2004, Kohatsu et al., 2006).

These interactions can induce innate immunity, which subsequently leads to the clearance of microorganisms or conversely enhances the infection (Sato et al., 2009). Interaction of Gal-3 with some pathogens may promote the infection. For example, binding of Gal-3 to *H. pylori* facilitated the bacterial infection by

promoting bacterial adhesion to the gastric epithelium (Fowler et al., 2006, Cerliani et al., 2011).

Gal-3 has also been shown to bind bacteria including *Escherichia coli* (Fermino et al., 2011), *Pseudomonas aeruginosa* and Neisseria gonorrhoeae (Gupta et al., 1997, John et al., 2002), and *Helicobacter pylori* (Fowler et al., 2006) mainly through bacterial lipopolysaccharide.

Gal-3 is also recognized by a number of commensal bacteria via high affinity interactions that may be involved in colonization resistance and host infection by limiting the availability of Gal-3 to pathogens (Kavanaugh et al., 2013). See Figure 1-11 that shows the interaction of galectins, including Gal-3 with different pathogens.

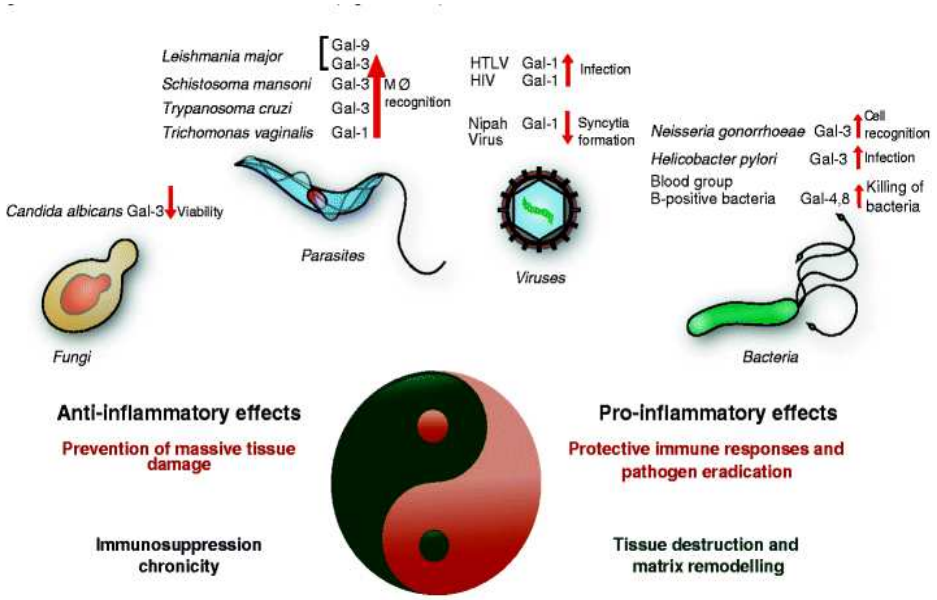


Figure 1-4 The interaction of galectins, including Gal-3 with different pathogens. Galectins recognize the glycan component of pathogens; this interaction may lead to pro-inflammatory responses and subsequent clearance of the pathogen or it may favour immune suppressive microenvironments that enhance chronic infection. *Candida albicans* for instance can be recognized by Gal-3 leading to fungal killing. Also, the recognition of some parasites, such as *Schistosoma mansoni* and *Trypanosoma cruzi*, by macrophages can be mediated by Gal-3, which subsequently leads to pathogen eradication. Gal-3 can also promote infections with *H. pylori* and *Neisseria gonorrhoeae* by enabling bacterial adhesion and bacterial host cell recognition, respectively. Adapted from (Cerliani et al., 2011).

Chapter two

Construction of plasmids to facilitate the expression of mutant forms of laminin receptor in bacterial and human expression systems

CHAPTER TWO

2 CONSTRUCTION OF PLASMIDS TO FACILITATE THE EXPRESSION OF MUTANT FORMS OF LAMININ RECEPTOR IN BACTERIAL AND HUMAN EXPRESSION SYSTEMS

2.1 Introduction

Numerous ribosomal subunit proteins in eukaryotes contribute to cellular processes in addition to their ribosomal function (Warner and McIntosh, 2009). One example is the mammalian ribosomal protein SA (or p40), which was initially termed the 37 kDa laminin receptor precursor as it was thought to be the precursor of the mature 67 kDa laminin receptor (LamR), a protein which enables cells to interact with components of the extracellular matrix in vertebrates (Nelson et al., 2008).

The most studied aspect of LamR is its laminin binding function. Synthetic peptides derived from the LamR primary sequence have been used to identify two putative laminin-binding regions: the first is referred to as Peptide G, comprising residues 161–180 (Castronovo et al., 1991a); the second site, typically referred to as the ‘direct binding region’, is the stretch of amino acids between 205 and 229 (Landowski et al., 1995b). Both peptides can bind laminin-1 and have bioactive properties *in vivo*; these peptides also display heparan sulfate (HS) binding properties (Guo et al., 1992, Taraboletti et al., 1993, Landowski et al., 1995b, Kazmin et al., 2000). Alongside its normal physiological functions, LamR has been strongly linked to bacterial infection; 67LR promotes the internalization of cytotoxic necrotizing factor 1 (CNF1) expressing *E. coli* (Chung et al., 2003, Kim et al., 2005, Wang and Kim, 2013). In addition, 67LR acts as a receptor for prion

protein (PrP) (Gauczynski et al., 2001) and is required for the internalization of some alphaviruses, such as the Sindbis virus (Wang et al., 1992). Interestingly, it has been reported that LamR mediates contact with the blood brain barrier for the bacterial pathogens *N. meningitidis, S. pneumoniae*, and *H. influenzae*; all of which can cause bacterial meningitis. Additionally, the bacterial ligands responsible for LamR binding were identified as pneumococcal CbpA, meningococcal PilQ and PorA and *H. influenzae* OmpP2 (Orihuela et al., 2009) and specifically PorA loop 4 and OmpP2 loop 2 (Abouseada et al., 2012).

Objective of the work described in this chapter

In order to characterise the bacterial LamR binding domains, the aim of work described in this chapter was to construct plasmids to facilitate expression of several mutant derivatives of LamR in both bacterial and human expression systems.

2.2 Experimental procedure

2.2.1 Bacterial strains, growth conditions, and media

E. coli strains and plasmids used are listed in Table (2-1). Bacterial strains were routinely grown at 37ºC in Luria-Bertani (LB) agar or in LB broth (Fisher Scientific) containing antibiotics where necessary (kanamycin: 100 µg ml^{-1}; ampicillin: 50 µg ml^{-1}). Broth cultures were incubated aerobically at 37ºC with shaking at 200 rpm.

Table 2-1 Bacterial strains and plasmids used in this study.

Strains/Plasmids	Descriptions	Source or Reference
***E coli* strain**		
***E. coli* JM109**	Transformation of pET28/LamR Transformation of pEF- BOS/LamR	Promega
BL21 (DE3) pLysS	Transformation of pET28/LamR	Promega
***E. coli* XL10-Gold**	Transformation of pEF- BOS/LamR	Promega
Plasmids		
pET28/LamR	• Human laminin receptor cDNA cloned into pET28a(+) • Carries kanamycin resistence cassette	Kindly provided by Dr. Noha Abouseada (Orihuela et al., 2009)
pEF-BOS/Fc	• Mammalian expression vector encoding Enhanced Green Fluorescent Protein (EGFP) Carries ampicillin resistence cassette	Kindly provided by Dr. Lee Weldon (Akbarzadeh et al., 2008)
pEF-BOS/LamR	• Human laminin receptor cDNA cloned into pEF-BOS/Fc	Kindly provided by Dr. Shaun Moroll

2.2.2 Plasmid DNA mini-/midi-/maxi- preparations

Plasmids were extracted using Qiagen plasmid purification kits (QIAprep Spin Miniprep kit; Plasmid Midi kit; Plasmid Maxi kit). The extraction of plasmid DNA follow the same procedure except a higher volume of buffers is needed during the Maxi-prep since there is more culture volume initially. The protocols

for mini; midi and maxi-prep were performed according to the manufacturer`s instructions and the purified plasmid DNA was stored at -20ºC.

2.2.3 Gel electrophoresis and gel extraction

PCR and restriction digestion products were analysed using 0.8-1% agarose gels (APPENDIX). Electrophoresis was carried out in 1 x Tris Acetate EDTA buffer (TAE buffer, Sigma)(APPENDIX) at 5 V cm^{-1}; DNA was stained with ethidium bromide (Sigma; final concentration: 10 mg ml^{-1}) and visualized using an ultraviolet trans-illuminator. The molecular weights of DNA molecules were estimated by comparison to the 1kb DNA ladder (New England Biolab). For gel extraction of DNA fragments, bands of interest were excised using a clean scalpel blade and extracted using the QIAquick Gel Extraction kit (Qiagen) according to the manufacturer's instructions.

2.2.4 Purification of PCR and Gel-extracted DNA products

PCR and restriction digestion products were purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. Briefly, 5 volumes of binding buffer (PBT) were added to the 1 volume of DNA and the mixture added to the column. This was then centrifuged (12 000 g) for 1 min and the flow-through was discarded. Then, 750 µl of washing buffer (PE) was added and centrifuged (12 000 g) for 1 min and flow-through was discarded. After that 50 µl of nuclease free deionised water was added and incubated for 2 min at room temperature. Finally, DNA was eluted by centrifugation for 1 min (12 000 g). Purified DNA fragments were stored at -20°C.

2.2.5 Quantification of DNA and protein

The concentration of DNA and purified proteins was quantified using a NanoDrop (ND-1000) spectrophotometer (Thermo Scientific).

2.2.6 Construction of plasmids to allow the expression of LamR in bacteria and human expression system

2.2.6.1 Mutations in pET28/LamR

The LamR expression vector pET28/LamR was kindly provided by Dr Noha Abouseada. Briefly, this contains the human LamR cDNA cloned into *Nde*I/*BamH*I digested pET28a (+) and encodes an N-terminal 6 × histidine-tagged protein (Figure 2-1). pET28/LamR was used as the DNA template for a series of inverse polymerase chain reaction (IPCR) reactions to introduce deletions and point mutations into the LamR coding sequence.

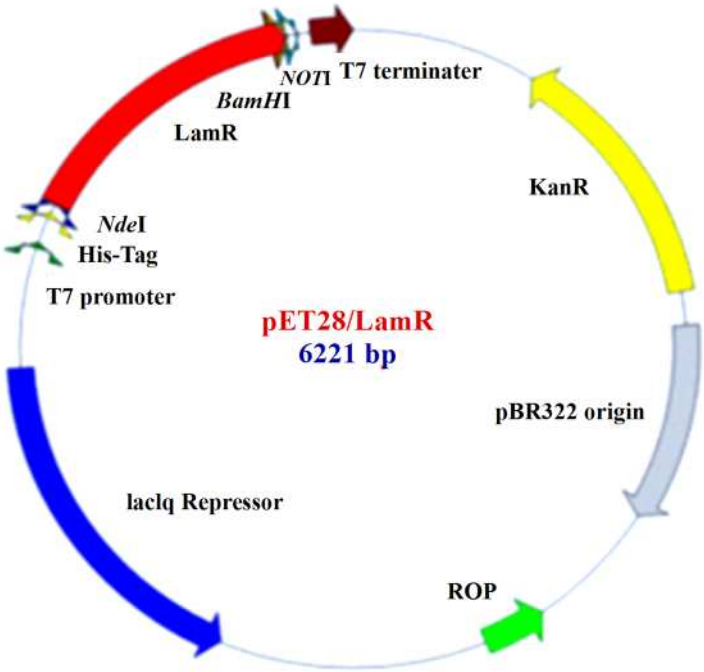


Figure 2-1 Schematic diagram of pET28/LamR plasmid map.

The bacterial expression vector contains the following functional elements: This plasmid harbours the human LamR cDNA flanked by *Nde*I and *BamH*I sites. The encoded recombinant LamR protein is histidine-tagged at the N-terminus and expressed under control of the T7 promoter (His-tag epitope allows protein detection and purification), the lactose repressor gene (lacI), the T7 promoter and terminator, kanamycin resistance gene (KanR), pBR322 origin of replication and the incorporation of the ROP sequence limits the plasmid copy number.

2.2.6.2 Deletion Mutations in pET28/LamR

2.2.6.2.1 Primer design

Several pairs of primers were designed for deletion mutations; *NheI* restriction sites were incorporated onto the 5' end of the forward and reverse primer sequences as shown in Table 2-2 All oligonucleotide primers were designed using Primer 3 software (http://frodo.wi.mit.edu/).

Table 2-2 Primers used to introduce deletion and point mutations into the LamR coding sequence.

Oligonucleotide sequences in blue represent primers to introduce deletions, sequences in brown represent primers designed for point mutations and oligonucleotide sequences in red represent *Nhe*I restriction sites.

Template Plasmids	Primer name	Oligonucleotide sequence 5' - 3'	Restriction site
Deletion Mutation			
pET28/LamR **pEF-BOS/LamR**	Del AA 206-229 For	GCGCGCTAGCGAATGGACTGCTCCCGC	*Nhe*I
	Del AA 206-229 Rev	CGCGCTAGCTCTGTAGAAGTACAGATCAGGC	*Nhe*I
	Del AA 244-295 For	GCGCGCTAGCTAAGCGGCCGCTCGA	*Nhe*I
	Del AA 244-295 Rev	GCGCGCTAGCAGGCTGAGTAGCAGTGAAC	*Nhe*I
	Del AA 262-283 For	GCGCGCTAGCCAGGCCACTGAATGGGTAG	*Nhe*I
	Del AA 263-283 Rev	GCGCGCTAGCGAATTGCTGAATAGGCACAGAG	*Nhe*I
Point mutations			
pET28/LamR **pEF-BOS/LamR**	R155A For	taacacagattctcctggcctatgtggacattgccatc	
	R155A Rev	gatggcaatgtccacataggccagaggagaatctgtgtta	
	K166A For	ccatcccatgcaacaacgcgggagctcactcagtgg	
	K166A Rev	ccactgagtgagctcccgcgttgttgcatgggatgg	
	Y139F For	cctctcacggaggcatcttttgttaacctaccta	
	Y139F Rev	taggtaggttaacaaaagatgcctccgtgagagg	
	Y156F For	ctcctctgcgctttgtggacattgccatcc	
	Y156F Rev	gatggcaatgtccacataggccagaggagaatctgtgtta	

2.2.6.2.2 Inverse Polymerase Chain Reaction (IPCR) to introduce deletion mutations

The IPCRs were performed in 300 µL reaction containing template DNA (10ng for LamR-pET28 template) -10µM- each of forward and reverse primers, 10µM dNTP mix, 5x Phusion HF reaction buffer and (1.0 units/50µl) Phusion DNA polymerase (Roche). The cycling reactions were conducted for 30S, at 98°C, followed by 34 cycles of 10 sec denaturation step at 98°C. The annealing temperature and extension time described in Table 2-3.

2.2.6.2.3 PCR site-directed mutagenesis

Primers were designed using the www.agilent.com/genomics/qcpd tool. Primers were designed to be between 25-45 bp in length, have a melting temperature (TM) between 75-85°C and GC content between 40-60%. Mutagenesis was carried out following the manufacturer's instructions using the Quick Change Lightning Site Directed Mutagenesis Kit (Agilent). Mutations were introduced by PCR using the QC Lightning Enzyme; the reagents used for PCR and the thermal cycles were described in Tables 2-4 and 2.5. PCR amplified DNA plasmids were selected by digesting parental methylated and hemi-methylated DNA plasmids with *Dpn*I enzyme at 37°C for 5 min. Digested PCR products were then used to transform chemically competent *E. coli* XL10-Gold ultra-competent cells. The transformation mixtures were spread on LB agar plates containing the appropriate antibiotic selection and grown overnight at 37°C. A single colony was selected and grown overnight in 10 ml LB medium containing the appropriate antibiotic selection and the DNA was extracted and sent for sequencing to confirm the mutation.

Table 2-3 PCR Site-directed mutagenesis reactions

Reagent	Volume	Final concentration
5 µl	10× reaction buffer	-
X µl	dsDNA template	10-100 ng
X µl	forward oligonucleotide primer	125 ng
X µl	reverse oligonucleotide primer	125 ng
1.5 µl	dNTP mix	0.2mM
1.5 µl	QuikSolution reagent	-
final volume of 50 µl	ddH_2O	-
1 µl (last add)	QuikChange Lightning Enzyme	-

Table 2-4 Cycling parameters of QuikChange Lightning reactions

Segment	Number of Cycles	Temperature	Duration
1	1	95°C	2 min
2	18	95°C	20 s
		60°C	10 s
		68°C	30 s/kb of plasmid length
3	1	68°C	5 min

2.2.6.2.4 Digestion

Following IPCR, the purified DNA was digested using *Nhe*I (NEB) according to the manufacturer's instructions. Then, DNA was extracted from gel slices containing the bands of interest using a QIAquick Gel Extraction kit (Qiagen) according to the manufacturer's instructions.

2.2.6.2.5 Ligation

Self-ligation reactions were performed using T4 ligase in a rapid ligation buffer (Fermentas) according to the manufacturer's recommended protocol. Briefly, a typical ligation reaction contained; 50 ng plasmid DNA, 1 µl T4 DNA ligase (5u μl^{-1}; Fermentas); 10 µl 5× rapid ligation buffer (Fermentas); and where required dH_2O was added to make a final volume of 50 µl. The mixture was incubated at 22°C for 24h. Ligation products were transformed into chemically competent *E. coli* JM109.

2.2.6.2.6 Transformation

E. coli JM109 chemically competent cells (Promega) were transformed as follows. Briefly, competent cells were thawed on ice and 50 µl aliquotted into two microcentrifuge tubes. 100 ng of plasmid construct was added to one tube, while the other served as a negative control. Both tubes were then incubated on ice for 20 min. Cells were then subjected to a heat shock treatment at 42°C in water bath for 50s. The cells were then immediately transferred to ice for 2 min. 400 µl of room temperature SOC medium (2% tryptone, 0.5% yeast extract, 10 mM sodium chloride, 10 mM magnesium chloride and 20 mM glucose; Sigma) was added to the cells and the tubes were incubated for 1 h with shaking at 250 rpm. Aliquots of the transformation reaction were then plated out on LB agar plates contain a

propraite antibiotic and incubated overnight at 37°C. Single colonies were picked from the plate, streaked onto LB agar plates and DNA was extracted as described in section 2.2.2 Screening for a successful clone was performed by colony PCR amplification of the desired DNA fragment and subsequent DNA sequencing.

2.2.6.3 Mutations in pEF-BOS/LamR

2.2.6.3.1 Deletion Mutations/Site-directed mutagenesis in pEF-BOS/ LamR

The mammalian expression vector, pEF-BOS/LamR, was kindly provided by Dr. Shaun Moroll and contained a wild-type copy of human LamR cDNA cloned into the *Nde*I and *Not*I sites (Figure 2-2). The expressed protein carries an N-terminal FC-tag [Fc fragment of human immunoglobulin G (IgG)]. Attempts to generate deletion mutations in pEF-BOS/LamR were unsuccessful. Therefore the wild-type copy of LamR was removed from pEF-BOS/LamR by *Nde*I and *Not*I digestion and replaced with mutated versions of the gene derived from pET28-based plasmids. Site-directed mutagenesis of pEF-BOS/LamR was performed for selected amino acid residues (Table 2-2) as described in 2.1.5.2.1.3.

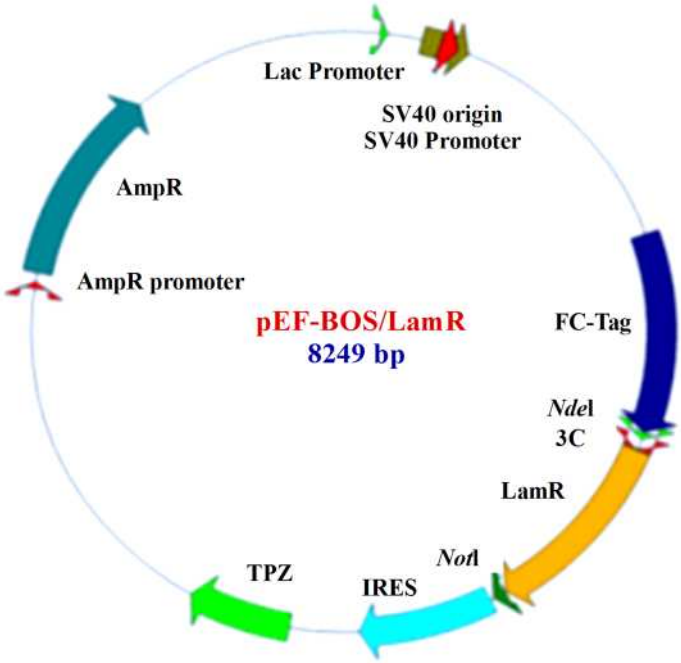


Figure 2-2 Schematic diagram of pEF-BOS/ LamR plasmid map.

The mammalian expression vector contains the following functional elements: This plasmid harbours human LamR cDNA cloned into the *Nde*I and *Not*I sites. The Simian virus 40 (SV40) polyoma origin of replication to allow the plasmid to replicate episomally in HEK293T cells that express the SV40 large T antigen. The N-terminal Fc-tag (the Fc-fragment of human antibody IgG) allows protein detection and purification, followed by 3C cleavage site, and gene encoding human laminin receptor (LamR). Downstream of the coding sequence, the internal ribosomal entry site (IRES) enables the co-translation of the enhanced green fluorescent protein (EGFP) and the selection of transfected cells. The TPZ encodes Enhanced Green Fluorescent Protein (EGFP) to monitor transfection efficiency. The ampicillin resistance gene (AmpR) was used for plasmid propagation in *E coli*.

2.2.6.3.2 DNA Ligation

A typical ligation reaction contained 100 ng insert, 50-100 ng plasmid DNA, 1 µl T4 DNA ligase (New England Biolabs), 1 µl 10× ligation buffer (New England Biolabs), and where required dH2O was added to make a final volume of 10µl. The ligation reaction was incubated at 16°C for 1h and then overnight at 4°C and finally transformed into the JM109 strain of *E. coli* as described previously.

2.3 Results

Human laminin receptor (LamR) has been recognized as an interaction partner for *N. meningitidis*, *S. pneumoniae*, and *H. influenzae*. Several regions of LamR were hypothesised to constitute the binding site for the bacterial ligands; these predictions were based on previous studies on LamR binding domains and the crystal structure of laminin receptor. Having identified likely binding regions, these were then deleted or modified by altering the LamR coding sequences in expression constructs, thus enabling allowing experiments to examine the effects on bacterial ligand binding to be undertaken.

2.3.1 Identification of LamR peptides and residues for deletion or mutation

Initially, five sites within the C-terminal portion of LamR were hypothesised as potential bacterial ligand binding regions: 161-182, 139-156, 263-282; 244-295 and 244-295 (Figure 2-3 and Figure 2-4). Based on published studies, each of these is a binding site for various ligands including host proteins, viruses or bacteria (see Figure 1-10).

However, based on the crystal structure of LamR, it was hypothesised that deletion of amino acids 161-182 and 139-156 may lead to drastic conformational changes within LamR since the majority of these amino acid residues are located within the globular structure of the protein (Lee Wheldon, personal communication). Therefore, modification of these two regions was restricted to the site-directed mutagenesis of specific surface-exposed amino acids that might interact with ligands including the meningococcal ligands. The IPCR strategy was used to create constructs encoding recombinant LamR (rLamR), rLamR$^{206-229}$, rLamR$^{263-282}$, and LamR$^{244-295}$ in both bacterial and human expression vectors, whilst site-directed mutagenesis was used to create the remainder.

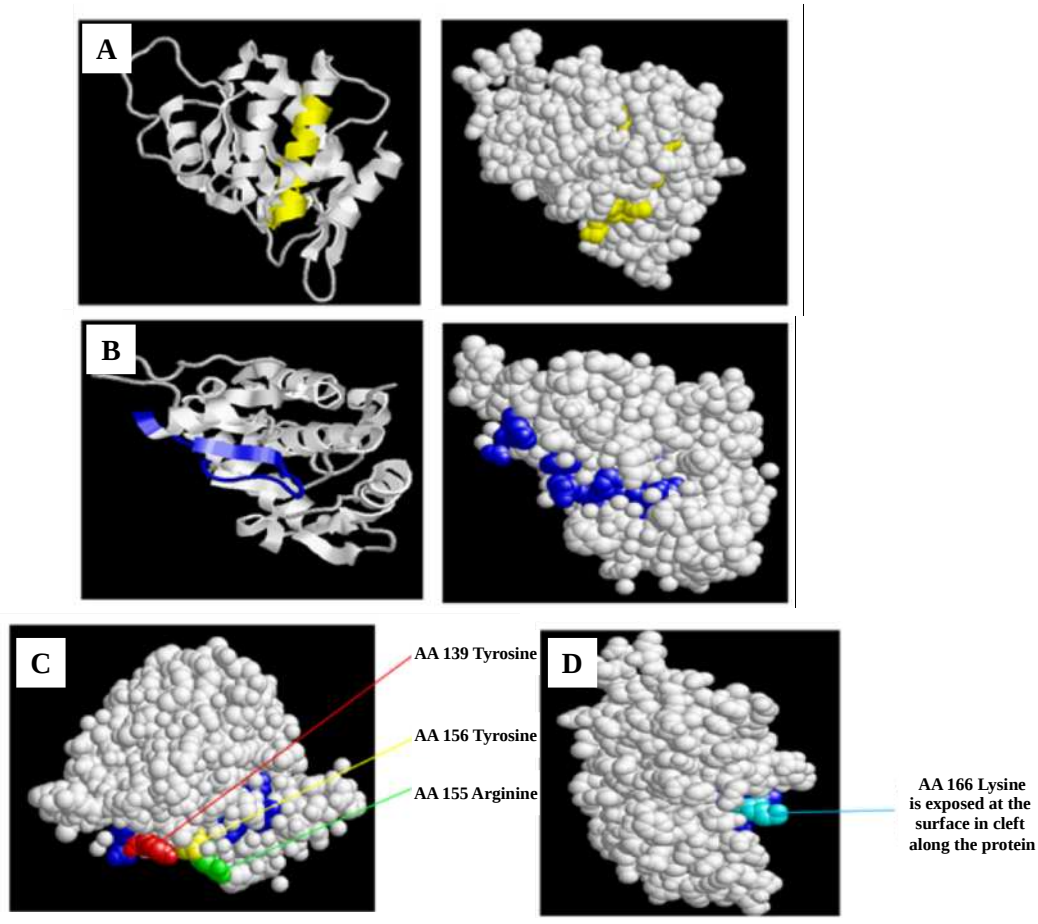


Figure 2-3 Crystal structure of 37LamR shows the regions or amino acids targeted for deletion or mutation.

The graphic figures were created using the RAZMOL program and explain the human LamR crystal structure ($LamR^{220}$)(Jamieson et al., 2008). A: aa161-182 are for the most part buried within the protein, B: similarly, aa139-156 are also predicted to largely buried in the protein, C and D: aa139 (Tyrosine), aa156 (Tyrosine), aa155 (Arginine) and aa166 (Lysine) residues are exposed at the cell surface.

- AA= Amino acids

LamR Mutation sites	Region	Reference
1- Del AA139-156	Dimerisation interface region	(Jamieson et al., 2008)
2- Del AA161-180	Heparin Binding Region	(Guo et al., 1992)
3- Del AA206-229	Direct Laminin Binding Region	(Landowski et al., 1995b)
4- Del AA244-295	Laminin Binding Region	(Nelson et al., 2008)
5- Del AA263-282	Proposed PorA/ Loop4 binding region	(Abouseada et al., 2012)

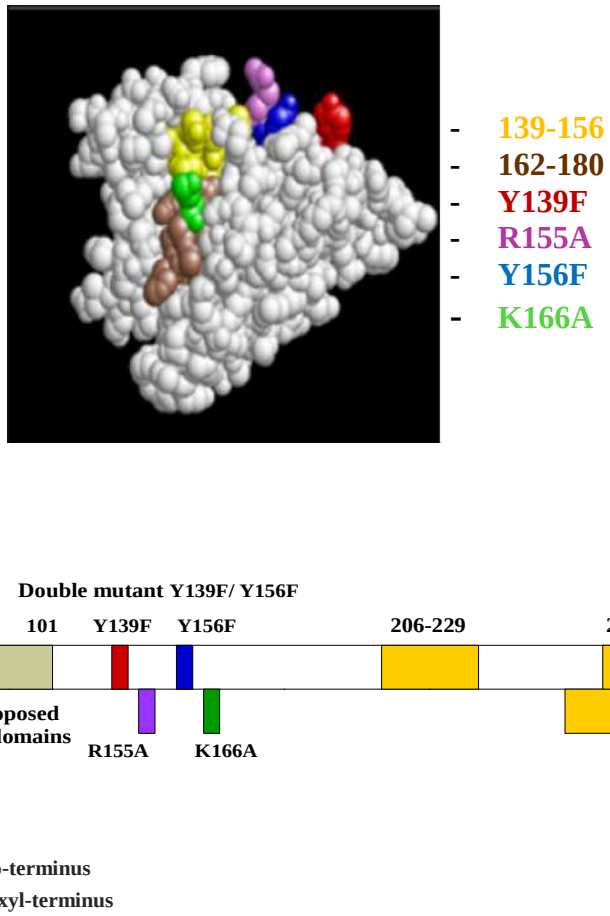


Figure 2-1 Schematic of the regions of LamR selected for deletion or mutation in order to establish their roles in the binding to various bacterial ligands.

2.3.2 Inverse PCR mutagenesis to delete DNA encoding amino acids 206-229, 263-282 and 244-295 from pET28/LamR

Mutation of LamR was achieved by inverse PCR deletion of selected regions encoding amino acids 206-229, 263-282 and 244-295 from pET28/LamR (Figure 2-1). Different primer pairs were designed for amplification of products (Table 2-2); these included *Nhe*I sites to facilitate re-ligation of digested amplicons. Initially, amplification steps were optimized in pilot experiments using different conditions, including different annealing temperatures and extension times (Figure 2-5). For example, IPCR using a temperature gradient to obtain the Δaa263-282 amplification product indicated that optimal yield was achieved using an annealing temperature of 57 or 58^{o}C (Figure 2-6). The reaction was then scaled up and the resulting amplicon digested with *Nhe*I. Digested and purified DNA was self-ligated using T4 DNA ligase (Figure 2-7) and transformed into JM109. PCR was used to confirm that the resulting clones contained inserts of the correct size. Subsequent DNA sequence analysis confirmed the identity of the clones. An identical approach was used to generate the remaining two deletions in the LamR coding sequence in pET28-LamR. Figure 2-8 explain all the steps followed to generate deletion mutations in LamR harboured on bacteria expression vector (pET28).

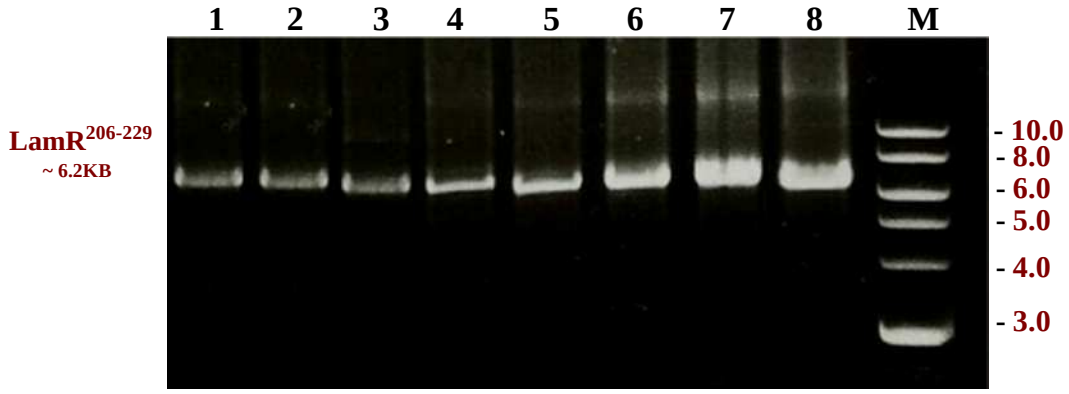


Figure 2-5 Inverse PCR amplicons produced from pET28-LamR template.

IPCR performed using a 67°C-57°C temperature gradient for annealing, and primers for the Δaa 263-282 amplicon (expected size = 6.2 kb) M: 1kb DNA marker, Lane 1: 67.0°C, Lane 2: 66.3°C, Lane 3: 65.1°C, Lane 4: 63.3°C, Lane 5: 61.0°C, Lane 6: 59.3°C, Lane 7: 58.0°C, Lane 8: 57.0°C. The same methodology was utilized to optimise the IPCRs required to obtain LamR$^{206-229}$ and LamR$^{243-295}$ (figures not shown).

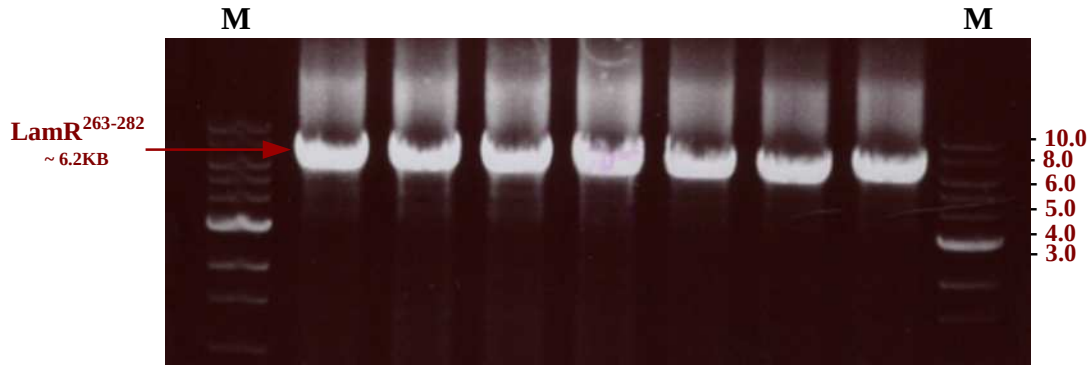


Figure 2-6 Agarose gel analysis showing inverse PCR amplicons produced from pET28 at optimal conditions.

IPCR performed at 58°C to generate deletion mutation (Δ aa 263-282) in pET28/LamR (6.2kb); M: 1kb DNA marker. WT pET28/LamR was subjected to IPCR using specific primers (Table 2.2), the primers was incorporating *Nhe*I restriction sites in both sides of the product. Same protocol was used to generate deletion mutations in both: LamR$^{206-229}$ and LamR$^{243-295}$ (figures not shown).

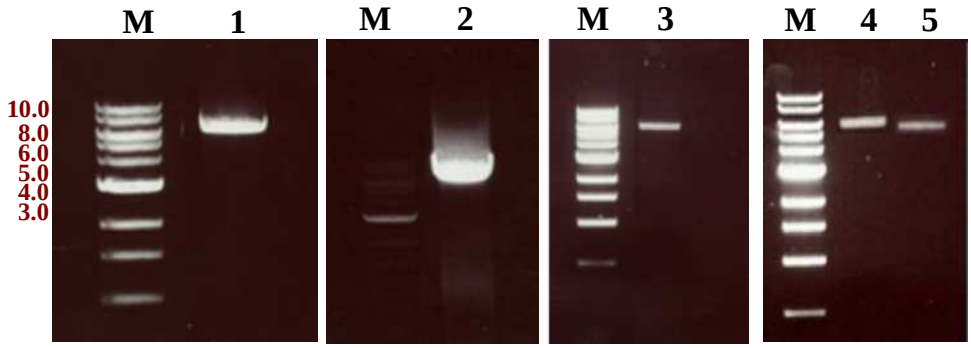
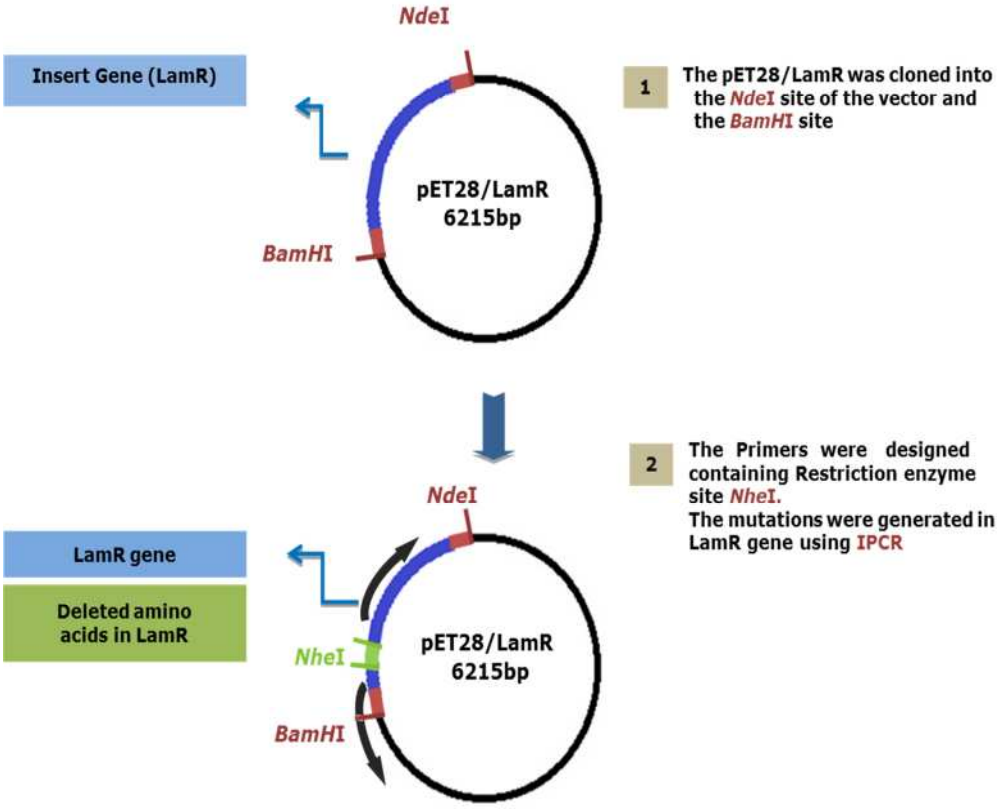


Figure 2-7 Purification, digestion and ligation of IPCR product for pET28/LamR (6.2 kb).

M: 1kb marker Lane 1: purified IPCR products, Lane 2: *NheI* digestion of pET28/LamR restriction site (6.2 kb). Lane 3: Purified product after digestion. Lane 4: purified digested IPCR products before ligation. Lane 5: purified digested IPCR products after self-ligation with T4 ligase enzyme.



3 **IPCR products were digested with Restriction enzyme site *Nhe*I**

4 **Digested products were self-ligated using T4 ligase enzyme**

5 **Ligated plasmid harbouring mutant LamR transformed into *E coli* by Transformation**

6 **Transformation of pET28/LamR into *E coli* BL21(DE3) plysS (expression host)**

7 **Isolation of the plasmid DNA (pET28) harbouring mutant LamR using Miniprep**

8 **Verification the identity of the transformed clones by sending to sequencing analysis**

9 **Expression of the recombinant protein was induced using IPTG**

Figure 2.8 Schematic diagram representing all the steps followed to generate deletion mutations in LamR harboured on bacteria expression vector (pET28).

2.4 Transformation of rLamR mutants clones into expression host

All ligated fragments were used to transform *E coli* JM109 competent cells. White-colonies were selected and PCR was used to confirm that the clones contained inserts of the correct size. Subsequent, sequence analysis confirmed the identity of the clones. To express the rLamR in bacteria, all obtained clones were used to transform the *E coli* expression host BL21(DE3) plysS. In this system

transcription of the target gene is controlled by the T7 promoter, and the production of the T7 RNA polymerase is encoded by the pLysS plasmid and is regulated by a *lac* Promoter derivative.

2.5 Inverse PCR mutagenesis to delete amino acids from the LamR coding sequence harboured on pEF-BOS/LamR

Amplification of IPCR products using pEF-bos/LamR as template was performed using primer pairs described in Table 2-2. As before, amplification steps were optimized in pilot experiments using a temperature gradient for the annealing steps (Figure 2-9) and again 58°C was determined to be the optimal annealing temperature. The reactions were then scaled up using an annealing temperature of 58^{o}C and amplified products with expected size of 8.2kb were visualized by agarose gel electrophoresis. The DNA yield of the amplicon was low but PCR fragments were nevertheless digested with *Nhe*I to yield the expected DNA fragments of 8.2 Kb (Figure 2-10). Due to the low DNA yield, an alternative strategy was performed to generate deletion mutations in pEF-BOS/LamR as described in section (2.6).

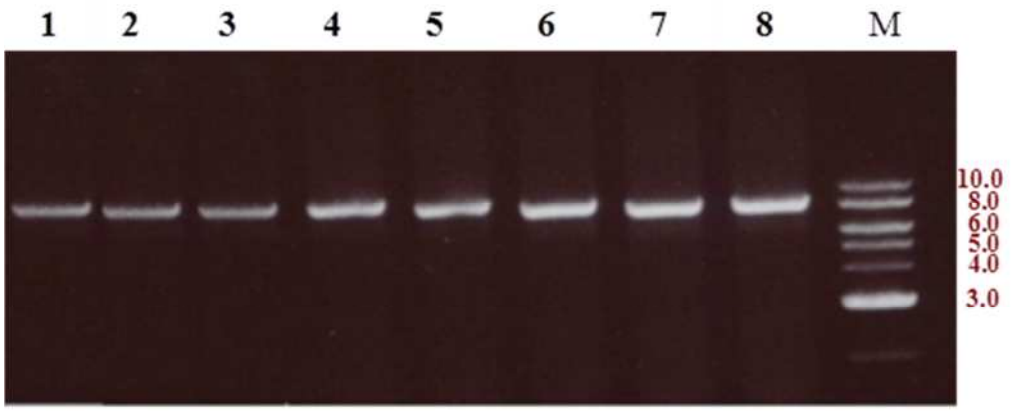


Figure 2-9 Inverse PCR amplicons produced from pEF-BOS-LamR template (~8.2kb).

IPCR performed using a 67°C-57°C temperature gradient for annealing, and primers for the Δaa 263-282 amplicon (expected size = 8.2 kb) M: 1kb DNA marker, Lane 1: 67.0°C, Lane 2: 66.3°C, Lane 3: 65.1°C, Lane 4: 63.3°C, Lane 5: 61.0°C, Lane 6: 59.3°C, Lane 7: 58.0°C, Lane 8: 57.0°C. The same methodology was utilized to optimise the IPCRs required to obtain LamR$^{\Delta 206\text{-}229}$ and LamR$^{\Delta 243\text{-}295}$ (figures not shown).

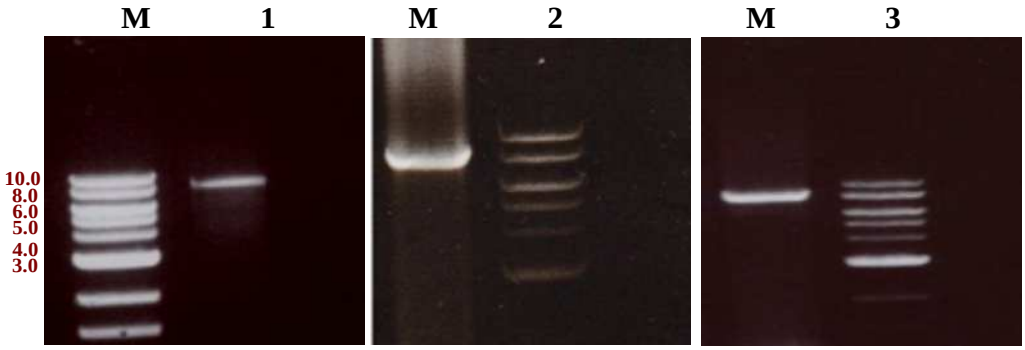


Figure 2-10 Purified and digested IPCR product for pEF-BOS/LamR.

M: 1kb DNA marker.Lane 1: purified IPCR products, Lane 2: Digested of IPCR product with *NheI* restriction site (~8.2kb). Lane 3: Purified product after digestion.

2.6 Strategy to generate deletion mutations in pEF-BOS/LamR

pET28/LamR-based deletion clones were used as the basis for pEF-BOS clones. Briefly, the mutated LamR sequences were removed from pET28 by digestion with *Not*I and *Nde*I and cloned into both pEF-BOS digested with the same enzymes (Figure 2-11). The resulting fragments which represent pEF-BOS/LamR (mutant clones) were analysed by agarose gel electrophoresis before and after ligation to verify the activity of the ligase (Figure 2-12). The ligated plasmid DNA was used to transform *E coli* JM109 and the identity of the resulting clones were confirmed by sequencing.

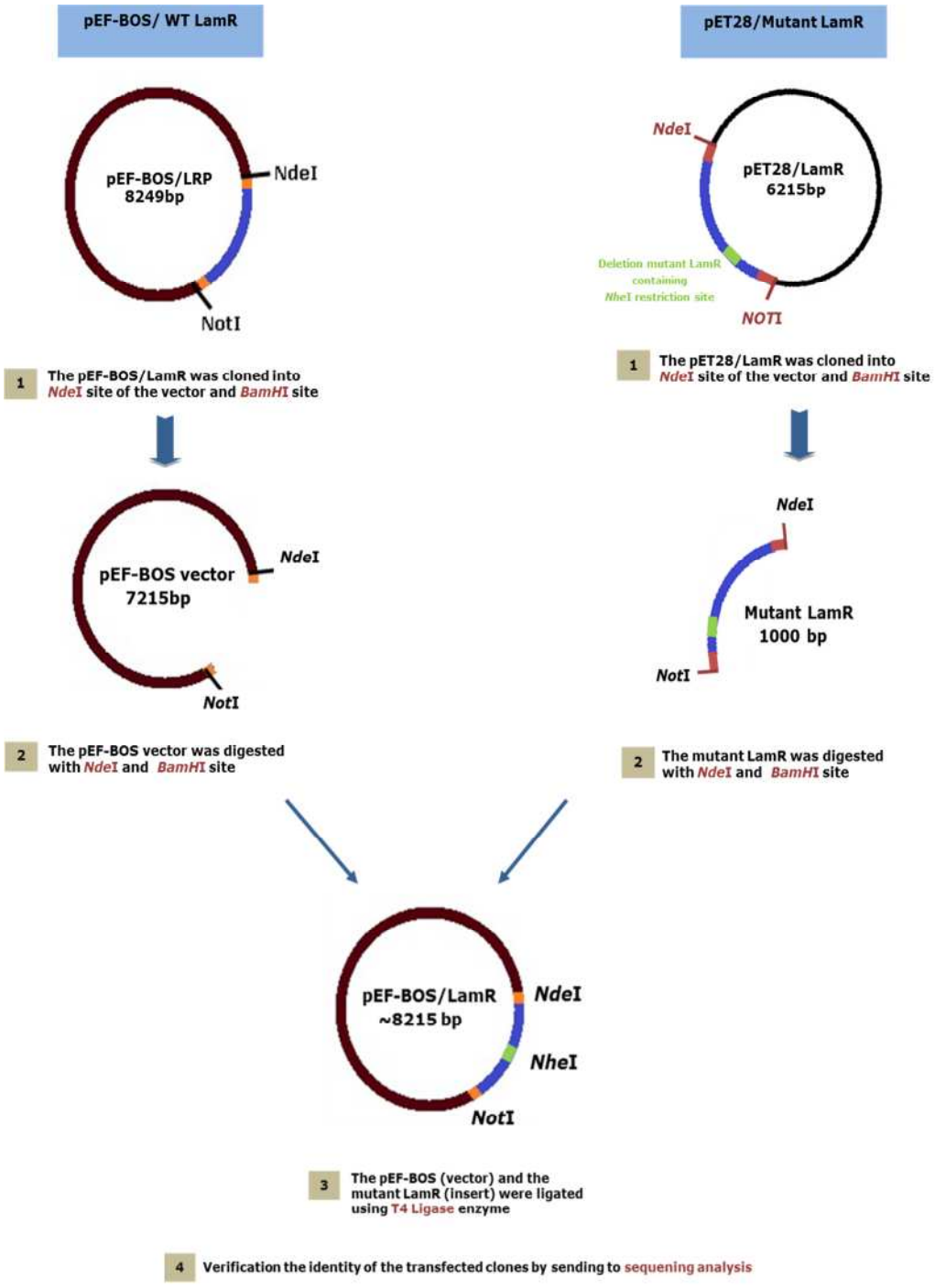


Figure 2-11 Schematic diagram representing the steps employed to sub-clone mutated LamR coding sequences into the human

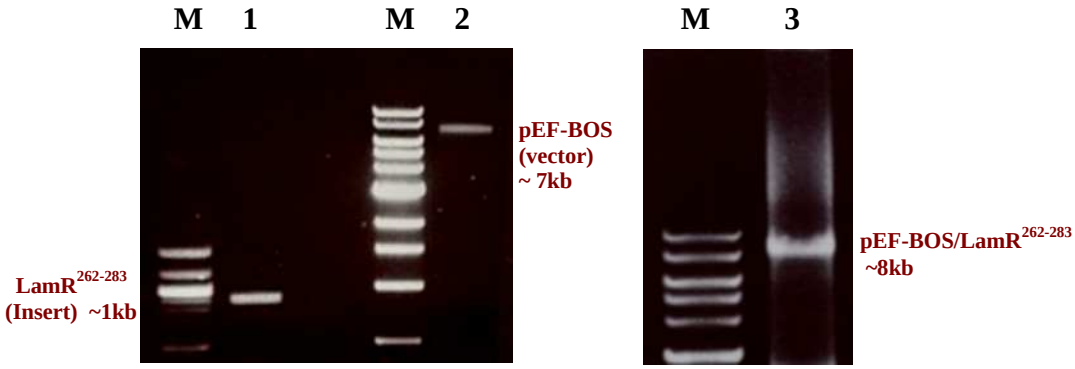


Figure 2-12 Agarose gel showing ligation of LamR$^{\Delta 263\text{-}282}$ with pEF-bos plasmid.
Lane 1: LamR$^{\Delta 263\text{-}282}$(1.2 kb), Lane 2: purified digested IPCR products after self-ligation with T4 ligase (7kb) M: 1kb marker.

2.7 Site-directed mutagenesis for pET28 and pEF-BOS

Site-directed was used to introduce point mutations into the LamR coding sequence carrying on both pET28/LamR and pEF-BOS/LamR as described in section (2.2.6.2.3). All mutations were confirmed by sequence analysis (Figure 2- and Table 2-5).

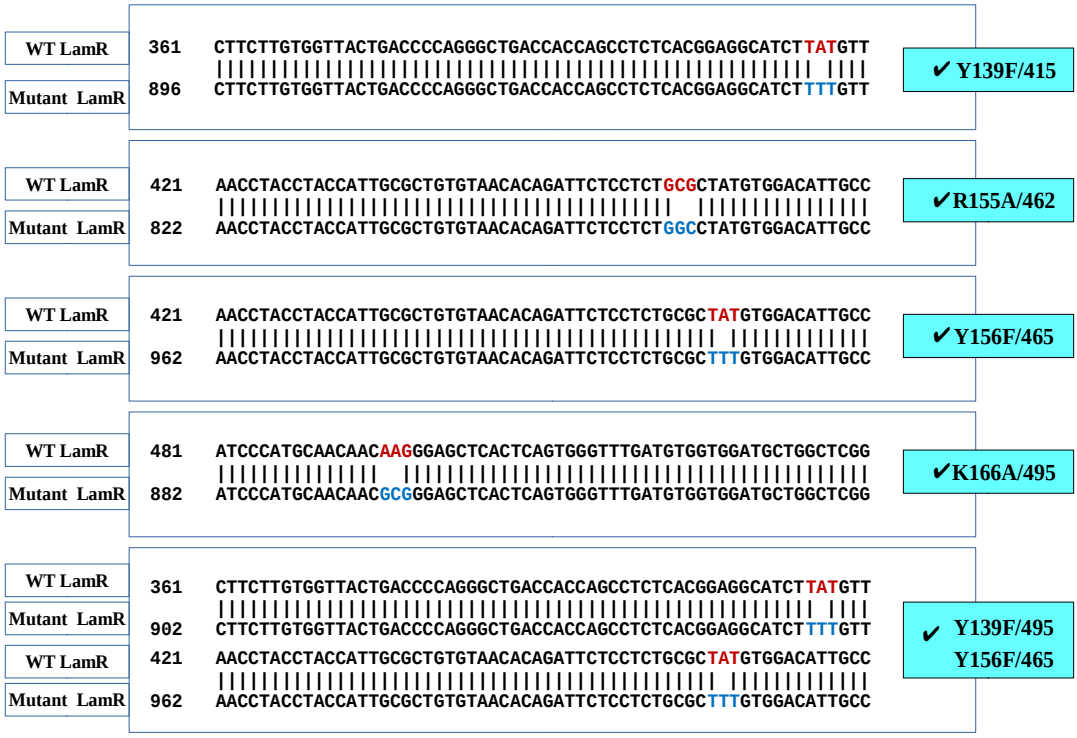


Figure 2-13 Sequence alignment showing substitution of codons in the LamR coding sequence following site directed mutagenesis.

Table 2-5 Deletion and Point mutants generated and confirmed by sequence analysis.

	Human expression clone	Bacterial expression clone
	pEF-BOS/LamR	**pET28/LamR**
	Deletion of amino acids	
1	206 – 229	206 - 229
2	243 – 295	243 - 295
3	263 – 282	263 - 282
	pEF-BOS/LamR	**pET28/LamR**
	Amino acid substitutions	
4	Y139F	Y139F
5	R155A	R155A
6	K166A	K166A
7	Y156F	Y156F
8	Y139F/Y156F	Y139F/Y156F

2.8 Discussion

The pathogenic bacteria utilise a number of mechanisms to cause disease in human hosts by expressing a wide range of molecules that bind host cell receptors to facilitate a variety of different host responses. Some of these receptors are pathogen-specific; others act as receptors for several pathogens (Huang and Jong, 2001, Pizarro-Cerda and Cossart, 2006). The non-integrin laminin receptor, LamR, has binding sites for a number of bacterial and viral pathogens, prion proteins as well as endogenous ligands (Nelson et al., 2008). Recently, LamR has been identified as a common portal of central nervous system (CNS) entry for the leading meningeal pathogens *S. pneumoniae*, *H. influenzae* and *N. meningitidis* (Orihuela et al., 2009, Abouseada et al., 2012).

In order to more deeply study the binding of LamR to its bacterial ligands, the work in this chapter aimed to construct plasmids encoding mutated versions of LamR for expression in both bacterial and cells. To this end, the plasmids vectors pET28 and pEF-BOS/Fc were used as vehicles for the expression of wild type and mutated versions of LamR. The former was employed to facilitate expression in a bacterial expression system, which offers relatively easy and rapid expression and purification of the recombinant proteins. However, rLamR may not be correctly folded or post-translationally modified in bacterial cells, and thus the derived proteins may not be biologically representative. To avoid these problems, a second plasmid (pEF-BOS/Fc) was employed to facilitate expression of in human cells where folding and post-translational modification are more likely to be similar to that found physiologically. However, expression and purification in mammalian cells is more complex and technically challenging.

As a first step toward identifying LamR binding domains for bacteria, several mutations were generated in LamR by IPCR and site-directed mutagenesis to create deletions and point mutations, respectively. Five deletion mutation constructs were generated by IPCR. Specifically, residues 161-182; 139-156; 206-229; 263-282 and 243-295 were deleted. The crystal structure of the LamR indicates that 161-182 and 139-156 regions were largely buried in the protein (Jamieson et al., 2011). As an alternative strategy to target sites that were predicted to have effects on the conformation of the protein, site-directed mutagenesis of single exposed amino acids within these regions was performed in attempt to minimise disruption to the structure of the resulting protein. Site-directed mutagenesis was successfully performed to create the following changes: Y139F; R155A; Y156F; K166A; and Y139F/Y156F using pET28 and pEF-BOS vectors to express mutant proteins in bacteria and human cells respectively. In terms of deletion mutations, several mutations were introduced into the LamR coding sequence harboured on the bacterial expression vector (pET28) using IPCR. The regions targeted for deletion encoded amino acid residues 206-229; 243-295, and 263-282. The ligation products were initially introduced into the *E coli* host strain JM109 due to its high efficiency of transformation before the constructs were transferred into the expression host *E coli* BL21 (DE3) plysS. This strain harbours the plysS plasmid, which encodes the T7 RNA polymerase gene required for induction of the gene encoded by the recombinant pET28 vector. As with pET28/LamR, similar deletion mutations were introduced into the LamR coding sequence harboured by expression vector pEF-BOS. Although, IPCR and digestion conditions were optimized, low DNA yields resulted in several unsuccessful digestion, ligation and transformation results. To address this

problem, mutant clones initially constructed in pET28/LamR were sub-cloned successfully into pEF-BOS and their identities confirmed by sequencing.

The precise contact sites between 67LamR and laminin have not been mapped. However, several studies results support the hypothesis that multiple laminin binding sites are present on LamR. In the current work, we examined mutation of a previously identified putative laminin and PrP binding sites (161-180 and 206-229) and one of the laminin binding sites (243-295). The residues 161-180, the so-called peptide G (IPCNNKGAHSVGLMWWAR) has been shown to possess diverse biological effects, it was identified as a binding epitope in assays that utilized peptide segments of LamR to bind laminin, also, binds directly to the sulphated polysaccharide heparin (Chang et al., 2010). A separate study using a yeast two-hybrid system recognized the same region as direct PrP-binding domain on LamR. In addition to laminin and PrP, heparin, *E coli* toxin (Chung et al., 2003) and VEE virus (Ludwig et al., 1996) have also been shown to target the peptide G region of LamR . Remarkably, it has been found that peptide G also increases and stabilizes the binding of laminin-1 on tumour cells (Magnifico et al., 1996). The crystal structure of human LamR revealed that almost all the G peptide is solvent buried by a surface flexible loop (residues 188-197), flanked by a C-terminus α-helix (residues 198-204), making it partially inaccessible to binding of laminin-1. In addition, the residues 139-156 were predicted to constitute the interface of a 67LamR homo-dimer; this makes these residues of particular interest for the study (Jamieson et al., 2008). However, these residues are largely buried within the protein structure. The only study to attempt a mutational approach to determine the laminin binding site identified Phenylalanine 32, Glutamine 35 and Arginine 155 using a truncated LamR37(1-220) recombinant

protein expressed in *E coli*; individual or mutations in combination resulted in a significant reduction in laminin binding capacity. However, introduction of these mutations into the full-length LamR (1-295) protein did not significantly affect laminin-1 binding, suggesting that they contribute to other LamR functions (Jamieson et al., 2011). In addition to residues 161-180 and 205-229, TEDWS-containing C-terminal repeats have been implicated as a binding epitopes for laminin (Kazmin et al., 2000). Notably, the green-tea-derived polyphenol, (-)-epigallocatechin-3-gallate (EGCG) has also been reported to interact with LamR mediating the anti-tumor effects of EGCG *in vivo* (Tachibana et al., 2004, Umeda et al., 2008). The extracellular region of human LamR, residues 161-170, Lysine 166 and 169 Histidine on LamR were identified as binding sites for EGCG (Fujimura et al., 2012).

In the current study, a mutagenesis strategy was devised to identify LamR domain functional sites and the principles that guided our approach were to avoid the mutagenesis of inferred buried hydrophobic residues. For that purpose, substitutions for surface exposed residues were chosen in an attempt not to disrupt the overall structure of the LamR protein. For this reason, tyrosine 139 and 156 were replaced by Phenylalanine. Although both aromatic amino acids are similar in their structure, the presence of the hydroxyl group in tyrosine in place of the ortho hydrogen on the benzene ring of Phenylalanine, making it much more likely to be involved in protein interactions. In addition, in the current study, Arginine 155 and Lysine 166 of LamR were mutated to alanine. Arginine and Lysine contain a positively charged amino group on their side chains and are quite frequently implicated in the active or binding sites of proteins; a mutation that

replaces Arginine or Lysine residues with polar short chain amino acids like alanine will thus facilitate the dissection of such interactions.

Three additional LamR regions including 206-229, 243-295 and 263-282 were also of interest as potential bacterium-interacting regions. As reported in previous studies, residues 205-229 of human LamR have been shown to bind to laminin, heparin and PrP. Modelling of the peptides derived from phage-display mapping has proposed that LamR 205-229 is mainly alpha helical with two faces: a polar heparan-sulfate-binding face and hydrophobic laminin binding face. A single study has focussed on residues 263-282 was conducted by Orihuela et al. (2009). In this study, evidence was presented supporting the hypothesis that pneumococcus, meningococcus, and *H. influenzae* target a common carboxyl-terminal recognition site on LamR (amino acids 263-282), since antibodies recognizing this sequence, or a synthetic peptide corresponding to $LamR^{263-282}$ could decrease bacterial adherence to microvascular endothelial cells. In a subsequent study, the binding of a synthetic peptide derived from the meningococcal PorA loop4 with synthetic LamR peptides, 161-182 and 263-282 was tested using ELISA assay. The synthetic loop4 bound the full length LamR as well as synthetic peptide (263-282), but not a synthetic peptide corresponding to residues 161-182 (Abouseada et al., 2012). These observations confirm the importance of the carboxyl terminus of LamR in meningococcal infection. However, *N. meningitidis* has two LamR ligands: PorA and PilQ; the relative contribution of these adhesins as well as their respective binding sites needs further investigation.

Concluding remarks

For the first time deletion and single mutant forms of full length LamR expressed in both bacterial and human expression vectors has been undertaken. In total, 18 plasmids were constructed successfully; the expression and purification of wild-type and mutated LamR will be discussed in detail in the next chapter.

Chapter three

Expression and purification of full-length human laminin receptor and its derivatives using bacterial and human expression systems

CHAPTER THREE

3 EXPRESSION AND PURIFICATION OF FULL-LENGTH HUMAN LAMININ RECEPTOR AND ITS DERIVATIVES USING BACTERIAL AND HUMAN EXPRESSION SYSTEMS

3.1 Introduction

Human LamR is encoded by the ribosomal protein SA (RPSA) gene which is composed of 5833 base pairs (Wewer et al., 1986). By fluorescence in situ hybridization, Jackers et al. localized *RPSA* to chromosome 3p21.3 (Jackers et al., 1996). The cDNA of *RPSA* is formed by the assembly of seven exons, six of which correspond to the coding sequence; LamR, as predicted from its cDNA sequence, is 295 amino acid residues long with a predicted molecular mass of 32.8kDa (Rao et al., 1989, Rea et al., 2012). LamR can be sub-divided in two main domains: an N-domain, comprising residues 1-209 (corresponding to exons 2-5 of *RPSA*) is highly conserved and homologous to the prokaryotic ribosomal protein S2 (RPS2); and a C-domain which displays more sequence divergence and is highly-negatively charged, and encompasses residues 210-295 (corresponds to exons 6 and 7 of *RPSA*) (Ardini et al., 1998, Nelson et al., 2008, Zidane et al., 2013). LamR has been identified as the p40S ribosome-associated protein (Siianova, 1992, Sorokin et al., 2000, Rosano and Ceccarelli, 2014); in fact phylogenetic analyses have suggested that LamR is a ribosomal protein that has acquired the function of a laminin receptor (Mecham, 1991, Ardini et al., 1998, Scheiman et al., 2010b). Using truncated $LamR^{1-220}$, Scheiman et al showed that the N-terminal domain of LamR was incorporated into ribosomes, suggesting that such a domain is sufficient for ribosomal binding (Jamieson et al., 2008).

The high resolution (2.15 Å) crystal structure of human $LamR^{1-220}$ has been solved by Jameson et al from crystals grown at 17^{o}C (Jamieson et al., 2008). The result showed that $LamR^{1-220}$ was structurally similar to prokaryotic ribosome protein SA, comprising 5 α-helices and 7 β-sheets. Although this structure provided insights into some LamR functions, in particular its binding to laminin, the absence of the unstructured C-terminus from the model means that some aspects are still unclear (Jamieson et al., 2008). In another study, recombinant C and N-terminal domains and full-length LamR were studied individually; Ould-Abieh and co-workers proposed multiple folding states for LamR domains and they found that the C-domain was intrinsically disordered, while the N-domain was mostly in an intermediate state of folding at 37°C as well as full-length LamR (Ould-Abeih et al., 2012).

It is clear now that LamR is a multifunctional protein with affinity for numerous ligands. Several investigations have examined the ligand-binding domains on the N and C-termini separately, either using synthetic peptides corresponding to certain sequences, or by truncated the LamR domain(s). Zidane et al (2013) found that laminin targets both N- and C-domains of purified recombinant 37LamR expressed in *E. coli*, while only the N-domain was found to be targeted by heparin, which competed for binding with laminin. Similar to heparin, green tea polyphenol epigallocatechin-3-gallate (EGCG) also exhibits N-domain binding only (Zidane et al., 2013). To characterize the role of LamR and its interaction with ligands expressed by different bacterial pathogens, the full-length wild type LamR and mutant derivatives of this protein detailed in chapter two were expressed in mammalian cells.

Both prokaryotic and eukaryotic expression systems can be used to express recombinant protein, but choosing the optimal system is vital to the success of an expression experiment (Yin et al., 2007). The advantages of prokaryotic systems is their low cost and easy handling, however the expression of eukaryotic proteins in a bacterial system is not ideal option (Khow and Suntrarachun, 2012, Wuest et al., 2012). Generally, bacterially expressed protein differs from mammalian produced protein in several ways, including the fact that bacteria lack the proper machinery to perform post-translation modifications in the produced protein. In *E coli* for example, the pathway for native disulphide bond formation involves the CXXC-containing Dsb family of proteins (Dsb-D) and may be analogous to the eukaryotic pathway (Roszczenko et al., 2012). However, due to the reducing environment of the bacterial cytoplasm, *E coli* is not favourable for the expression of proteins containing disulphide bonds leading to the formation of inactive inclusion bodies (Baneyx and Mujacic, 2004). Therefore, additional *in vitro* refolding is necessary to acquire biologically functional proteins (Yamaguchi and Miyazaki, 2014). In contrast, mammalian cells usually allow the formation of the disulphide bonds which are crucial for proper protein folding, stability, and activity. Overall, generation of protein in a soluble form is the preferred choice (Fakruddin et al., 2013). Therefore, using eukaryotic systems is the only way to express correctly post-translationally modified proteins (Heras et al., 2004, Baneyx and Mujacic, 2004).

Objective of the work described in this chapter

In order to study LamR, the aim of work described in this chapter was to express wild type full-length and mutated LamR in both bacterial and mammalian cells and purified under non-denaturing conditions.

3.2 Experimental procedures

3.2.1 Expression of the rLamR in a bacterial host system

E. coli BL21 (DE3) pLysS containing pET28/LamR was inoculated into 10 ml of LB medium (Fisher) containing kanamycin (100 µg ml^{-1}) or chloramphenicol (25 µg ml^{-1})(Sigma) and incubated overnight at 37°C with shaking. One litre of LB broth containing appropriate antibiotics was then inoculated with the overnight starter culture. The optical density (OD) of grown bacteria was monitored using a UV-160A spectrophotometer (Shimadzu) until it reached 0.6 (logarithmic phase). Next, bacteria were induced using 1 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Promega) for up to 24 h. After harvesting cells by centrifugation (9,000 × *g* for 10 min, 4°C) using an AllegraTM X-22R centrifuge (Beckman Coulter), cells were either stored for purification of rLamR or resuspended in 1× SDS sample buffer, boiled for 5 min at 95°C and resolved by SDS-PAGE.

3.2.2 Purification of rLamR under denatured conditions

HisPur Cobalt resin batch purification was used to purify rLamR under denature conditions. Cells from 50 ml *E. coli* cultures were re-suspended in 5 ml of lysis buffer (8M urea, 0.1M NaH_2PO_4, 0.01M Tris.HCl [pH 8.0]), followed by incubation at room temperature for 30 min with shaking. Then the mixture was sonicated in an ice bath for 15 cycles of 10 s with 5 s intervals in between using a SoniPrep Sonicator 150 (MSE). The lysate was then centrifuged at 10,000 × *g* for 30 min at 4°C to pellet cellular debris, followed by incubation of the supernatant (soluble phase) with 20 mM imidazole (Qiagen) and HisPure^{TM} cobalt resin (Fisher Scientific) overnight at 4°C. This was then passed through a gravity

column (Evergreen) and using washing buffer (8M urea, 0.1M NaH_2PO_4, 0.01M Tris.HCl, [pH 6.3]). The protein was finally eluted using elution buffer (8M urea, 0.1M NaH_2PO_4, 0.01M Tris.HCl, [pH 4.5]). Buffer exchange was performed using PD-10 desalting columns (GE Healthcare), replacing the acidic urea buffer with PBS (pH 7.2). Protein concentration was estimated using a Nanodrop ND-1000 spectro-photometer (NanoDrop Technologies) by measuring the absorbance at 280 nm. Proteins were stored at -20°C.

3.3 Expression of rLamR in mammalian cells

3.3.1 Mycoplasma Detection

PCR Mycoplasma Test Kit II (AppliChem) was used to screen for the presence of mycoplasma contamination in cultured cells following manufacturers' instructions.

3.3.1.1 Culture of human embryonic kidney cells (HEK293T)

Human embryonic kidney cells (HEK293T) were grown as monolayer cultures in 6-well plates, 100mm dishes and 225cm^2 tissue culture flasks (Corning) for small scale, test and large-scale transfections, respectively at 37°C, in a humidified atmosphere in the presence of 5% CO_2. Cells were grown in Dulbecco's modified Eagle's Medium (DMEM; Gibco) supplemented with 1% (v/v) antibiotic/antimycotic solution (Sigma), 10% (v/v) fetal bovine serum (FBS, Sigma) and 0.5% L-Glutamine (Sigma). Media was replaced every two days and cells were split when they reach 80% confluence. On the day before transfection, cells were split in order to reach 40-50% confluency on the day of transfection.

3.3.1.2 Harvesting and passaging cell cultures

Cells were harvested from the cell culture vessels by Trypsin-EDTA treatment. Briefly, the medium was removed from the flask and Trypsin-EDTA added (5 ml/75 cm^2 flask) and the flask incubated at 37°C for approximately 5 min. After detachment of the cells, the trypsin was neutralised with 5 ml media containing 10% (v/v) FBS and the cell suspension was transferred to a 15 ml centrifuge tube. Cells were centrifuged at room temperature for 5 min at 350 × *g*. If being passaged, the cell pellet was resuspended in 5 ml growth medium and an appropriate fraction transferred to a new flask containing growth medium.

3.3.1.3 Cryopreservation and thawing of cell cultures

After trypsination and addition of prewarmed DMEM media, cells were transferred to a falcon tube and centrifuged at 350 × *g*. In order to prevent the formation of ice crystals within the cells, cultured cells were cryopreserved by resuspending the harvested pellet in 1 ml of media containing 20% DMSO. Then, the cell mixture was transferred into cryogenic vials and immediately placed in a -80°C freezer overnight before transfer to a -150°C freezer for long-term storage. To reconstitute cultures, cryovials removed from -150°C or liquid nitrogen storage were thawed at room temperature (RT), washed once with fresh media to remove the freezing medium, and then resuspended in 10 ml of fresh media.

3.3.1.4 Determination of cell numbers and cell viability

Cells counted on a hemocytometer using a light microscope and the cell viability was estimated by a trypan blue exclusion method, thereby determining the live/dead ratio of cells and the total cell concentration per milliliter.

3.3.1.5 Transfection of HEK293T cells using calcium phosphate precipitation

For small-scale transfection; cells were grown in 10 cm^2 (1 well of a 6-well plate) to 40-50% confluence before transfection. Transfection mixes were prepared by dilution of 3.3µg DNA in 8.6µl 2M $CaCl_2$ and the volume made up to 83µl by adding dH_2O. After mixing, the DNA/$CaPO_4$ precipitation was dropped into 83µl of 2× Hepes-buffered saline (HBS and the mix incubated for 5-10 minutes at room temperature (a fine opaque precipitate was observed at this stage). Then the mixture was added drop wise to each well containing cells and incubated at 37°C, 5% CO_2 for 18-24h. After overnight incubation, the medium was replaced with Ultra CHO cell medium (Lonza) and cells incubated for a further 48h. Cells were then observed by fluorescence microscopy and images recorded using a Nikon fluorescence microscope. Cells were harvested 72h post transfection for protein purification; culture supernatants were removed and centrifuged at 350 × *g* for 10 min at 4°C and the supernatant transferred to a clean tube. The total protein extracts from cells were obtained by lysis (all lysis steps were performed on ice or at 4°C to prevent proteolytic degradation of the target) in the radio immunoprecipitation assay buffer [RIPA, 85% v/v 1M TE, 3.8% v/v 4M NaCl, 10% v/v 10% NP40 (nonyl phenoxypolyethoxylethanol), 1% v/v 10% SDS, 5 mg ml^{-1} deoxycolate] supplemented with Phosstop (Merck Millipore) and complete Mini EDTA-free protease inhibitor cocktail tablets (Roche) for 10 min on ice, then the cell debris removed by centrifugation at 20,000 ×*g* for 10 min at 4°C and the clarified lysate, transferred into fresh tubes. The protein samples were boiled for 5 min at 95°C in 1 × SDS-PAGE loading buffer (APPENDIX). After optimising the protocol in 6-well plate format, transfection was scaled-up to other

dish formats including 75cm^2 and 225cm^2 for test and large scale transfections, respectively.

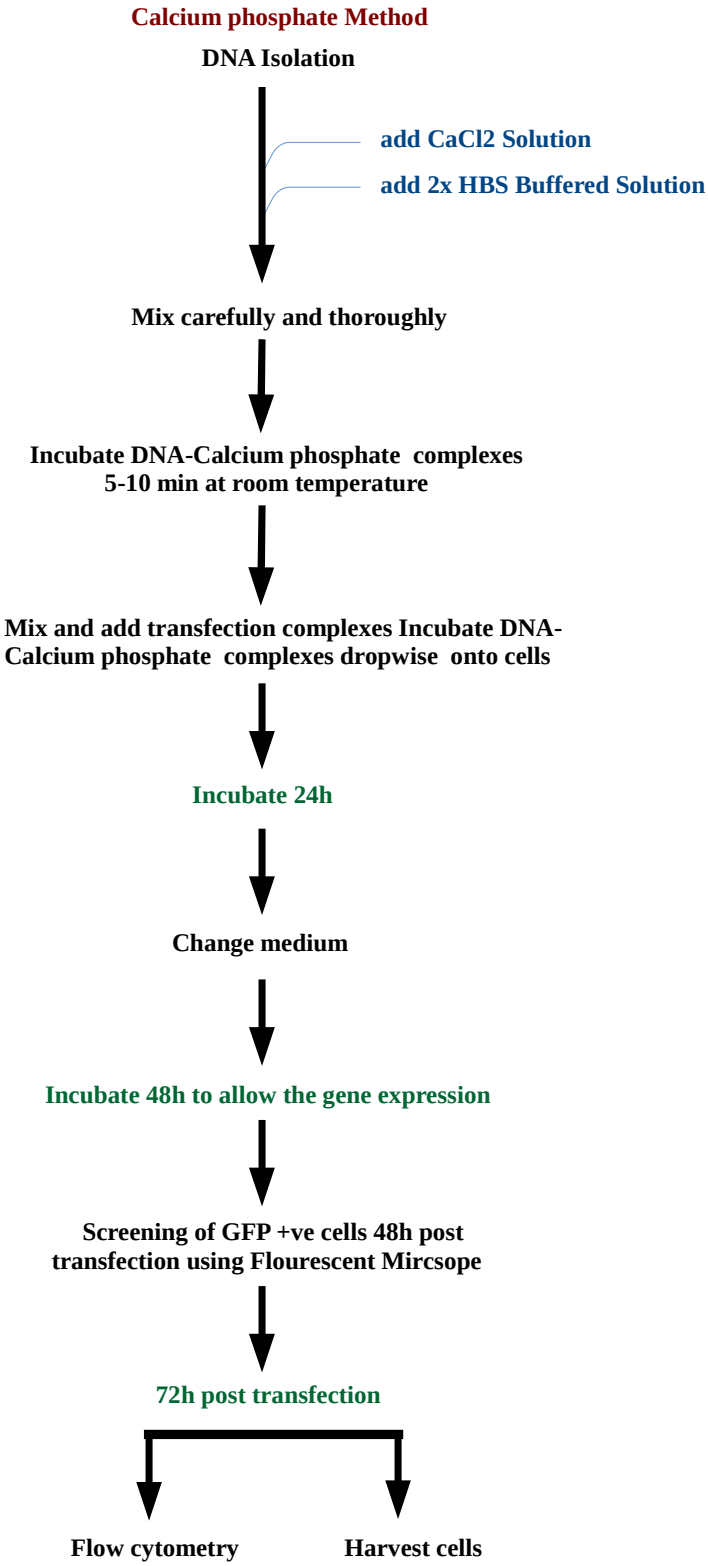


Figure 3-1 Schematic diagram representing optimised protocol for transient transfection of HEK293T cells using the CaPO4 precipitation method.

3.3.1.6 Fluorscent microscopy screening and flowcytometry assay

Expression of eGFP-tagged proteins (wild type and mutated rLamR) was initially detected by fluorescence microscopy (Nikon). The transfection efficiency was measured quantitatively using flow cytometry as described in chapter five.

3.3.1.7 Protein purification and concentration

3.3.1.7.1 Protein A affinity purification of rLamR under native conditions

Fc tagged rLamR was purified under native conditions by protein A affinity chromatography using HiTrap protein A-sepharose HP columns and the AKTA PrimePlus purification system, according to the manufacturer's instructions. Briefly, the clarified lysates were centrifuged at 20,000 × *g* for 10 min at 4°C, and lysates were mixed with an equal volume of binding/wash buffer (20mM Na_3PO_4 [pH 7.3], 150mM NaCl) using a 50 ml super loop (GE Healthcare Biosciences). The clarified lysate was then passed through 1 ml HiTraP^{TM} Protein A HP column, the column prepacked with 1 ml HiTrap Protein A Sepharose^{TM} High Performance (GE Healthcare Biosciences) connected to an ÄKTAprime Plus liquid chromatography system (GE Healthcare Biosciences) and equilibrated with 10 column volumes of binding/wash buffer. The column was loaded with 15 ml sample/binding buffer mix at a flow rate of 1 ml/min over 15 min. The fusion protein was eluted by using 0.1 M glycine (pH 2.5), and the pH neutralized with 1 M Tris.HCl (pH 8.8). Eluted proteins were then dialysed into PBS using Float-A-Lyzer G2 100-kDa dialyzers (Sigma) and stored at -80°C. The Prime view 5.0 software (Amersham Biosciences) was used to analyse and view the whole chromatography process.

3.3.1.7.2 BCA protein assay

Proteins were quantified with the BCA (Bicinchoninic Acid) assay protein quantitation kit (Thermo Scientific): a colorimetric assay which involves the reduction of Cu^{2+} to Cu^{+} by the peptidic bonds of proteins. Bicinchoninic acid chelates Cu^{+} ions with very high specificity to form a water-soluble purple colored complex. The reaction was measured by the optical absorbance at 562 nm. Protein concentrations were calculated in reference to a standard curve obtained with BSA. The working reagent was prepared by mixing 50 volumes of BCA reagent A with 1 volume of BCA reagent B. 100 µl of the standard protein fractions and samples were mixed with 2 ml of working reagent, respectively, incubated at 37°C for 30 min, and measured at 562nm on a spectrophotometer (Unicam Helios Instruments). The average absorbance values for the standard curve were plotted, and the linear regression equation for the best fit line through the points was calculated by Microsoft Excel. Average sample absorbance values in the range of the standard curve were interpolated to determine the protein concentration.

3.3.1.7.3 SDS-PAGE analysis and immunoblotting

Proteins were electrophoretically separated by 12% SDS-PAGE (APPENDIX) using Mini-Protean III equipment (BIO-RAD) at a constant 30mA per gel with a 125V voltage limit for approximately 2 h. Protein samples were resuspended in 1× SDS sample buffer and heated at 95°C for 5 min. 10 µl of sample was loaded into each well alongside 10 µl of pre-stained broad range protein markers (New England Biolabs). Separated proteins were visualized by staining with SimpleBlue™SafeStain™ (Invitrogen) according to the manufacturer's

instructions. For immunoblotting, proteins were transferred onto nitrocellulose membrane (0.22µm; BIO-RAD) in semi-dry blotting buffer (constituents) using a Trans-Blot SD semidry transfer cell (BIO-RAD) at a constant current of 19mA for 19 min. After blocking the membrane with 5% BSA/PBS for 1 h at RT or overnight with shaking at 4°C, membranes were probed with mouse anti-pentahistidine (Qiagen; 1:2000 dilution) or anti-37 laminin receptor precursor (anti-37LRP [A7]) mouse monoclonal IgG antibody (Santa Cruz Biotechnology; 1:2000 diluted) or goat anti-human IgG-Fc HRP conjugate (Millipore; 1:40000) for 1 h at RT and then washed 5 times with washing buffer (PBS-Tween 0.05%). After washing, the membrane was probed with anti-mouse IgG HRP-conjugate (Sigma; 1:30000 diluted) for 1 h at RT prior to being developed with luminata western HRP substrate (Millipore).

3.4 Results

3.4.1 Expression of wild type and mutated rLamR in bacteria

3.4.1.1 Full-length wild type rLamR can be produced in *E. coli*

In efforts to identify the bacteria binding region(s) on LamR, the first step was to express and purify rLamR for use in subsequent binding assays. pET28/LamR was transformed into E. coli BL21 (DE3) pLysS; rLamR expression was under the control of the T7 promoter and was induced by IPTG. Time course experiments demonstrated that the optimal expression was achieved by addition of a final concentration of 1mM IPTG when the cells had reached an OD600nm of 0.6 with further incubation for 3 h at 37°C (Figure 3-2). SDS-PAGE analysis revealed the production of an IPTG-induced recombinant protein with un-expected size approximate 46 kDa which was larger than expected rLamR (~37 kDa). The optimised protocol was used to produce both rLamRΔ206-229 and rLamRΔ263-282 in bacterial cells (Figure 3 4). The protein identity for each wild type (Figure 3-3) and mutated rLamR (Figure 3-5) were confirmed by immunoblot analysis with mouse anti-pentahistidine. Expression experiments using empty plasmid pSAT-9 (encoding meningococcal fructose-1,6-bisphosphate aldolase; rFBA) (Tunio et al., 2010) and pET28 were included in this experiment as positive and negative controls respectively.

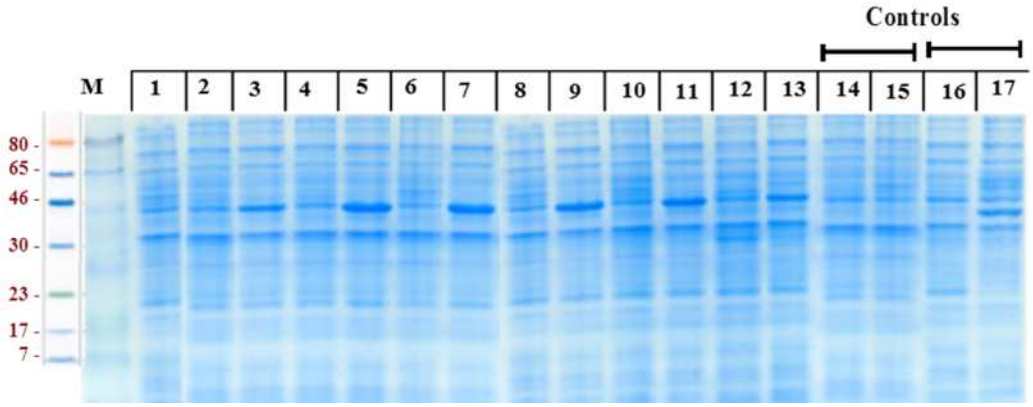


Figure 3-2 SDS-polyacrylamide gel (12%) demonstrating rLamR expression by *E. coli* BL21 (DE3) pLysS cells harbouring pET28/LamR or control plasmids before and after induction for different times with 1mM IPTG.

M: ColorPlus Prestained Protein Marker (7-175 kDa), Lane1: pre induction WTLamR in 0 h incubation Lane2: pre induction WTLamR after 1h incubation; Lane3: after 1h induction (+IPTG) of WTLamR; Lane4: pre induction WTLamR after 2hrs incubation, Lane5: after 2hrs induction of WTLamR, Lane 6: pre induction WTLamR after 3h incubation, Lane7: after 3h induction of WTLamR, Lane 8: pre induction WTLamR after 4h incubation, Lane 9: after 4h induction of WTLamR, Lane10: pre induction WTLamR after 5h incubation, Lane11: after 5h induction of WTLamR, Lane12: pre induction WTLamR after 24h incubation, Lane13: after 24hr induction of WTLamR, Lane14: pre induction BL21 (DE3) pLysS cells harbouring empty pET28 vector after 3hr induction (-ve control), Lane15: after 3h induction of BL21 (DE3) pLysS cells harbouring empty pET28 vector, Lane16: pre induction of BL21 (DE3) pLysS cells harbouring pSAT-9/rFBA (WT) after 5h induction (-ve control), Lane17: after induction of BL21 (DE3) pLysS cells harbouring pSAT-9/rFBA (WT) after 5h incubation

The size of the expressed protein: WTLamR= **46 KDa**
The size of the expressed protein: rFBA (WT)= **39 KDa** (Positive control)

- rFBA= Recombinant fructose 1, 6 bisphospate aldose

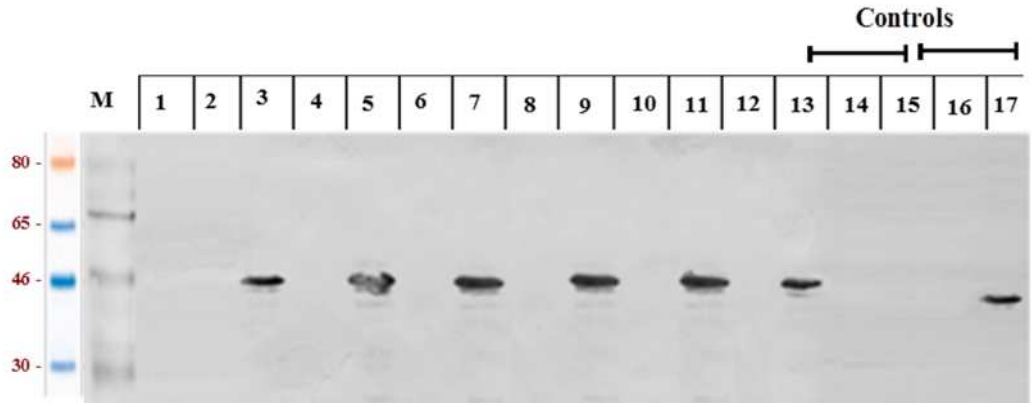


Figure 3-3 Immunoblot analysis demonstrating the yield of rLamR expression before and after induction with 1mM IPTG with different incubation times using anti-pentahistidine antibody.

M: ColorPlus Prestained Protein Marker (7-175 kDa), Lane1: pre induction WTLamR in 0 h incubation Lane2: pre induction WTLamR after 1h incubation; Lane3: after 1h induction (+IPTG) of WTLamR; Lane4: pre induction WTLamR after 2hrs incubation, Lane5: after 2hrs induction of WTLamR, Lane 6: pre induction WTLamR after 3h incubation, Lane7: after 3h induction of WTLamR, Lane 8: pre induction WTLamR after 4h incubation, Lane 9: after 4h induction of WTLamR, Lane10: pre induction WTLamR after 5h incubation, Lane11: after 5h induction of WTLamR, Lane12: pre induction WTLamR after 24h incubation, Lane13: after 24hr induction of WTLamR, Lane14: pre induction BL21 (DE3) pLysS cells harbouring empty pET28 vector after 3hr induction (-ve control), Lane15: after 3h induction of BL21 (DE3) pLysS cells harbouring empty pET28 vector, Lane16: pre induction of BL21 (DE3) pLysS cells harbouring pSAT-9/rFBA (WT) after 5h induction (-ve control), Lane17: after induction of BL21 (DE3) pLysS cells harbouring pSAT-9/rFBA (WT) after 5h incubation

The size of the expressed protein: WTLamR= **46 KDa**

The size of the expressed protein: rFBA (WT)= **39 KDa** (Positive control)

- rFBA= Recombinant fructose 1, 6 bisphospate aldose

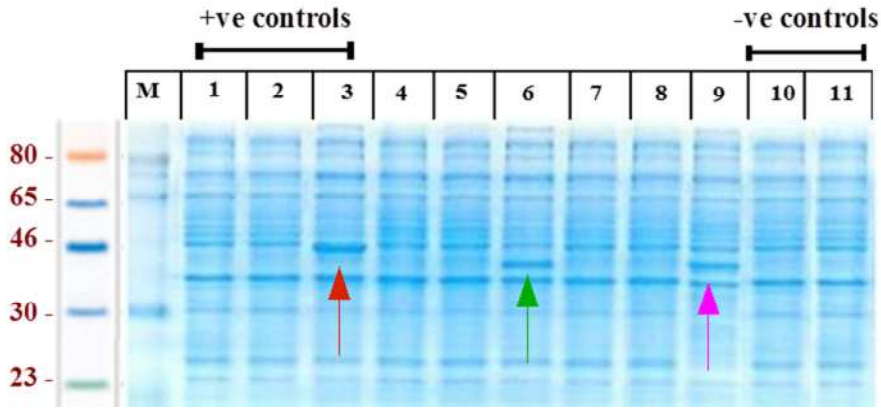


Figure 3-4 Expression of rLamR$^{\Delta 206-229}$ and rLamR$^{\Delta 263-282}$ with and without induction with 1mM IPTG for 3 h.

M: ColorPlus Prestained Protein Marker (7-175 kDa), Lane1: pre induction WTLamR after 0h incubation Lane2: pre induction WTLamR after 3h incubation; Lane 3: after 3h induction of WTLamR (+ve controls); Lane4: pre induction rLamR$^{\Delta 206-229}$ after 0hr incubation, Lane5: pre induction rLamR$^{\Delta 206-229}$ after 3h incubation, Lane6: after 3h induction of rLamR$^{\Delta 206-229}$, Lane7: pre induction rLamR$^{\Delta 263-282}$ after 0h incubation, Lane8: pre induction rLamR$^{\Delta 263-282}$ after 3h incubation, Lane9: after 3h induction of rLamR$^{\Delta 262-282}$, Lane10: pre induction empty pET28 vector after 3hr induction (-ve control), Lane11: after 3hr induction of empty pET28 vector (-ve control),

The size of the expressed protein: WT LamR= **46 KDa**

The size of the expressed rLamR$^{\Delta 206-229}$= **39 KDa**

The size of the expressed rLamR$^{\Delta 263-282}$= **39 KDa**

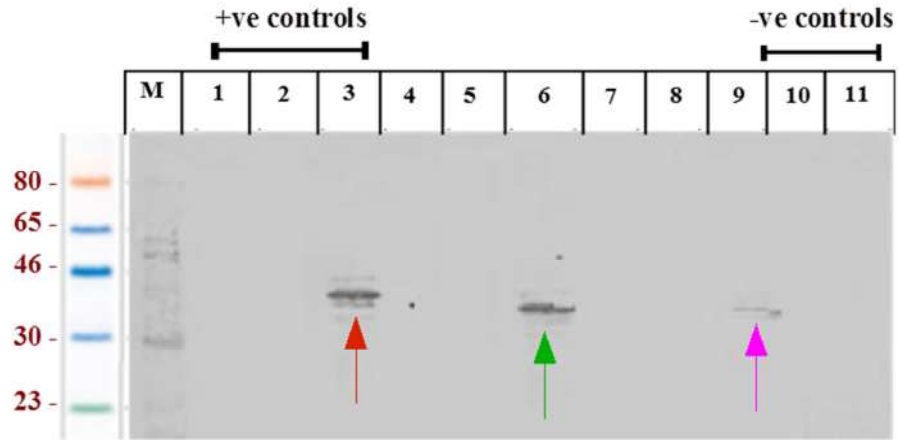


Figure 3-3 Immunoblot analysis demonstrating the yield of rLamR$^{\Delta 206-229}$ and rLamR$^{\Delta 263-282}$ rLamR with and without induction with 1mM IPTG for 3h incubation using anti-pentahistidine antibody.

M: ColorPlus Prestained Protein Marker (7-175 kDa), Lane1: pre induction WTLamR after 0h incubation Lane2: pre induction WTLamR after 3h incubation; Lane 3: after 3h induction of WTLamR (+ve controls); Lane4: pre induction rLamR$^{\Delta 206-229}$ after 0hr incubation, Lane5: pre induction rLamR$^{\Delta 206-229}$ after 3h incubation, Lane6: after 3h induction of rLamR$^{\Delta 206-229}$, Lane7: pre induction rLamR$^{\Delta 263-282}$ after 0h incubation, Lane8: pre induction rLamR$^{\Delta 263-282}$ after 3h incubation, Lane9: after 3h induction of rLamR$^{\Delta 262-282}$, Lane10: pre induction empty pET28 vector after 3hr induction (-ve control), Lane11: after 3hr induction of empty pET28 vector (-ve control),

The size of the expressed protein: WT LamR= **46 KDa**

The size of the expressed rLamR$^{\Delta 206-229}$= **39 KDa**

The size of the expressed rLamR$^{\Delta 263-282}$= **39 KDa**

3.4.2 Expression of wild type and mutated rLamR in human cells

3.4.2.1 Cell cultures were free of Mycoplasma

In order to express rLamR in human host cells, HEK293T cell line was selected for transient transfection. Before using HEK293T for cell cultures, the cells were screened for Mycoplasma contamination by PCR; the agarose gel of PCR products indicated that HEK293T cells were clear of any Mycoplasma contamination. The positive control showed a strong 270-bp band verifying that all PCR reagents are working efficiently and can generate a band, the internal control DNA produced a 191 base pair band indicating no inhibition of the PCR reaction. The HEK293T cell sample showed no band confirming that the cell is free of Mycoplasma (Figure 3-6).

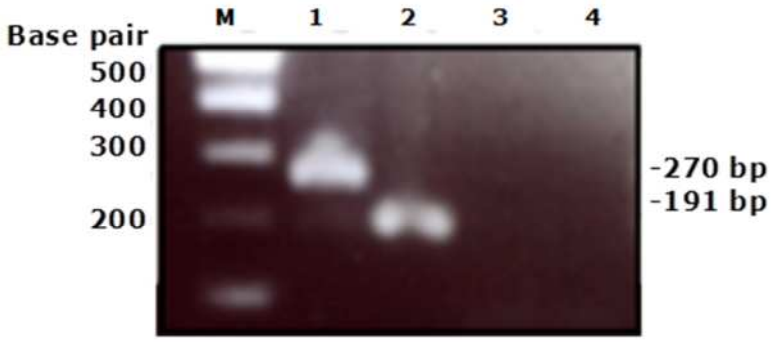


Figure 3-4 Agarose gel (1.5%) showing PCR products from different controls and HEK293T cells.

Lane1: DNA marker (100bp DNA ladder), Lane2: Positive control (270bp band), Lane3: Internal control (191bp band), Lane4: HEK293T cell (no band has been detected).

3.4.2.2 Optimizing conditions for the successful transfection of HEK293Tcells

Examination of the literature revealed that calcium phosphate precipitation and lipofectamine-based transfection are the most successful methods that have been reported to transfect HEK293T cells. To confirm this, the transfection efficiencies

of three transfection reagents: calcium phosphate; jetPRIME and *Trans-IT*2020 were compared. For *Trans-IT*2020 and jetPRIME, transfections were performed according to the suppliers instructions; however, the $CaPO_4$ precipitation protocol was optimized using different concentrations of plasmid DNA (1-5µg) (data not shown). Transfection efficiecny was determined in small-scale transfection (SST; using 6-well plates) experiments based on the expression levels of eGFP (encoded by pEF-BOS-based constructs) as measured by flow cytometry at 24, 48 and 72h post transfection. For each transfection, un-transfected cells were used as a -ve control. The results demonstrated that eGFP was successfully expressed in HEK293T cells following transfection and reached highest levels of expression at 72h post transfection for all three methods. Flow cytometry results revealed that transfection of cells using $CaPO_4$ and *Trans-IT*2020 resulted in higher and similar transfection efficiencies of 81.5% and 73.5%, respectively, compared to jetPRIME, (Figure 3-7). Since it yielded the highest transfection efficiency, $CaPO_4$ was used for subsequent experiments. The expression of the proteins was detected using immunoblott assay as described later in (section 3.4.2.3.2).

3.4.2.3 Large-Scale transient transfection using $CaPO_4$ precipitation method

3.4.2.3.1 HEK293T cells can be efficiently transfected in a large-scale using $CaPO_4$ precipitation method

After establishing an optimised protocol that provided a high level of expression for rLamR using calcium phosphate in a small scale (data not shown), the protocol was scaled up to perform medium-scale transfections (MST) in 75cm^2 (data not shown), then large-scale transfections (LST) in 225cm^2-tissue culture flasks in

order to produce high quantities of protein. Similar to SST, transfection of HEK293T cells in MST and LST led to high levels of eGFP+ve cells as determined by fluorescent microscopy and flow cytometry indicating that the optimized protocol was reproducible on a larger scale. As before, pEF-BOS lacking *RPSA* (Fc-stop) was used as a +ve control, whilst non-transfected cells were used as a -ve control (Figure 3-8).

3.4.2.3.2 Immunoblotting analysis confirmed the expression of rLamR

Immunoblot analysis was performed by probing whole cell lysates with anti-37LRP [A7] (which binds to the peptide 253-289 of LamR) and anti-Fc antibodies to detect Fc-tagged rLamR. Weak bands were observed in culture supernatants obtained from cells transfected with constructs to express rLamR (results not shown). Immunoblot analysis of whole cell lysates for wild type rLamR was performed in the presence or absence of the reducing agent, 2-mercaptoethanol (2-Me) and the results showed that both endogenous LamR (eLamR; 38 kDa) and rLamR (78 kDa) were detected. It was observed that the amount and size of wild type LamR was not affected regardless of the presence or absence of the reducing agent (Figure 3-9A).

Note that in some samples, such as rLamR$^{\Delta 263\text{-}282}$ and LamR$^{\Delta 243\text{-}295}$, rLamR was not detectable using anti-37LRP antibody since the binding sites for the antibody had been deleted in these proteins (Figure 3-9B). rLamR could also be detected using anti-Fc antibodies confirming that the rLamR proteins were Fc-tagged (Figure 3-9C). Controls used in these immunoblotting analyses (such as Fc stop and Fc-3ATM) were included as they lack rLamR, but contain Fc-tagged proteins. Non-transfected cells were used as a further control to show the absence of rLamR, but presence of eLamR. Collectively these results showed the expression of the rLamR in only transfected cells and eLamR in all transfected and non-transfected cells.

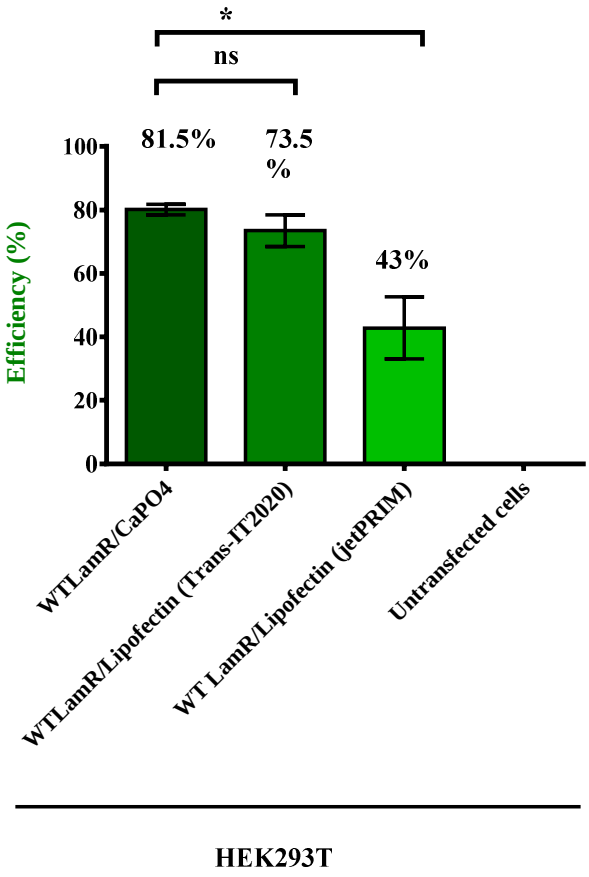


Figure 3-7 Optimisation the transfer of plasmid DNA into HEK293T cells using different transfection reagents.

HEK293T cells transfected using CaPO4; *Trans-IT*2020 and jetPRIME. The transfection efficiency of each method was screened 72 h post transfection by measuring the fluorescent intensity of cells as the plasmid DNA encodes eGFP. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. *Significant at $P \leq 0.05$.

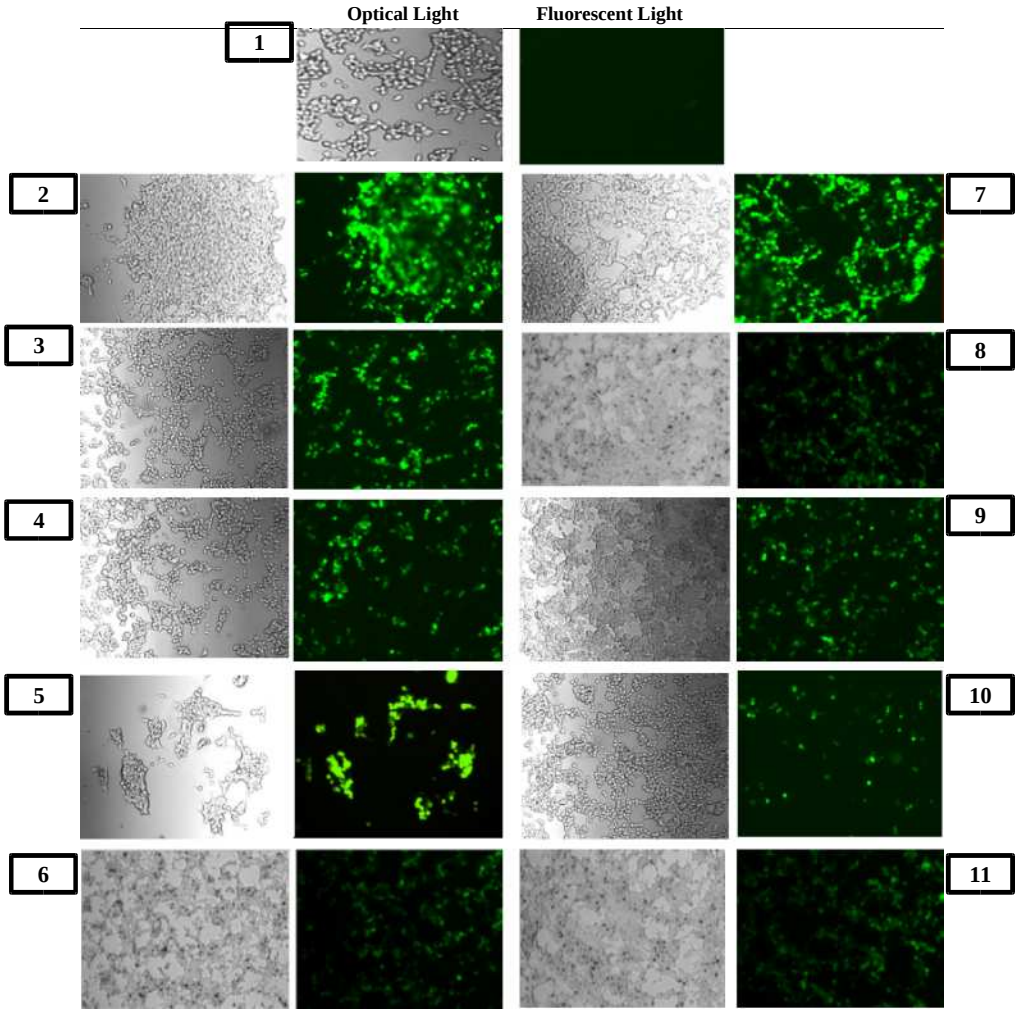


Figure 3-8 Fluorescent microscopy screening 48 h post LST (large-scale transfection) for eGFP signal.

For each image, the optical and fluorescent light imaging of cells are shown; GFP signals (green) indicating successful expression in HEK29T cells, 1: non-transfected cells; 2: WT rLamR; 3: rLamR$^{206\text{-}229}$; 4: rLamR$^{243\text{-}282}$; 5: rLamR$^{263\text{-}295}$; 6: Fc-stop (+ve control); 7: rLamR^{Y139F}; 8: rLamR^{R155A}; 9: rLamR^{Y156F}; 10: rLamR^{K166A}; 11: rLamR$^{Y139F/Y156F}$.

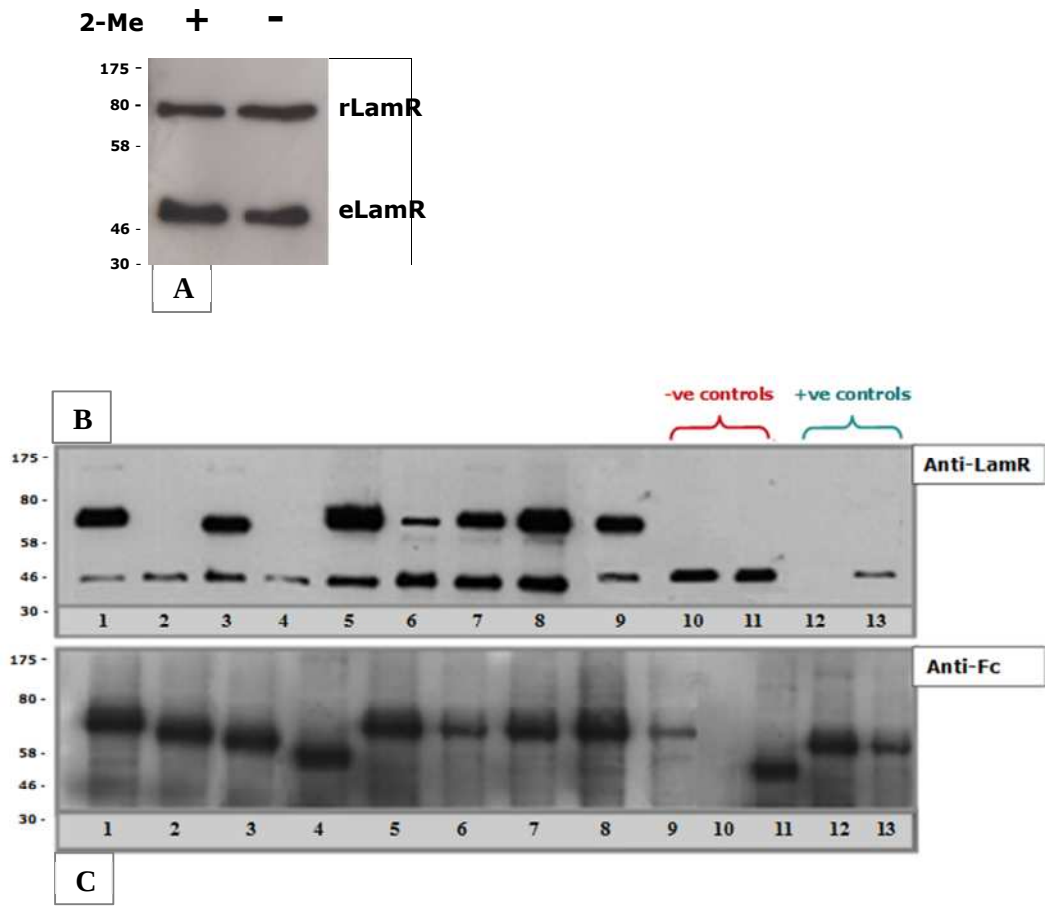


Figure 3-9 Agarose Immunoblotting analysis for the presence of LamR in whole cell lysates (WCL) of HEK293T cells 72 h post large-scale transfection.

Immunoblotting analysis performed using) anti-37LRP [A7] (A & B and anti-Fc antibodies (C). In A, the test was performed under reducing (2-Me (+)) or non-reducing (2-Me (−)) conditions using anti-37LRP [A7] antibody; the results showed eLamR and rLamR with expected size of 38 kDa and 78 kDa, respectively. (B & C) The test was performed under reducing conditions. In both B & C, Lane 1: WT LamR; Lane 2: $rLamR^{263-282}$; Lane 3: $rLamR^{206-229}$; Lane 4: $rLamR^{243-295}$; Lane 5: $rLamR^{Y139F/Y156F}$; Lane 6: $rLamR^{k166A}$; Lane 7: $rLamR^{155A}$; Lane 8: $rLamR^{Y156F}$; Lane 9: $rLamR^{Y139F}$; Lane 10: Non transfected cells (-ve control); Lane 11: Fc stop (-ve control); Lane 12: Fc-3ATM (culture supernatant); Lane 13: Fc-3ATM (WCL).

- 2-Me indicates 2-mercaptoethanol
- Binding sites of anti-37LRP [A7] with LamR= 253-289

3.4.2.3.3 Purification of highly pure and soluble rLamR proteins using protein A affinity chromatography

Purification of rLamR and its mutant derivatives from whole cell lysates was achieved under native conditions using the ÄKTA prime plus liquid chromatography system (HPLC) as described in section (3.3.1.7.1). The purification of each mutant protein was similar to that of wild type LamR and most mutated Fc-tagged LamR purified proteins, as well as the wild type, were collected in fractions 7 and 8. These results indicated that the mutations did not interfere with protein folding or stability (Figure 3-10, 3-11 and 3-12). The fractions were analysed by SDS-PAGE and immunoblotting with anti-LRP [A7] and anti-Fc antibodies. Proteins obtained from a purification procedure are assayed to estimate the yield. Protein concentration was determined by reference to a standard curve of the purified protein. Proteins purified include: wild type LamR; rLamR$^{206\text{-}229}$; rLamR$^{263\text{-}282}$; rLamR$^{243\text{-}295}$; rLamR^{Y139F}; rLamR^{155A}; rLamR^{Y156F}; rLamR$^{Y139F/Y156F}$; rLamR^{k166A}; Fc-stop and 3ATM proteins.

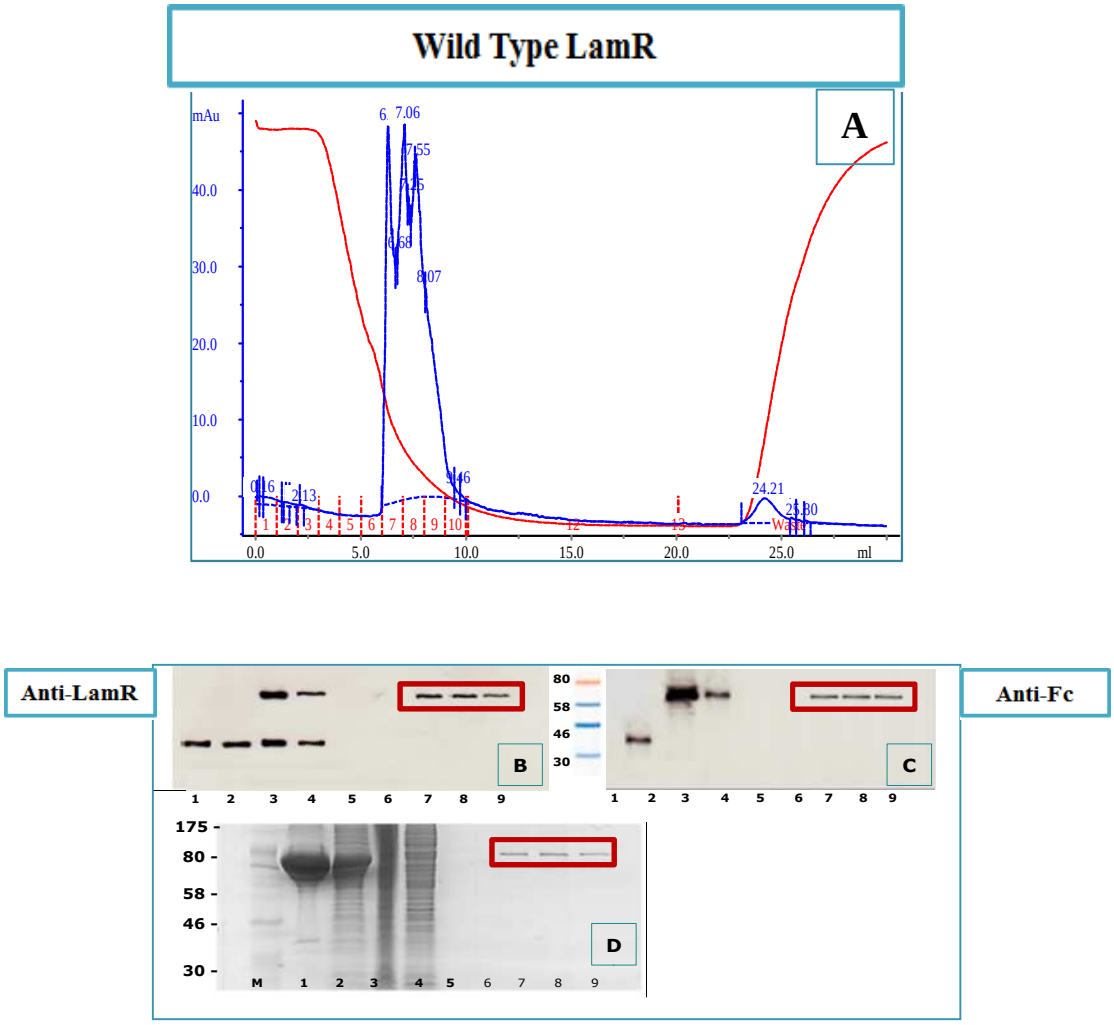


Figure 3-5 Purification of wild type rLamR.

(A) Chromatogram of purification of WT rLamR by step elution. rLamR binds to Protein A-Sepharose inside the column via its Fc tag, started to elute after passage of 6 ml elution buffer (peak of UV absorbance), purified rLamR was collected in fractions 7,8 & 9. A declining UV absorbance (blue line) indicated completion of the elution process. Immunoblot analysis of collected fractions of purified protein probed with (B) Anti-37LRP [A7] and Anti-Fc antibodies (C) and SDS-PAGE analysis of purified protein (D). Whole cell lysate of non-transfected cells; Fc-stop or rLamR transfected cells showed band with 38 kDa for eLamR with anti-37LRP [A7] antibody. While with anti-Fc antibody, only Fc-stop and Fc-tagged rLamR showed corresponding bands. Lane 1: Non-transfected cells (-ve control); Lane 2: Fc stop transfected cells (-ve control); Lane 3: Precolumn whole cell lysate; Lane 4: Flow through; Lane 5: Washing from column; Lane 6: Eluted sample, fraction 6; Lane 7: purified rLamR, fraction 7; purified rLamR, fraction 8; Lane 9: Eluted sample, fraction 8. The red box indicates bands corresponding to rLamR.

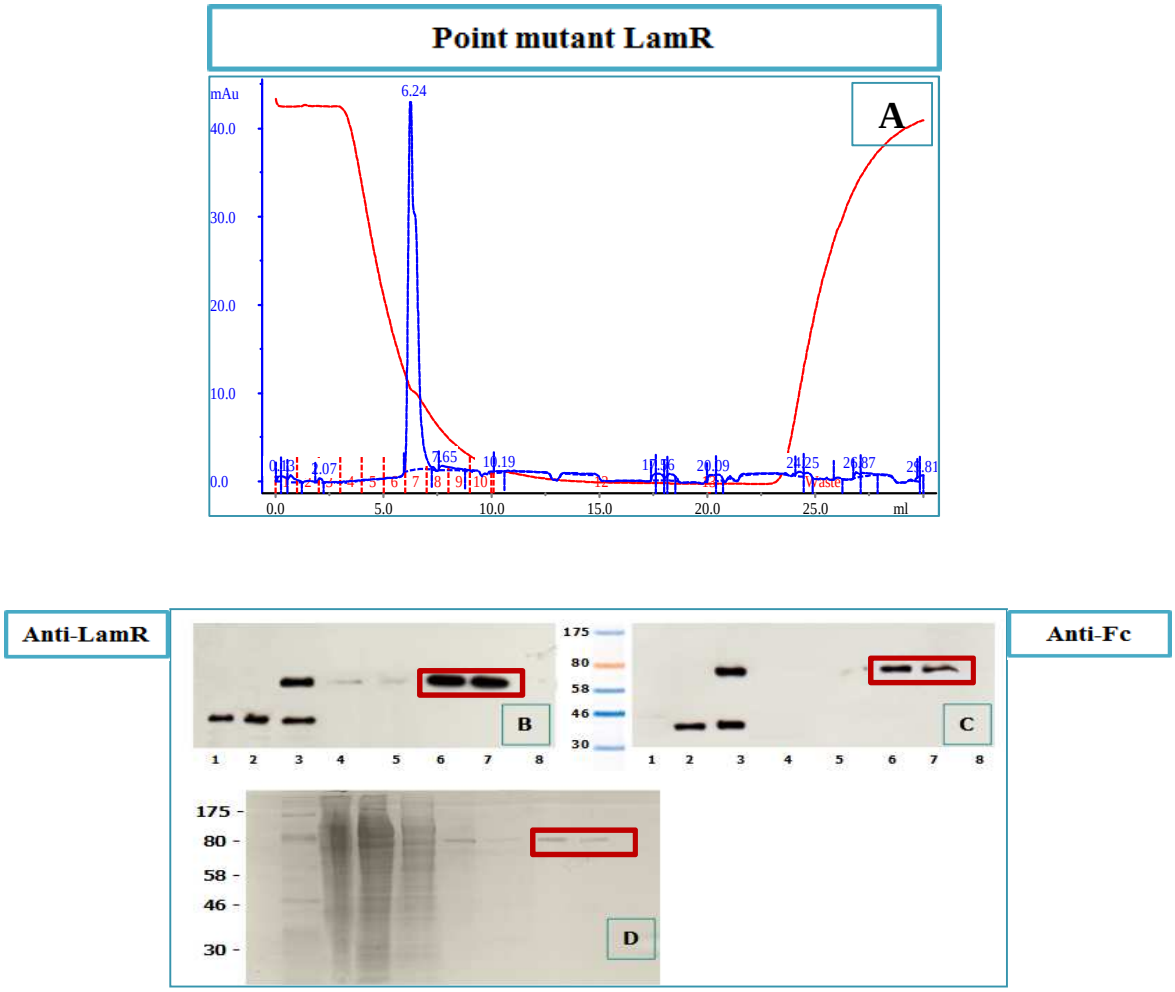


Figure 3-6 Purification of mutated rLamR.

(A) Chromatogram of purification of wild type rLamR by step elution. rLamR binds to Protein A-Sepharose inside the column via its Fc tag, started to elute after passage of 6.24 ml elution buffer (peak of UV absorbance), purified rLamR was collected in fractions 6 & 7. A declining UV absorbance (blue line) indicated completion of the elution process. Immunoblot analysis of collected fractions of purified protein probed with (B) Anti-37LRP [A7] and Anti-Fc antibodies (C) and SDS-PAGE analysis of purified protein (D). Whole cell lysate of non-transfected cells; Fc-stop or rLamR transfected cells showed band with 38 kDa for eLamR with anti-37LRP [A7] antibody. While with anti-Fc antibody, only Fc-stop and Fc-tagged rLamR showed corresponding bands. Lane 1: Non-transfected cells (-ve control); Lane 2: Fc stop transfected cells (-ve control); Lane 3: Precolumn whole cell lysate; Lane 4: Flow through; Lane 5: Washing from column; Lane 6: Eluted sample, fraction 6; Lane 7: purified rLamR, fraction 7; purified rLamR, fraction 8; Lane 9: Eluted sample, fraction 8. The red box indicates bands corresponding to rLamR.

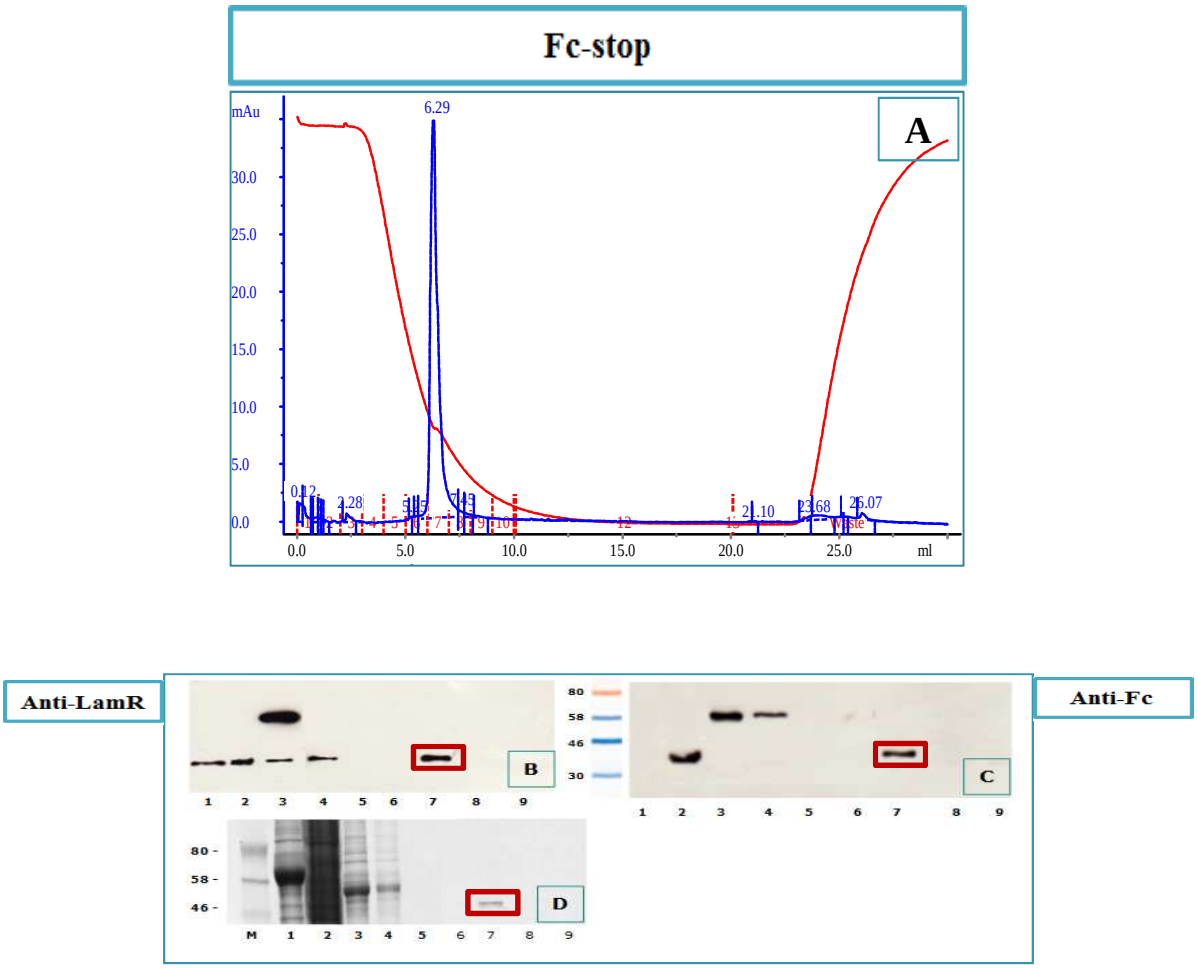


Figure 3-7 Purification of Fc-stop.

(A) Chromatogram of purification of WT rLamR by step elution. rLamR binds to Protein A-Sepharose inside the column via its Fc tag, started to elute after passage of 6.29 ml elution buffer (peak of UV absorbance), purified rLamR was collected in fractions 7. A declining UV absorbance (blue line) indicated completion of the elution process. Immunoblot analysis of collected fractions of purified protein probed with (B) Anti-37LRP [A7] and Anti-Fc antibodies (C) and SDS-PAGE analysis of purified protein (D). Whole cell lysate of non-transfected cells; Fc-stop or rLamR transfected cells showed band with 38 kDa for eLamR with anti-37LRP [A7] antibody. While with anti-Fc antibody, only Fc-stop and Fc-tagged rLamR showed corresponding bands. Lane 1: Non-transfected cells (-ve control); Lane 2: Fc stop transfected cells (-ve control); Lane 3: Precolumn whole cell lysate; Lane 4: Flow through; Lane 5: Washing from column; Lane 6: Eluted sample, fraction 6; Lane 7: purified rLamR, fraction 7; purified rLamR, fraction 8; Lane 9: Eluted sample, fraction 8. The red box indicates bands corresponding to rLamR.

3.5 Discussion

It is becoming increasingly apparent that LamR is prominently involved in many processes: the formation of organs and tissues (Rea et al., 2012), tumor growth and metastasis (Scheiman et al., 2010a), and also in the penetration of the central nervous system by different pathogenic bacteria (Kim et al., 2005, Orihuela et al., 2009, Abouseada et al., 2012, Wang and Kim, 2013) and viruses (Wang et al., 1992, Ludwig et al., 1996, Thepparit and Smith, 2004, Akache et al., 2006, Kielian et al., 2010). However, more thorough studies of LamR are needed. Examination of the literature reveals that full length, truncated or synthetic peptides corresponding to LamR have been expressed in bacterial systems and mostly under denaturing conditions. The purification and crystallisation of recombinant human 67LamR was reported several years ago (Siianova, 1992, Sorokin et al., 2000). However, this has not been followed by a description of the high-resolution structure, and has the structure been solved by other groups. Later, a high-resolution crystal structure of truncated LamR^{220} was solved for the first time by Jamieson et al. (2008). In this study, several different-length constructs were overexpressed in *E. coli*, including residues 1-295 (full-length), residues 1-220, and residues 1-195. Of these, LamR^{220} was purified in amounts suitable for crystallisation trials. Although the crystal structures of 37kDa LamR resolved many questions regarding the N-terminal domain and the details of LamR-laminin interactions, this structure includes only residues 1-220. Thus, the exact structure of the C-terminus is largely unknown (Jamieson et al., 2008).

The production of full length LamR protein in its native host in sufficient quantity and of appropriate quality is an essential pre-requisite for structural studies and to gaining a full understanding of LamR functions. We hypothesised that LamR

protein may possibly be subjected to posttranslational modification, thus, the structure and binding activity of *E. coli* and human expressed LamR may be variable. The work described in this chapter was aimed to express full length wild type LamR protein and its mutant derivatives in a mammalian cell system and to purify them under native conditions; this was hypothesised to produce protein that was properly folded, processed and active. In addition, LamR protein was also produced in bacteria and purified under denaturing conditions for comparative purposes. Initially, the wild type LamR and its mutant derivatives, $LamR^{\Delta 229\text{-}244}$ and $LamR^{\Delta 245\text{-}295}$ were expressed in *E coli* BL21(DE3) pLysS using pET28-based vectors which encode N-terminally His tagged proteins. The expression was induced by IPTG, and LamR protein expressed under control of the T7 promoter. *E coli* BL21(DE3) pLysS contains gene encoding T7 lysozyme which lowers the background expression level of target genes without interference with the level of expression achieved following induction with IPTG (Rosano and Ceccarelli, 2014). Wild type rLamR and its mutant derivatives including $rLamR^{\Delta 206\text{-}229}$ and $rLamR^{\Delta 263\text{-}282}$ were successfully expressed in bacteria; the expression of wild type rLamR yielded a protein with a molecular weight of approximate 43 kDa which was higher than that predicted on the basis of the length of the *RPSA* cDNA (~37 kDa). Compared to wild type rLamR size (43 kDa), expected bands of 39 kDa were observed for each $rLamR^{206\text{-}229}$ and $rLamR^{263\text{-}282}$.

Although, the theoretical molecular weight of LamR is 37 kDa, the aberrant migration of LamR on SDS-PAGE has been noted in several previous studies. Our results are consistent with reports that described a band of 43 kDa for rLamR expressed in *E. coli* which was reactive with anti-LamR antibody (Landowski et al., 1995a, Landowski et al., 1995b). Electrophoretic estimation of the LamR

molecular weight has ranged up from 37 to 43 kDa; the anomalous electrophoretic mobility of rLamR is possibly a result of its particular physicochemical properties. Since rLamR expressed in *E. coli* may not be correctly folded or post-translationally modified, and thus may not be biologically representative (Sahdev et al., 2008, Lambertz et al., 2014).

Depending on the objective of the experiment, foreign nucleic acids can be introduced into mammalian cell culture either stably or transiently. In transient gene expression (TGE), the transfected genetic material express only for a short time and is not integrated into the genome, but remains as an episomal DNA element until it is degraded. However, the high copy number of the transfected genetic material leads to high levels of protein expression for the period that the DNA exists in the cell (Adamson et al., 2011). Transient expression also permits determination of the strength of individual promoter sequences and effects of mutation and DNA deletion and rearrangement on gene expression. The transcriptional promoters used for TGE are mainly from viral sources, but cellular promoters are also employed. The immediate early cytomegalovirus (CMV) promoter/enhancer from mouse (mCMV) or human CMV (hCMV) and the simian virus40 (SV40) early promoter/enhancer are frequently used elements. In the current work the pEF-BOS/Fc-LamR construct was employed to allow expression in mammalian cells. The expressed gene is under the control of SV40 (viral promoter) (Mizushima and Nagata, 1990); the vector also contains TPz, with enhanced Green Fluorescent Protein (eGFP) as a transfection indicator. eGFP is a mutant form of GFPS65T/F64L which has emerged in recent years as a powerful reporter molecule for monitoring gene expression. Such mutations allowed GFP to rapidly fold and mature even when fused to poorly folding peptide, also it

offers higher-intensity emission after blue-light excitation with respect to wild-type GFP (Patterson et al., 1997, Lippincott-Schwartz et al., 2000, Pedelacq et al., 2006). The pEF-BOS plasmid also contains an IRES sequence to aid expression of the recombinant protein at higher levels (Goldman et al., 1996).

In order to express rLamR in mammalian cells, transient gene expression (TGE) was used (Meissner et al., 2001, Jager et al., 2013). A number of methods are currently available for TGE, prominent among these methods is calcium phosphate precipitation ($CaPO_4$); $CaPO_4$ forms a fine precipitate by interacting with DNA allowing the formation of small complexes which are internalized into mammalian cells by endocytosis, thus transfecting them. Although this procedure can be routinely used to transfect a wide variety of cell lines, $CaPO_4$ precipitation has been shown to yield superior results with the HEK293T cell line, although, optimization was required to obtain the best results using this method (Jager et al., 2013). More importantly, HEK293 cells were utilized for this study as they share many similarities and features with neuronal cells (Shaw et al., 2002). In the present study, we have modified and optimized a protocol which provides a very reproducible and efficient calcium phosphate transfection method in HEK293T cells. Initially an attempt was made to enhance TGE production by increasing the amount of transfected plasmid DNA (data not shown). However, this approach did not result in increased recombinant protein levels; on the contrary, transfection with an excess of plasmid DNA had a negative impact on the level of protein production.

The rLamR eGFP levels was monitored using fluorescent and flow cytometry at different time points including 24; 48; 72 and 96 h post transfection to determine the optimal protein expression, the higher level of expression was observed after

72 h of transfection, therefore, the transfected cells were collected at 72 h post transfection. In contrast to *E. coli*-produced LamR, immunoblot analysis of Fc-tagged rLamR in HEK293T cells revealed *ca.* 37- and 78-kDa bands at the expected size for endogenous and recombinant LamR, respectively, using both anti-LamR and anti-Fc antibodies. The results suggested that LamR is a stable protein and it retains its size and amount under the denaturing and reducing conditions of SDS-PAGE as noted in the review (Landowski et al., 1995a). Furthermore, protein expression of each mutant was similar to that of wild type LamR, which indicates that the mutations do not affect the biochemical properties of the LamR protein. The results presented in this chapter suggested that either substitution or deletion of amino acids residues from the C-terminus of the LamR do not affect the rest of the protein. Based on previous study conducted by Jamieson et al (2008), a mutation of C-terminal domain of LamR was believed to not affect the protein structure. This hypothesis was supported by deletion of 75 amino acid residues from the C-terminus of LamR which called $LamR^{220}$. In his study, $LamR^{220}$ exhibited the same affinity of binding to laminin as full length LamR. Our observations are consistent with that hypothesis.

After optimisation of the expression conditions for rLamR in mammalian cells, we looked towards finding a suitable purification method to purify rLamR under native conditions. Several investigators have attempted to purify bacterially expressed full length rLamR under non-denaturing conditions. In one study, several attempts to purify the full length rLamR under native conditions were not-successful, so their protocol instead was based on the total denaturation of the *E. coli* proteins, extraction of the recombinant protein by affinity chromatography, then renaturation of rLamR on the column prior to elution. However, renaturation

of denatured proteins may not accurately reproduce the native state. Other investigators attempting to purify full-length 37LamR and truncated LamR ($LamR^{220}$) under non-denaturing have found that only the truncated protein was shown to be soluble. It should be noted that, in the present study, several attempts to purify His-tagged LamR under native conditions were unsuccessful.

The current study was undertaken to express full length rLamR protein in a human system, taking advantage of the Fc-domain amino acids which mimic Protein A (PrA) of *Staphylococcus aureus* and provide tight binding of protein A with the hinge region on the Fc fragment of human immunoglobulin G (IgG) (Feng et al., 2012). This strategy allows the production of recombinant protein on a large scale as the Fc-tag confers a greater stability and improves the solubility of the partner protein both i*n vitro* and *in vivo*. Indeed, Fc fusion proteins have been recently exploited to enhance the expression of proteins in mammalian cells that are otherwise difficult to produce (Zhang et al., 2010). Another major advantage of using Fc fusion proteins is that the Fc tag facilitates purification through protein A chromatography to purify large quantities of proteins. In the current study, the purification of the Fc-tagged rLamR was performed using the ÄKTAprime plus liquid chromatography system. The washing/binding buffer contains sodium phosphate for protein stabilization; glycine-HCl (pH 2.5) buffer was used for dissociation of the Fc fusion protein from the column. The low pH of elution buffer may damage the protein (Bornhorst and Falke, 2000), this was avoided by adding Tris.HCl (pH 8.5) immediately to the purified protein to neutralize the pH, then after, the protein was directly dialyzed with PBS. SDS-PAGE and immunoblotting analysis results confirmed the purity and molecular mass of the purified proteins.

Concluding remarks

For the first time, full length wild type and mutated rLamR were purified from mammalian cells under native conditions. This work will be highly relevant to other researchers wishing to study LamR in relation to viruses, bacteria, laminin or cancer. Also, this work may provide a useful approach to purify a full length LamR protein for crystallisation, as the only crystal structure obtained to date lacks the C-terminal domain. The following chapter will describe work utilizing the purified rLamR proteins in interaction studies to identify regions involved in bacterial ligand binding.

CHAPTER FOUR

Localisation of the *N. meningitidis*, *H. influenzae* and *S. pneumoniae* binding domains within human LamR

CHAPTER FOUR

4 LOCALISATION OF THE *N. MENINGITIDIS' H. INFLUENZAE* AND *S. PNEUMONIAE* BINDING DOMAINS WITHIN HUMAN LAMR

4.1 Introduction

The ability of bacterial species to colonize and infect host cells is critically dependent upon their capacity to adhere to cellular surfaces of the host via host receptors (Webb and Kahler, 2008). For instance, the bacterial pathogens which infect the central nervous system (CNS) have the capability to adhere to host receptors to cross the blood brain barrier (BBB) and gain entry to the CNS, where they can proliferate (Doran et al., 2003). The mechanisms by which bacterial pathogens invade and traverse the BBB are not fully understood. However, previous findings suggest that four common meningeal pathogens: *N. meningitidis*; *S. pneumoniae*; *H. influenzae* and *E. coli* K1 co-opt the 37/67 kDa laminin receptor (LamR) located on the surface of human brain microvascular endothelial cells (HBMECs) for host cell adhesion and bacterial translocation across the BBB to invade the vascular endothelium of the BBB and cause meningitis (Chung et al., 2003, Orihuela et al., 2009, Abouseada et al., 2012).

The process of bacterial infection involves bacterial adherence to host cell surfaces followed by cellular invasion. Having shown that these bacterial pathogens recruit human LamR, receptor activity-directed affinity tagging (ReTagging) and mutagenesis studies conducted by colleagues in our research group indicated that the corresponding bacterial LamR-binding ligands were pneumococcal CbpA, meningococcal PilQ and PorA, and OmpP2 of *H. influenzae* (Orihuela et al., 2009). A subsequent study conducted by Abouseada et al., (2012)

in our group showed that the regions of PorA and OmpP2 that interacts with LamR are located on Loop4 of PorA and Loop2 of OmpP2 in *N. meningitidis* and *H. influenzae*, respectively (Abouseada et al., 2012).

Meningococcal PorA is a major outer membrane porin; it has eight predicted surface-exposed loops, the two largest of which harbour variable regions: VR1 (loop 1) and VR2 (loop 4) (van der Ende et al., 2000, Luijkx et al., 2008). These loops exhibit a high level of heterogeneity and are utilized in the determination of the meningococcal subtypes. Meningococcal PilQ is a conserved secretin that is fundamental for pilus extrusion and is naturally expressed at very high levels and forms a pore through which Tfp (type IV pilus) emerges onto the bacterial surface (Martin et al., 1993, Haghi et al., 2012). The meningococcal PilQ oligomer, which is organised as a ring, consists of 12 identical subunits. One set of pilus-associated genes, *pilM*, *pilN*, *pilO*, *pilP*, *pilQ*, is organised in an operon (Georgiadou et al., 2012). The protein products of some of these genes, when mutated, can give rise to a non-piliated phenotype that can be reversed in strains deficient for pilus retraction (Martin et al., 1993, Drake et al., 1997, Tonjum and Koomey, 1997). The CD46 receptor was first proposed as the receptor for *N. meningitidis* via the adhesive pilus tip formed by the minor pilin PilC1. However, this finding was not confirmed by subsequent studies (Kallstrom et al., 1997, Kirchner et al., 2005). More recently, Morand and colleagues found that pilus-mediated meningococcal adhesion to eukaryotic epithelial/ endothelial cells not restricted only to PilC1, their study results suggested the role of the meningococcal PilC2 variant in efficient adherence of *N. meningitidis* to endometrium-derived ME180 cells (Morand et al., 2009).

The choline-binding protein, CbpA is a major pneumococcal adhesin as well as being a component of the pneumococcal cell wall; it has eight choline-binding repeats (Rosenow et al., 1997, Brooks-Walter et al., 1999, Janulczyk et al., 2000). CbpA binds carbohydrates on pulmonary epithelial surface and CbpA-deficient mutants were found to be defective in colonization of the nasopharynx and failed to bind to various human cells *in vitro* (Zhang et al., 2000, Lu et al., 2003).

H. influenzae type b (Hib) porin, also called OmpP2, is the most abundant protein in the outer membrane (OMP), where it exists as a trimer. Secondary structure predictions suggest that Omp2 exists as β-barrel structure containing eight extracellular connecting loops (L1-L8) which differ in their lengths (Hiltke et al., 2002, Hiltke et al., 2003, Cantisani et al., 2012). OmpP2 plays fundamental roles in pathogenicity and in protection and thus represents a potential targets for therapeutic intervention or vaccine development (Cantisani et al., 2012). The contribution of Omp2 in the pathogenesis of bacterial meningitis through binding to LamR has been reported recently (Orihuela et al., 2009, Abouseada et al., 2012).

All identified LamR-ligands play roles in bacterial pathogenesis. For instance, both meningococcal PorA and OmpP2 of *H. influenzae* are abundance proteins containing surface-exposed loop structures and play vital roles in adhesion and invasion of host cells (van der Ende et al., 2000, Galdiero et al., 2003, Andersen et al., 2003, Delbaz et al., 2013). Further, meningococcal PilQ is an essential component of the type IV pilus, while CbpA is a major pneumococcal adhesin, both play critical roles in bacterial binding to host cells (Luo et al., 2005, Berry et al., 2012). Regardless of the bacterial species, each of these pathogens target the

laminin receptor via different surface proteins and share a common property of being able to cross the BBB after LamR has been engaged.

Objective of the work described in this chapter

The aim of work described in this chapter was to localise the domains of LamR responsible for binding the identified ligands in the three meningeal pathogens *N. meningitidis, S. pneumonia* and *H. influenzae*.

4.2 Experimental procedures

4.2.1 Enzyme-linked immunosorbant assay (ELISA)

All ELISA assays were performed using 96-well plates (NUNC Immobilizer Amino) treated with 5µg ml^{-1} of protein A (Sigma), which was prepared in sodium carbonate buffer (0.1M Sodium Carbonate (Na_2CO_3) and 0.1M Sodium Bicarbonate (NaH_2CO_3); pH 9.37). Various recombinant proteins were made up in PBS and 100 µl of adsorbent solution/suspension was added to each well.

4.2.1.1 Digoxigenin-labelling based ELISA for protein-whole bacterial cell interactions

All DIG-labelling was carried out using the Digoxigenin-3-0-succinyl-ε-aminocaproic acid-N-hydroxy-succinimide ester (Roche) in accordance with the manufacturer's instructions. Briefly, bacteria were harvested from an overnight chocolate agar plates and suspended in PBST (0.05% v/v), washed three times with the same buffer and finally resuspended in sodium carbonate buffer (APPENDIX). The OD_{600} was measured and 20ng (2µl of 10 ng $µl^{-1}$ of DIG) was added to 1ml of bacterial suspension with OD_{600}=1. The bacterial suspensions were incubated for 3h in the dark at room temperature (RT) on a shaking platform. Bacteria were washed three times with PBST and resuspended in 1% BSA/PBS (w/v). The OD_{600} was adjusted to 0.02 before use. Plates were incubated overnight at 4ºC, plates were washed five times and then incubated at RT for 1h with monoclonal anti-Digoxin-alkaline phosphatase antibody (produced in mouse and diluted 1:5000, Sigma). Fresh buffers and labelled bacterial cells were prepared for each experiment. After washing five times in PBS-T, 100µl of substrate2, 2'-azino-bis; 3-ethylbenzthiazoline-6-sulphonic acid (ABTS;

Chemicon international ES004) substrate dissolved in alkaline phosphatase buffer (7.879g Tris-HCl, 2.92g NaCl, 0.1016g $MgCl_2.6H_2O$ in 500 ml of H_2O, [pH: 9.5]) were added and incubated at RT for 1h. Finally, plates were read with an ELISA reader (BioTek EL800) at an absorbance of 405nm.

4.2.1.2 ELISA (Protein-protein interaction)

Purified proteins were diluted in PBS at concentration (5µg ml^{-1}) and 100µl added to protein A-treated wells. After overnight incubation in protein A at 4ºC, control wells were coated with BSA diluted in PBS or 5µg ml^{-1} Fc-stop and 3ATM (negative controls). Plates were incubated overnight at 4ºC with shaking then washed five times with PBS-T (0.05% v/v) and blocked with 1% BSA/PBS (w/v) overnight at 4ºC. After incubation, the plates were washed again five times with PBS-T, followed by addition of 5µg ml^{-1} of purified recombinant proteins overnight at 4ºC. The plate was washed five times with 350µl PBS-T with (10s soak time). One hundred microliters of the Laminin-R Antibody (A-7) mouse monoclonal IgG2b/anti-37 LRP [A7] antibody (Santa Cruz Biotechnology; 1:1000 dilution) or rabbit polyclonal anti-PorA antibody (in house; 1:8000 dilution) in PBS/1% BSA were added to each well and plates were incubated overnight at 4ºC. Plates were washed five times, and then incubated at RT for 1h with secondary antibodies: Alkaline phosphatase-conjugated anti-mouse IgG (produced in goat and diluted 1:20000; Sigma) or alkaline phosphatase-conjugated anti-rabbit IgG (produce in goat and diluted 1:16000; Sigma). After washing five times in PBS-T, 100µl of substrate2, 2'-azino-bis; 3-ethylbenzthiazoline-6-sulphonic acid (ABTS; Chemicon international ES004) substrate dissolved in alkaline phosphatase buffer (Section 4.2.1.1) were added and incubated at RT for 1h. Finally, plates were read with an ELISA reader at an absorbance of 405nm.

4.2.1.3 Purification and refolding of recombinant His-tagged meningococcal PorA protein

His-tagged recombinant PorA was purified under denaturing conditions and refolded into its native trimers by immobilized metal ion affinity chromatography (IMAC) using an AKTA PrimPlus (GE Healthcare) following the manufacturer's instructions. After harvesting the cells, the cell pellets were thoroughly resuspended in freshly prepared lysis buffer (50mM Tris-HCl, 8M urea, 100mM NaH_2PO_4, pH 8; 1% Triton X100 was also added for solubilizing the membrane). The mixture was sonicated (15s on, 10s off for 5min on ice) using a SoniPrep Sonicator 150 (MSE). Then, the cell lysate was centrifuged at 10,000 × *g* for 30min, using an Allegra x-22R centrifuge (Beckman Coulter). The cleared lysate (supernatant) was added to HisTrap FF crude column and mounted to the AKTA PrimPlus system and equilibrated with the washing/binding buffer (50mM Tris-HCl, 8M Urea, 100mM NaH_2PO_4 pH 8 and 10mM imidazole). The sample soluble lysate was then injected into the column at a flow rate of 0.5 ml min^{-1}. Finally, elution buffer (8M Urea, 100mM NaH_2PO_4, 500mM Imidazole) was applied to the column to elute the bound His-tagged recombinant protein. The purified protein was then collected from the corresponding fraction(s). To remove the detergent from purified protein and refold the protein to its nature trimers, the protein was resuspended in dialysis Buffer (50mM Tris-HCL, 150mM NaCl, 0.1% Triton X-100, 0.25% Zwittegen 3-12). Gel filtration was performed to ensure the refolding of the protein and whether the protein aggregated or not.

4.2.1.4 Purification of recombinant His-tagged meningococcal PorA protein under denaturing conditions

Immobilized metal ion affinity chromatography (IMAC) was used to purify the His-tagged recombinant proteins, using an AKTA PrimPlus (GE Healthcare) and following the manufacturer's instructions. The stored cell pellets from 150ml culture of *E coli* JM109 was resuspended with lysis buffer (8M urea, 10mM Tris-HCl, 100mM NaH_2PO_4, pH 8). Sonication on ice was carried out to disrupt the cells for 10min in short bursts (30s on and off), using an MSE SoniPrep 150 sonicator. The lysate was centrifuged at 4,200 × *g* for 15min, using an Allegra x-22R centrifuge (Beckman Coulter). The supernatant was collected for purification. Briefly, a HisTrap FF crude column was mounted to the AKTA PrimPlus system and equilibrated with the washing/binding buffer (8M urea, 10mM Tris-HCl, 100mM NaH_2PO_4, 20mM imidazole, pH 6.3). The soluble lysate sample was then injected into column at a flow rate of 0.5ml/min. Finally, elution buffer (8M urea, 10mM Tris-HCl, 100mM NaH_2PO_4, pH 4.5) was applied to the column to elute the bound His-tagged recombinant protein. The purified protein was then collected from the corresponding fraction(s) by monitoring the purification chromatogram. Throughout the purification process, aliquots were taken and subjected to SDS-PAGE and immunoblot analysis.

4.2.1.5 Desalting and concentration of purified protein

After mounting on the AKTA PrimPlus system (GE Healthcare), a Hitrap desalting column was equilibrated with desalting buffer (PBS). The purified protein was then injected into column, where it was combined and concentrated using 30KDa membrane cut-off Vivaspin tubes (Sartorius) by centrifuging at 4,200 × *g*.

4.2.1.6 SDS-PAGE and immunoblot analysis

The identity of purified proteins was confirmed by SDS-PAGE and immunoblot analysis. Briefly, proteins were electrophoretically separated under reducing conditions. The protein mixture separated by SDS-PAGE (APPENDIX) was transferred onto a nitrocellulose membrane in semi-dry blotting buffer using a Trans-Blot SD semi-dry transfer cell (BioRad) at 15 V for approximately 20min. The membrane was blocked by incubating with 5% (w/v) skimmed milk powder in 20ml PBS (Oxoid, England)(APPENDIX). Then the membrane was probed with the primary antibodies: rabbit polyclonal anti-PorA antibody (in house; 1:5000 dilution) or mouse anti-pentahistidine (Qiagen; 1:2000 dilution) antibodies. After washing in PBS containing 0.05% Tween-20 (PBST) (Sigma) three times, each for 15min, the membrane incubated with the secondary antibodies: horseradish peroxidase (HRP) conjugated anti-rabbit IgG (Sigma; 1:20000 dilution) or HRP-conjugated anti-mouse IgG (Sigma; 1:30000 dilution). The primary and secondary antibodies were diluted in the same buffer as used for blocking and incubated for 1h at RT with shaking. The membranes were then developed with luminata western HRP substrate (Millipore).

4.3 Results

The aim of the work presented in this chapter was to investigate the binding-domain interactions of human LamR protein with three bacterial species that are the most common agents of meningitis: *N. meningitidis*; *H. influenzae* and *S. pneumoniae*. Using an ELISA approach, microtiter well plates were treated with 5mg ml^{-1} protein A. Treating of wells with protein A facilitated the binding of the Fc-tag of recombinant proteins onto the well surface to optimally display the immobilized LamR protein (Figure 4-1). Wells were coated with rLamR (recombinant 37kDa laminin receptor protein, expressed in human cells and purified under native conditions). Wild type and mutant strains of the three bacterial species were surface-labelled with DIG and added to triplicate wells; after incubation, unbound bacteria were washed away and bound bacteria were detected using an anti-DIG antibody.

In each experiment, BSA coated wells were included as a control to measure background levels of binding. Equal amounts of labelled bacteria were added to the BSA-coated wells, and mean values obtained from labelled bacteria-BSA wells were subtracted from the values of rLamR-labelled bacteria wells to correct for non-specific background binding. Two other proteins: 3 ATM (Fc-FGFR2 IIIa TM; FGFR2 is Fibroblast Growth Factor 2) and Fc-tag only (termed Fc-stop; a recombinant protein comprising the fragment crystallisable region [Fc region] of the IgG antibody used to tag the rLamR protein), which had been cloned in the same vector and purified by the same method as hLamR, were used as negative controls. All recombinant proteins: wild type and mutated rLamR; Fc-stop and 3ATM were used to coat wells at equi-molar concentrations. Labelled-bacteria were added to coated wells, anti-Dig antibody was added and the plates incubated

for 1h at RT, and protein-bound bacteria detected 1h after adding alkaline phosphatase (AP) (APPENDIX) substrate in an ELISA plate reader. The binding-level of bacterial cells to recombinant proteins was evaluated by measuring the OD at 405nm. Each experiment was performed in 3 technical replicates and repeated at least 3 times.

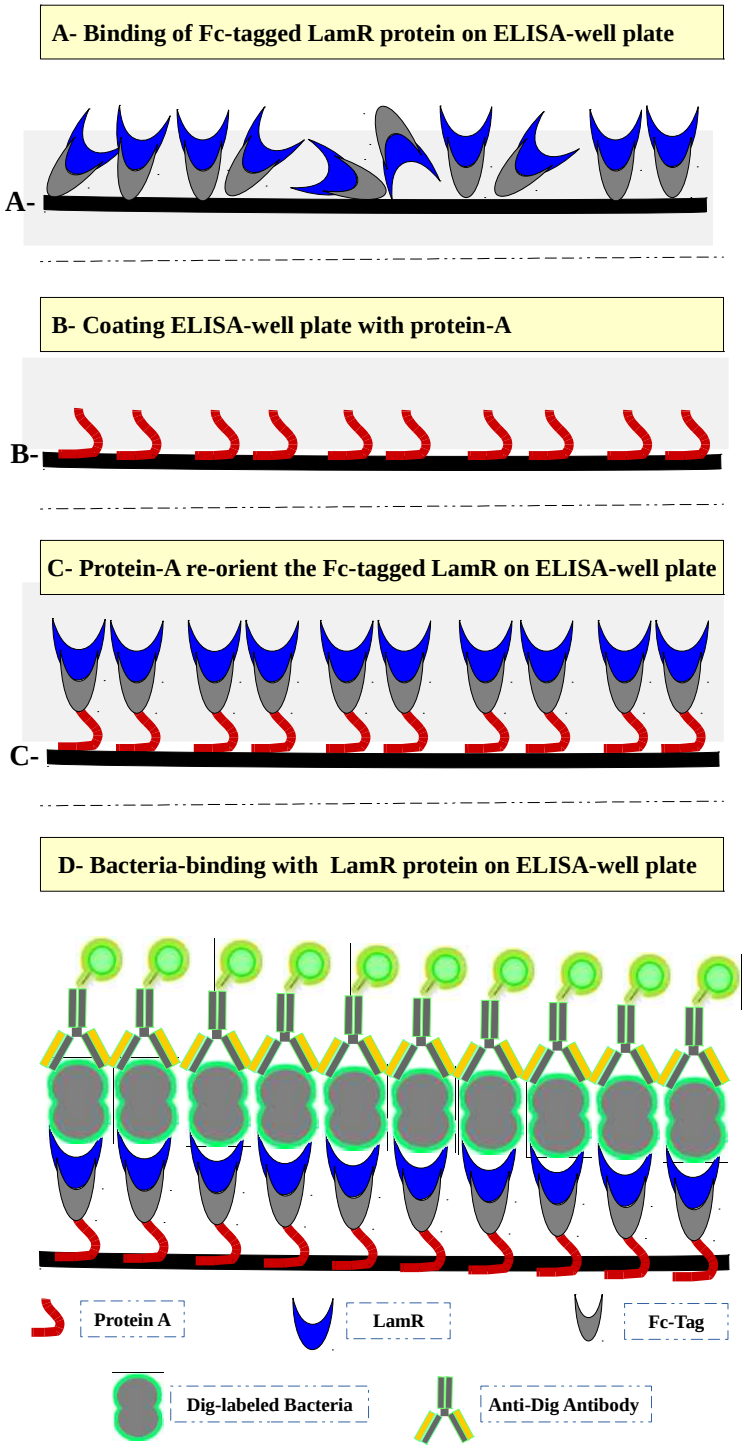


Figure 4-1 Schematic of re-orientation of Fc-tag proteins using protein-A.

ELISA plates in (A) In un-treated ELISA plates, the recombinant proteins will be coated in a random orientation. (B) Coating ELISA-well plate with protein-A which has a high affinity to bind Fc fragment of antibody (C) Protein-A re-orient the Fc-tagged LamR on ELISA-well plate to allow (D) more specific binding between LamR and bacteria.

4.3.1 Human produced LamR (hLamR) and bacterial produced LamR (bLamR) exhibit similar binding-affinity to the group B MC58 meningococcal strain

As an initial step towards determining the region(s) of LamR responsible for the interaction with bacteria, DIG-label based ELISAs were performed as described earlier (section 4.3) to confirm previously published results showing that LamR binds to *N. meningitidis* (Orihuela et al., 2009). In our results, *N. meningitidis* bound to both hLamR and bLamR at similar levels and the binding was significant (***$P \leq 0.001$; Figure 4-2) in comparison to the negative controls: BSA which used for each protein; Fc-stop; and 3 ATM proteins. The data obtained in the current experiment confirmed previous studies where the bLamR exhibited a binding affinity to *N. meningitidis* (Orihuela et al., 2009). Further, our results showed that there were no significant differences between hLamR and bLamR in respect of binding to *N. meningitidis*.

In order to optimise the LamR-concentration used, different concentrations of rLamR (2.5 μg ml^{-1}; 5 μg ml^{-1} and 10 μg ml^{-1}) were used to coat the wells. Also, to show the specificity of LamR-*N. meningitidis* binding, an isolate of *Staphylococcus aureus* was also included. Binding of *N. meningitidis* to rLamR was statistically significant for all concentrations used, and significantly higher than *S. aureus* binding, which was negligible (***$P \leq 0.001$; Figure 4-3). No significant differences in bacterial binding to LamR were observed between the three tested LamR-concentrations.

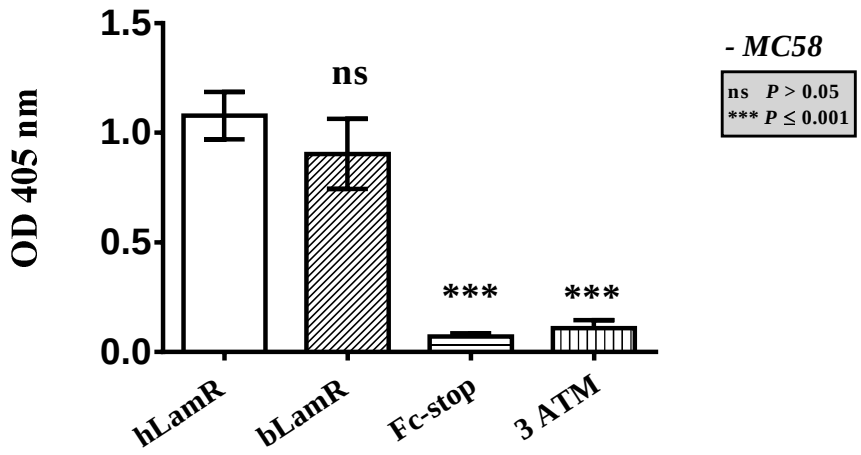


Figure 4-2 Binding of immobilised hLamR and bLamR to *N. meningitidis* MC58.
Protein A treated wells were coated with hLamR or bLamR at a concentration of 5µg ml^{-1}. Fc-stop and 3ATM-coated wells were included as negative controls. BSA-coated wells were included to correct for non-specific background binding, and the mean value obtained from the BSA-bacteria coated wells was subtracted from all other data points. Wells were subsequently incubated with DIG-labelled MC58 bacteria followed with anti-DIG antibody. Bound bacteria were detected after 1h of adding AP substrate in an ELISA plate reader. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ***Significant at $P \leq 0.001$ compared to hLamR.

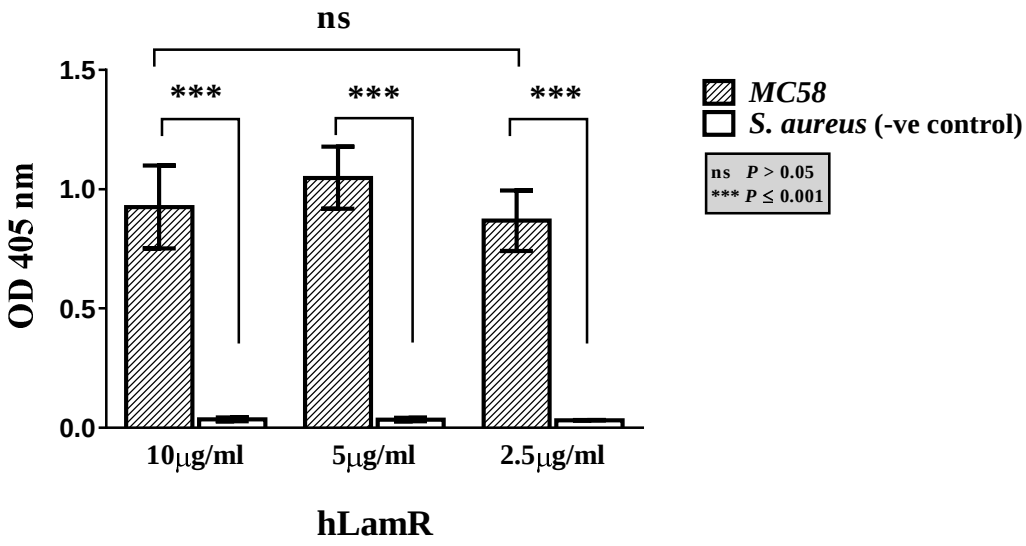


Figure 4-3 Binding of MC58 *N. meningitidis* and *S. aureus* to various concentrations of hLamR.

Protein A treated wells were coated with various concentrations (10μg ml^{-1}; 5μg ml^{-1} and 2.5μg ml^{-1}) of recombinant hLamR. BSA-coated wells were included to correct for non-specific background binding. An isolate of *S. aureus* was included as a negative control. After coating the wells with hLamR or BSA, the wells were subsequently incubated with DIG-labelled bacteria followed with anti-DIG antibody. Bound bacteria were detected after 1h of adding AP substrate in an ELISA plate reader. Specific binding of bacteria to hLamR-coated ELISA plates was determined by subtracting the absorbance in BSA-coated wells from that in protein-coated wells. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ns at $P > 0.05$; ***Significant at $P \leq 0.001$.

4.3.2 Meningococcal PorA and PilQ were confirmed as LamR-binding ligands

Meningococcal PorA and PilQ were previously identified as LamR binding ligands of *N. meningitidis* (Orihuela et al., 2009). Prior to investigating the amino acid residues that may be involved in binding of hLamR to the *N. meningitidis* ligands, DIG-label-based ELISA was undertaken to determine whether specific mutants of *N. meningitidis* strain MC58: Δ*porA*, Δ*porAL4*, Δ*pilQ* and Δ*porA/pilQ* could bind hLamR. Wild type MC58 was used as a comparative positive control;

Fc-stop and 3ATM were used as negative controls. BSA coated wells were included as a measure for background as described previously (section 4.3). Deletion of either *porA* or *pilQ* resulted in a significant reduction (*$P \leq 0.05$; **$P \leq 0.01$; respectively) in binding of hLamR to the mutant cells compared to the wild-type bacteria; a similar reduction was observed in the *porA*/*pilQ* double-knock out mutant (Figure 4-4).. This finding confirms the previously described finding that PorA and PilQ bind bLamR (Orihuela et al., 2009).

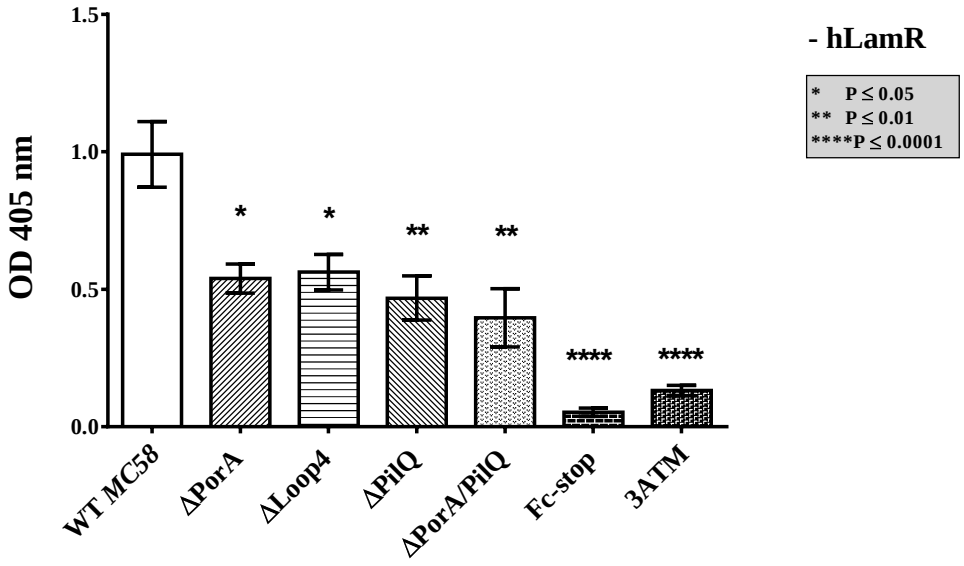


Figure 4-4 Binding of *N. meningitidis* MC58 and corresponding isogenic mutants to immobilised hLamR.

Protein A-treated wells were coated with hLamR at a concentration of 5μg ml^{-1}. Fc-stop and 3ATM-coated wells were included as negative controls. BSA-coated wells to correct for non-specific background binding. After coating the wells with hLamR or BSA, the wells were subsequently incubated with DIG-labelled MC58 bacteria followed with anti-DIG antibody. Bound bacteria were detected after 1h of adding AP substrate in an ELISA plate reader. Specific binding of DIG-labelled bacteria to hLamR-coated ELISA plates was determined by subtracting the absorbance in BSA-coated wells from that in protein-coated wells. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. *Significant at $P \leq 0.05$; ** $P \leq 0.01$; *** at $P \leq 0.001$.

4.3.3 Mutations in hLamR result in a significant reduction in binding to *N. meningitidis*

To better understand LamR interactions with *N. meningitidis*, we investigated the interaction of DIG-labelled wild type *N. meningitidis* MC58 and the constructed mutants hLamR mutants ($rLamR^{\Delta 206-229}$; $rLmaR^{\Delta 262-282}$; $rLamR^{\Delta 243-295}$; $rLamR^{Y139F}$; $rLamR^{R155A}$; $rLamR^{Y156F}$; $rLamR^{K166A}$; $rLamR^{Y139F/Y156F}$) using ELISA as described previously (section 4.3). In these assays, wells were treated with 5μg ml^{-1} protein A, then Fc-tagged proteins were added at equi-molar

concentrations. The non-specific binding sites were blocked and wells subsequentlly challenged with DIG-labeled bacteria. After rigorous washing, anti-DIG antibody was added to detect the interaction between the bacteria and rLamR. Wild type hLamR was used as a comparative positive control as it demonstrated specific binding with bacteria (section 4.3.1). Fc-stop and 3ATM were consistently used as negative controls. In total, 8 mutants were examined for their ability to bind wild type MC58; deletion of LamR amino acid residues: 206-229 and 263-282 resulted in an significant reduction (***$P \leq 0.001$) of binding affinity to bacteria, which indicated the involvement of amino acid residues in these regions in bacterial binding. Also, substitution of LamR residues: Arginine 155 and Tyrosine 156 which are surface-exposed amino acid residues in smaller, but nethertheless still significant reduction (*$P \leq 0.05$; **$P \leq 0.01$; respectively) in bacterial binding activity. Substitution of Lysine166, Tyrosine 139 and double mutation of Tyrosine 156 and 139 of LamR did not significantly effect bacterial binding. The data imply that regions from 206-229 and 263-282 and amino acid residues Arganine155 and Tyrosine 139 of LamR comprise bacteria binding sites (Figure 4-5).

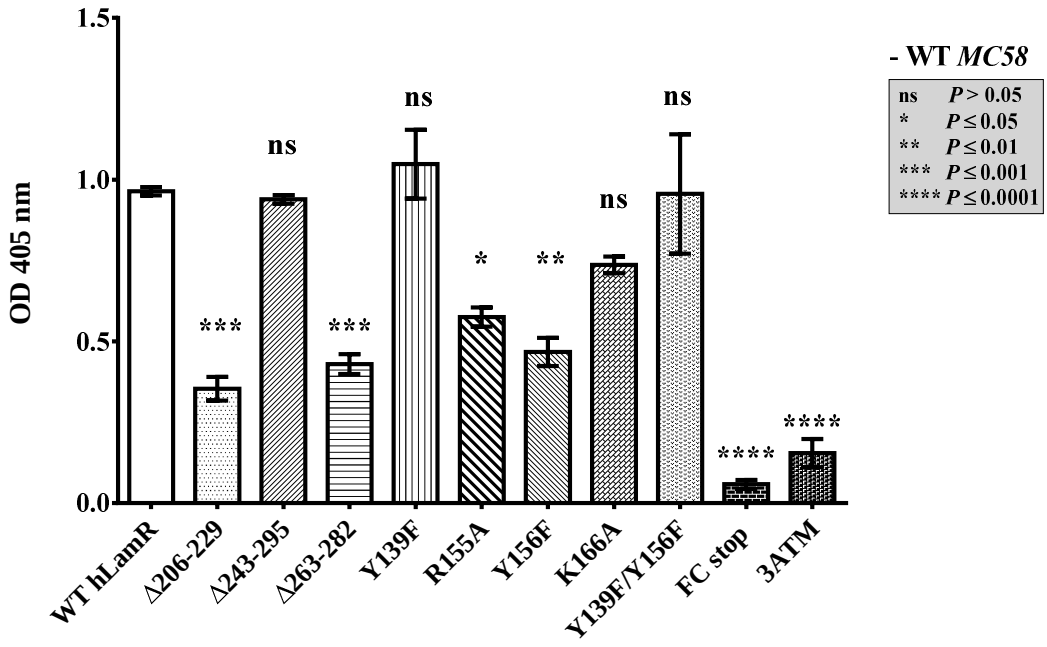


Figure 4-5 Mutations in hLamR resulted in a marked reduction in binding by *N. meningitidis* MC58.

Protein A-treated wells were coated with hLamR and its derivatives at 5 μg ml^{-1}. Fc-stop and 3ATM-coated wells were included as negative controls. BSA-coated wells to correct for non-specific background binding. After coating the wells with hLamR or BSA, wells were subsequently incubated with DIG-labelled MC58 bacteria followed with anti-DIG antibody. Bound bacteria were detected after 1h of adding AP substrate in an ELISA plate reader. Specific binding of DIG-labelled wild type MC58 to rLamR-coated ELISA plates was determined by subtracting the absorbance in BSA-coated wells from that in protein-coated wells. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ns at P >0.05; *Significant at $P \leq 0.05$; ** $P \leq 0.01$; *** at $P \leq 0.001$; **** at $P \leq 0.0001$.

4.3.4 hLamR peptide, 263-282 as a binding site for meningococcal PilQ

We previously demonstrated that meningococcal mutants lacking PilQ have a significantly reduced ability to bind hLamR (Section 4.3.2). To further identify the LamR residues that may be involved in LamR-PilQ binding, wild type hLamR and its mutant derivatives were tested for their ability to bind DIG-labelled bacteria expressing no PorA or a PorA derivative in which the fourth

extracellular loop had been deleted (Δ*porA* and Δ*porA*L4, respectively) in ELISA experiments. Wild type hLamR was used as a comparative positive control; Fc-stop and 3ATM were used as negative controls. In comparison to the wild-type strain, only deletion of amino acid residues 263-282 in the C-terminal portion of LamR resulted in a significantly reduction in binding to MC58 ΔPorA and MC58 $PorA^{\Delta L4}$ (Figure 4-6; A & B). The reduction in hLamR binding by either ΔPorA or PorAΔL4 was similar indicating that the majority of the LamR binding activity of PorA was restricted to the fourth extracellular loop. This agrees with a previous study by Abouseada and colleagues (Abouseada et al., 2012).

These results suggested that these particular residues are potentially involved in binding of human LamR to the other identified meningococcal LamR-binding protein: PilQ (Figure 4-6; A & B).

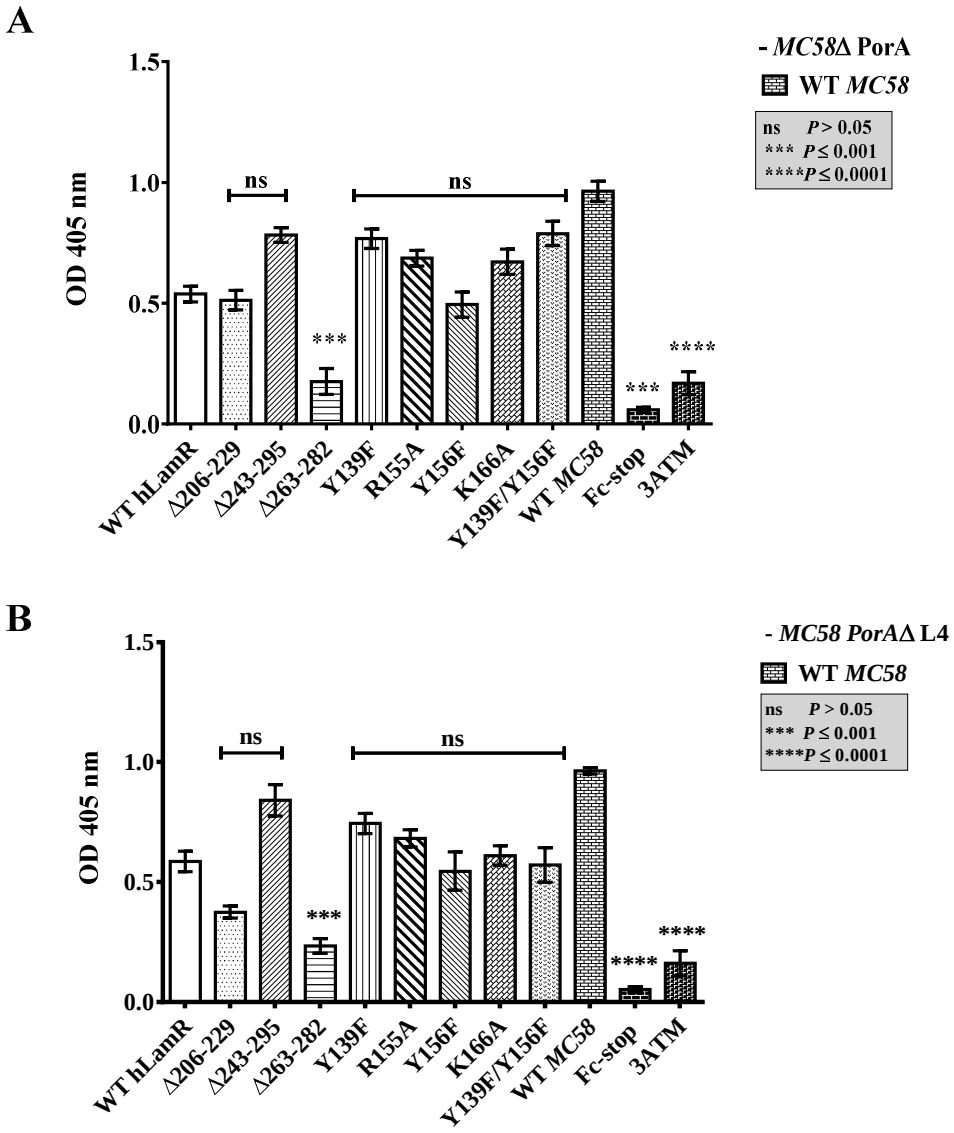


Figure 4-6 Binding of MC58 ΔPorA and PorAΔL4 to hLamR identifies the carboxyl terminal peptide, 263-282 as a meningococcal PilQ-binding site.

Protein A treated wells were coated with hLamR and its derivatives at concentration of 5μg ml^{-1}. Fc-stop and 3ATM-coated wells were included as negative controls. BSA-coated wells to correct for non-specific background binding. After coating the wells with hLamR or BSA, the wells were subsequently incubated with DIG-labelled (A) PorA-deficient (B) L4 of PorA-deficient MC58 bacteria, followed with anti-DIG antibody. Bound bacteria were detected after 1h of adding AP substrate in an ELISA plate reader. Specific binding of bacteria to protein-coated ELISA plates was determined by subtracting the absorbance in BSA-coated wells from that in protein-coated wells. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ns at P >0.05; ***Significant at $P \leq 0.001$; **** at $P \leq 0.0001$ compared to binding to wild type hLamR.

4.3.5 hLamR peptide, 206-229 as a binding site for meningococcal PorA

Previous studies with synthetic peptides corresponding to $LamR^{263-282}$ demonstrated that this region in LamR may be involved in binding of LamR to meningococcal PorA (Orihuela et al., 2009). Further investigations were therefore conducted with wild type and LamR mutants expressed in human cells and purified under native conditions for their ability to bind to DIG-labelled *N. meningitidis* lacking PilQ in ELISA experiments. Among 8 hLamR mutant proteins tested, $LamR^{\Delta 263-282}$ revealed a significant ($*P \leq 0.05$) reduction in its ability to bind ΔPilQ *N. meningitidis*, suggesting that these amino acid residues are important for binding the bacteria and confirming the previous observations. Interestingly, a significant ($*P \leq 0.05$) reduction in bacterial binding was also observed in both mutant constructs $LamR^{\Delta 206-229}$ and $LamR^{Y156F}$, demonstrating that additional meningococcal PorA-binding sites may be present on LamR (Figure 4-7). Further investigations using PorA or $PorA^{L4}$ synthetic peptides are required to confirm the results.

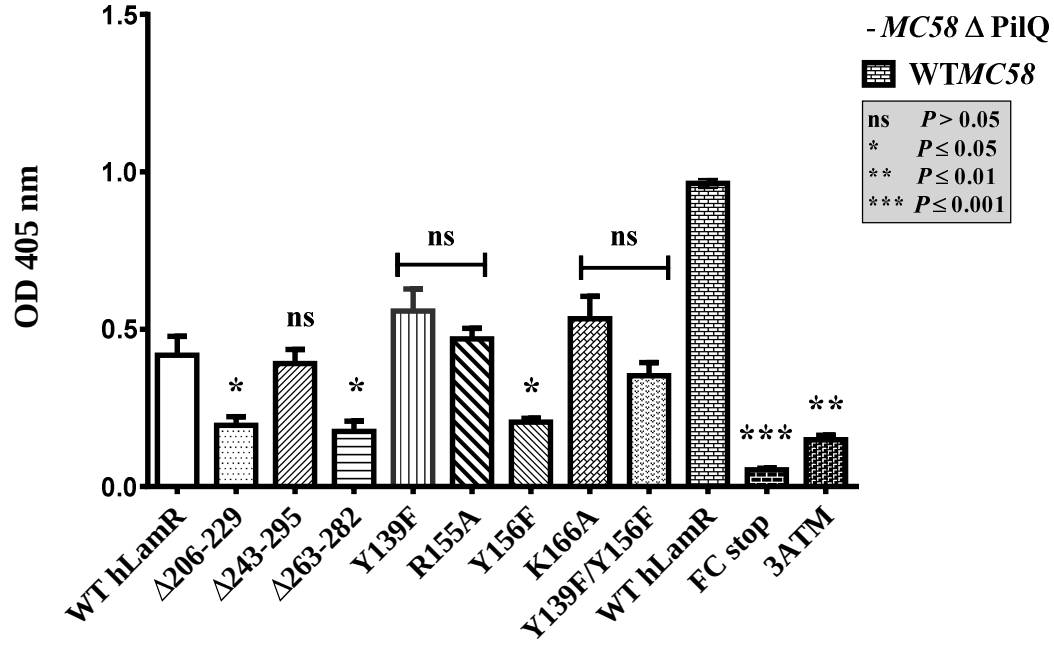


Figure 4-7 The peptide, 206-229 in hLamR identified as a binding site for *N. meningitidis* MC58 PorA, in addition to the 263-229 region.

Protein A-treated wells were coated with hLamR and its derivatives mutants at concentration of 5 μg ml^{-1}. Fc-stop and 3ATM-coated wells were included as negative controls. BSA-coated wells to correct for non-specific background binding. After coating the wells with hLamR or BSA, the wells were subsequently incubated with DIG-labelled MC58 lacking PilQ followed with anti-DIG antibody. Bound bacteria were detected after 1h of adding AP substrate in an ELISA plate reader. Specific binding of bacteria to protein-coated ELISA plates was determined by subtracting the absorbance in BSA-coated wells from that in protein-coated wells. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ns at P >0.05; *Significant at $P \leq 0.05$; ** $P \leq 0.01$**;** *** at $P \leq 0.001$ compared to binding to wild type hLamR.

4.3.6 Double PorA and PilQ knock out of *N. meningitidis* exhibits significantly reduces binding to hLamR

Previously; our data showed that a single ablation knock out of either PorA (Δ*porA*) (Figure 4-6) or PilQ (Δ*pilQ*) (Figure 4-6) resulted in a reduction in hLamR binding compared to wild type MC58. To further show the importance of these two meningococcal proteins as LamR ligands, DIG-labelled *N. meningitidis*

lacking both PorA and PilQ were tested for their ability to bind to hLamR and its mutant derivatives. Although a significant reduction in binding to hLamR was observed compared to that exhibited by the wild type MC58, no significant differences were observed between the binding of the double knockout to the wild type and different mutated versions of hLamR (Figure 4-8). These results provide evidence that meningococcal PorA and PilQ are the major meningococcal-LamR ligands and confirmed the previous results. These results also suggested that no additional LamR-binding proteins were present on the meningococcal surface.

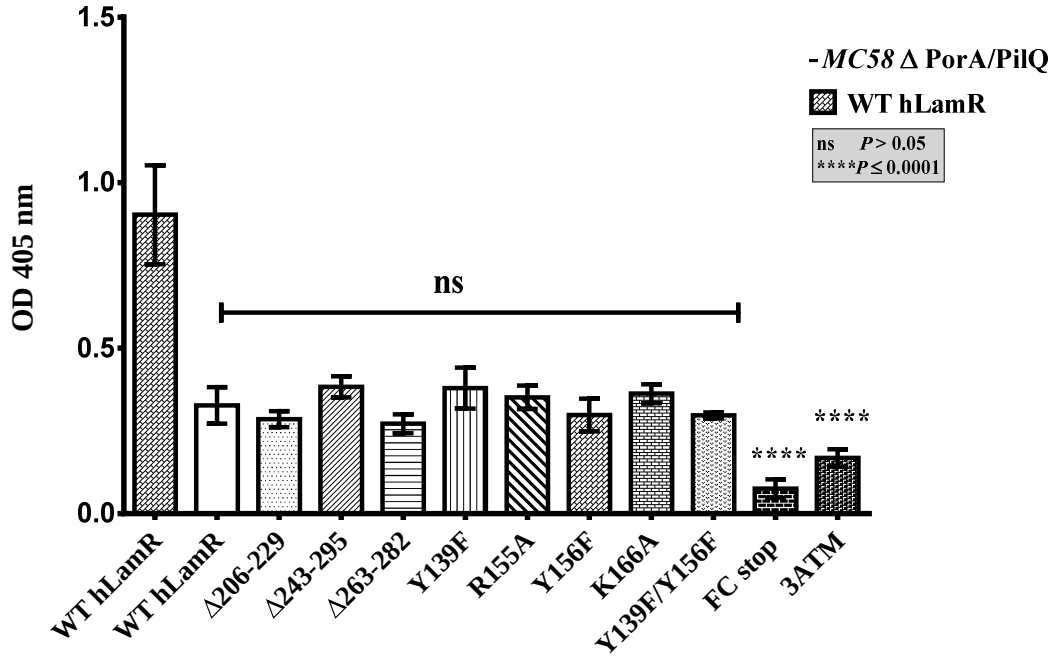


Figure 4-8 Double knockout of *porA* and *pilQ* in *N. meningitidis* significantly reduces the binding ability of *N. meningitidis* MC58 to bind to wild type and mutants hLamR proteins.

Protein A-treated wells were coated with hLamR and its derivatives at concentration of 5 μg ml^{-1}. Fc-stop and 3ATM-coated wells were included as negative controls. BSA-coated wells to correct for non-specific background binding. After coating the wells with hLamR or BSA, the wells were subsequently incubated with DIG-labelled MC58 lacking PorA and PilQ followed with anti-DIG antibody. Bound bacteria were detected after 1h of adding AP substrate in an ELISA plate reader. Specific binding of bacteria to protein-coated ELISA plates was determined by subtracting the absorbance in BSA-coated wells from that in protein-coated wells. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ns at P >0.05; ****Significant at $P \leq 0.0001$ compared to binding to wild type *N. meningitidis* MC58.

4.3.7 ELISA results reveal significant interaction of two proteins, human hLamR and meningococcal rPorA

4.3.7.1 Expression and purification of recombinant PorA

In order to test the binding of recombinant proteins LamR and PorA, firstly rPorA was expressed in *E coli*. Briefly, the pQE30/PorA construct was introduced into the *E. coli* strain JM109 and expression of rPorA protein was induced with IPTG. Optimal expression was achieved by addition of a final concentration of 0.5 mM IPTG when the cells had reached an OD_{600} of 0.6 incubated for 1 h at 37°C. The results of SDS-PAGE after induction revealed the production of recombinant protein with an expected size approximate 45 kDa. The empty pQE30 vector and rLamR were used as negative and positive controls, respectively. The expressed protein was confirmed as rPorA by immunoblot analysis with both anti-PorA and anti-His antibodies (Figure 4-9). The recombinant PorA was purified using IMAC under denaturing conditions, solubilized supernatants added to the His Trap Ni Sepharose resin, and the a single band corresponding to the expected protein size was obtained (Figure 4-10). Purified protein was then immediately removed from the urea buffer into PBS using desalting columns.

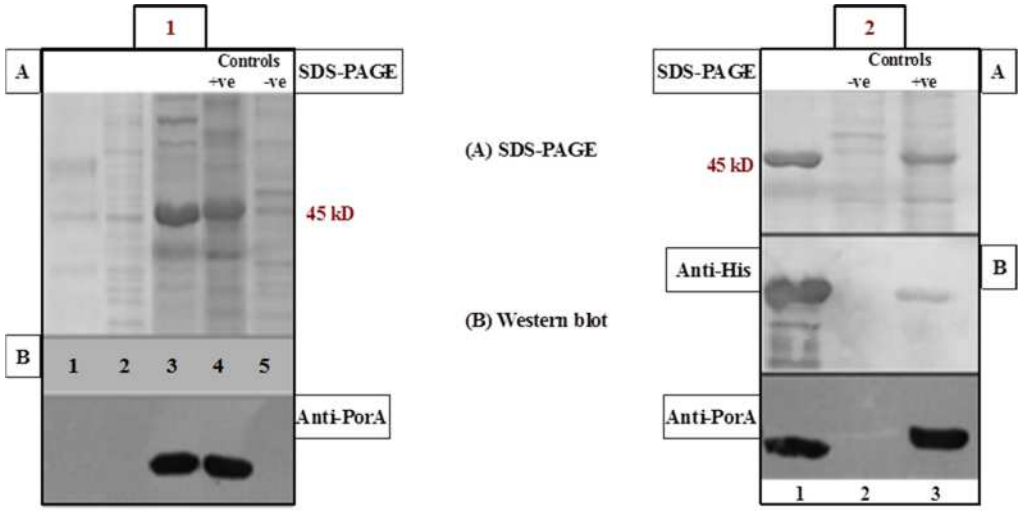
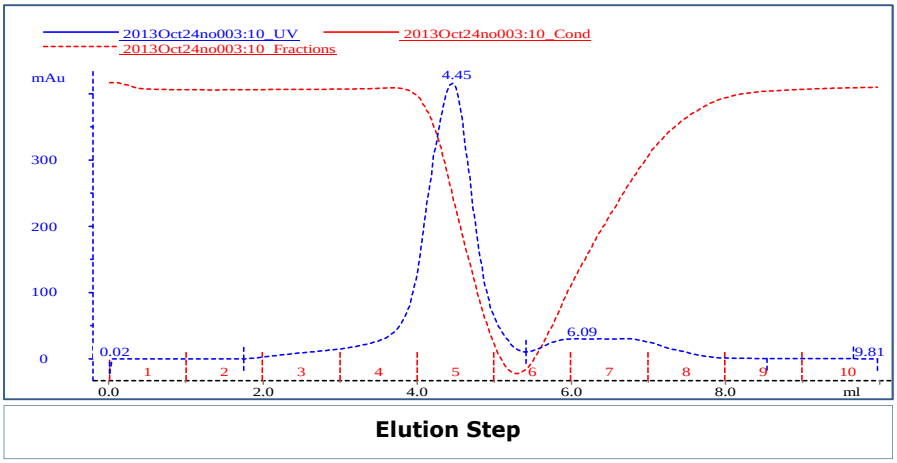


Figure 4-9 Expression of recombinant PorA in *E coli*.

SDS-PAGE (A) and immunoblot using PorA and penta-his antibodies (B) showing rPorA expression following induction with 0.5mM IPTG (panel 1) and purification using IMAC (panel 2). Panel 1; Lane 1, protein marker; Lane 2, non-induced *E. coli* JM109 (pQE30); Lane 3, 3h induced *E. coli* JM109 (pQE30); Lane 4, 3h induced *E. coli* JM109 contains pET28/LamR (+ve control); Lane 5, 3h. Induced strain JM109 with pQE30 lack rPorA (-ve control). Panel 2; Lane 1: purified rPorA; Lane 2: pQE30 lack rPorA (-ve control); Lane 3: purified rLamR (+ve control).



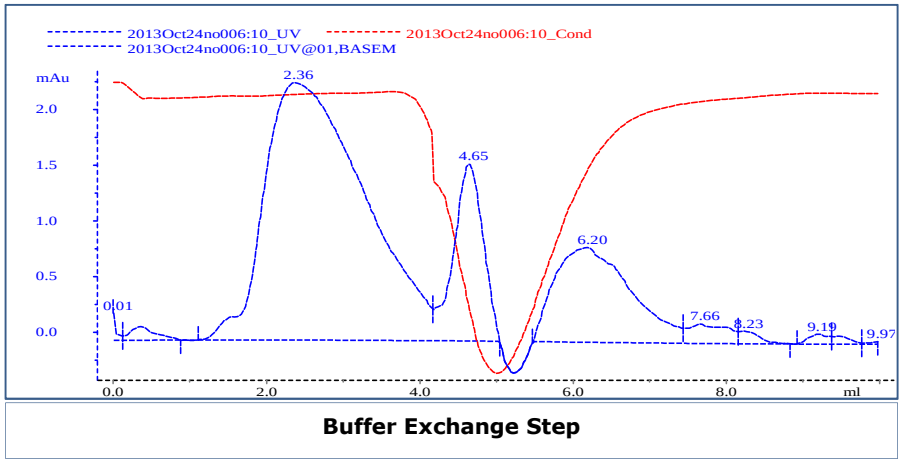


Figure 4.1 Chromatogram of purification of rPorA by step elution and Buffer exchange.

(A) Elution step: rPorA bound to the Ni-via the 6×His tag, stars to elute with the passage of elution buffer; the highest concentration of protein was eluted after passage of 4.45 ml elution buffer (second peak of UV absorbance) and was collected in fractions 4-6. After that a plateau was reached where the eluted rPorA concentration decrease. A declining UV absorbance (blue line) indicated completion of the elution process. (B) Buffer exchange: According to the molecular weight, rPorA appeared first (first blue peak of UV absorbance) and was collected in fractions 2 to 4 followed by lower molecular weight molecules present in the elution buffer used to elute rPorA (lower blue peaks of UV absorbance).

4.3.7.2 Interaction of recombinant hLamR with rPorA

In order to further dissect and confirm the contribution of defined amino acids within hLamR to the interaction with PorA, binding of natively purified human rLamR protein with rPorA was tested using ELISA.

4.3.7.3 Optimizing the ELISA reaction conditions

The optimal primary and secondary antibody dilutions for the ELISA were first determined. Each dilution was tested on the same type of sample, to keep the experimental conditions standardized. In these experiments, rLamR was used to coat protein A-treated solid phase-microplate wells by adding 5µg ml^{-1} rLamR diluted in PBS. After blocking and washing, an equal molarity of rPorA was added and later challenged with different concentrations of anti-PorA antibody and corresponding secondary antibodies. Plates were read every15 min between 1 and 6h. Wells coated with BSA only was used as a negative control for non-specific interactions with the solid phase. The optimal antibody concentrations were found to be 1:8000 and 1:16000 for primary and secondary antibodies, respectively.

After optimisation of ELISA conditions 5µg ml^{-1} of rLamR was immobilized onto protein A-treated ELISA wells and challenged with rPorA. The interaction was detected using anti-PorA antibody; the plate was read after 1 h incubation with the substrate. Significant binding of hLamR to rPorA to hLamR was observed (***$P < 0.001$; Figure 4-11A) compared to negative controls. Similar results were obtained when rPorA was used as the solid phase and this was probed with soluble rLamR. (***$P < 0.001$; Figure 4-11B).

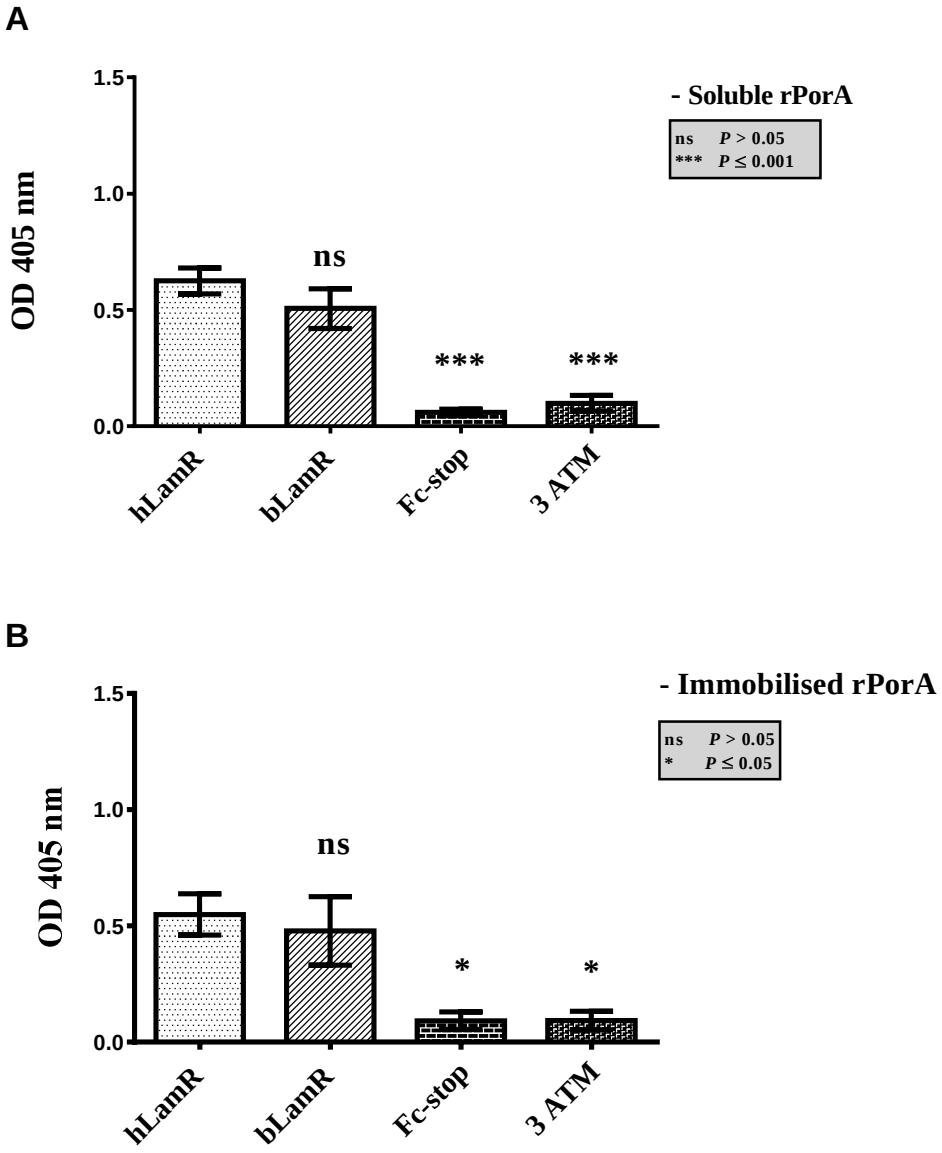


Figure 4-11 Binding of recombinant hLamR and bLamR to soluble (A) or immobilized (B) meningococcal rPorA protein.

Protein A treated wells were coated with rLamR or rPorA at concentration of 5 μg ml^{-1}. Fc-stop and 3ATM-coated wells were included as negative controls. BSA-coated wells to correct for non-specific background binding. Specific binding of recombinant purified proteins (bLamR and hLamR) to rPorA were determined either by using anti-PorA antibody (A) or anti-LamR antibody (B). The mean value obtained from each rPorA-BSA hLamR-BSA were subtracted from mean obtained from respective test wells. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ns at P >0.05; *Significant at $P \leq 0.05$;*** at $P \leq$ 0.001compared to hLamR.

4.3.7.4 Interaction of rPorA with recombinant hLamR and its mutant derivatives

Our previous data demonstrated that amino acid residues 206-229, 263-282 and Tyrosine 156 of LamR were involved in the interaction of hLamR with an *N. meningitidis* MC58 derivative lacking PilQ (section 4.3.5). In order to provide confirmatory evidence for the direct interaction of hLamR with PorA, and also to confirm the role of specific amino acid residues of LamR in the interaction, the binding ability of mutant rLamR proteins to wild type rPorA was examined using the same ELISA conditions as described previously (section 4.3.7.3). The experimental conditions were optimised using either soluble rLamR or immobilized rLamR as anti-37LRP [A7] antibody can detect only sites (253-289); it will not facilitate detection the possible interaction of PorA-with deletion mutant that lack these residues. For this reason, ELISA assays were performed using immobilized hLamR molecules or derivatives which were challenged with rPorA and the interaction detected by anti-PorA antibody after 1h incubation with the substrate. Similarly to our previous DIG-based ELISA data with whole bacteria and rLamR protein, the highest reduction in binding levels to rPorA was observed using $LamR^{\Delta 206\text{-}229}$; $LamR^{\Delta 263\text{-}282}$ and $LamR^{Y156F}$. Also, $LamR^{R155A}$ and $LamR^{K166A}$ showed a reduction in binding, but to a lesser extent (Figure 4-12). These data confirmed our previous findings showing the involvement of amino acid residues 206-229; 263-229 and Tyrosine 156 of LamR in binding to PorA. To examine the interaction of PilQ, the second meningococcal LamR ligand, ELISA was performed to detect the interaction of rLamR and rPilQ. Our data confirmed the previous results where the LamR region 263-282 was identified as a meningococcal PilQ-binding domain (data not shown). However, the protein

quantity was not enough for three separate experiments and the available time did not allow further expression and purification of PilQ protein, ELISA experiments to detect the interaction of rLamR-rPorA proteins were performed only twice and ideally they should be assessed at three different occasions to allow us to estimate

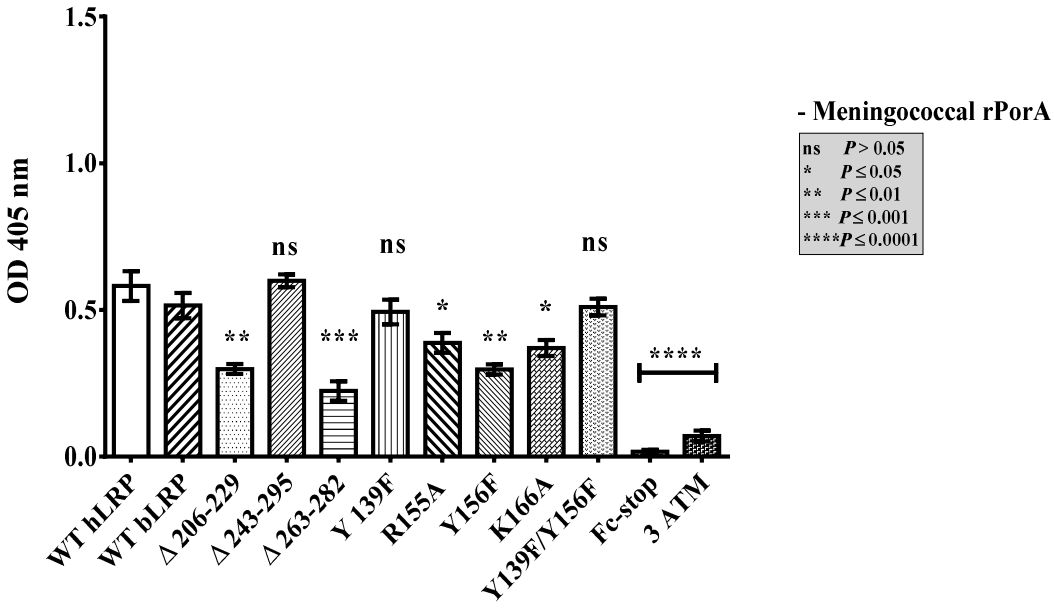


Figure 4-12 Binding of meningococcal rPorA hLamR and its derivatives confirms the identified LamR-PorA binding sites.

Protein A-treated wells were coated with hLamR at concentration of 5 µg ml-1. Fc-stop and 3ATM-coated wells were included as negative controls. BSA-coated wells were included to correct for non-specific background binding. Specific binding of rPorA to immobilised recombinant hLamR were determined using anti-PorA antibody. The mean value obtained from BSA coated wells was subtracted from rLamR-coated wells. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ns at P >0.05; *Significant at $P \leq 0.05$; ** $P \leq 0.01$; *** at $P \leq 0.001$ compared to binding to wild type hLamR.

its statistical significance.

4.3.7.5 *H. influenzae* OmpP2 confirmed as a LamR-binding ligand

In addition to *N. meningitidis,* it has been reported previously that LamR binds *H. influenzae* and specifically, the second extracellular loop of its porin OmpP2 (OmpP2/L2) was identified as a LamR ligand (Orihuela et al., 2009, Abouseada et al., 2012). In order to determine the region(s) within LamR which contribute to binding OmpP2, DIG-label based ELISA was conducted as previously described (section 4.3.1). For this purpose, the binding affinity of rLamR produced in human cells to *H. influenzae in vitro,* DIG-labelled ELISA was examined; bacteria produced LamR (bLamR) was included for comparison purpose; Fc-stop and 3ATM were included as negative controls as described previously. Recombinant LamR expressed in either *E. coli* or human cells bound *H. influenzae* cells and no significant difference between bLamR and hLamR binding capability to *H. influenzae* was observed (Figure 4-13). Also, various concentrations (2.5 µg ml^{-1}; 5µg ml^{-1} and 10µg ml^{-1}) of recombinant hLamR were used as the solid phase in ELISA experiments to examine their abilities to bind *H. influenzae*. In our results, *H. influenzae* bound rLamR significantly more than *S. aureus,* which was included as a negative control ($P \leq 0.001$; Figure 4-14), and the binding was slightly increased at a rLamR concentration of 5µg ml^{-1} compared to others, although this difference did not reach significance. rLamR produced in *E. coli* was previously shown to bind to *H. influenzae* in ELISA assays (Abouseada et al., 2012).

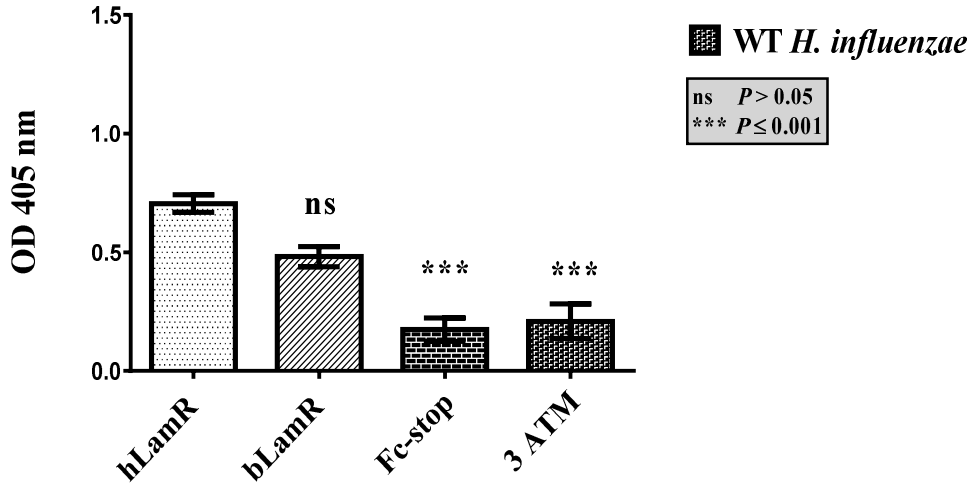


Figure 4-13 Binding of immobilised hLamR and bLamR to *H. influenzae*.

Protein A-treated wells were coated with hLamR or bLamR at a concentration of 5 µg ml^{-1}. Fc-stop and 3ATM-coated wells were included as negative controls. BSA-coated wells to correct for non-specific background binding. Wells were subsequently incubated with DIG-labelled *H. influenzae* bacteria followed with anti-DIG antibody. Bound bacteria were detected after 1 h of adding AP substrate in an ELISA plate reader. Specific binding of bacteria to protein-coated ELISA plates was determined by subtracting the absorbance in BSA-coated wells from that in protein-coated wells. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ns at $P > 0.05$; ***Significant at $P \leq 0.001$ compared to binding to wild type hLamR.

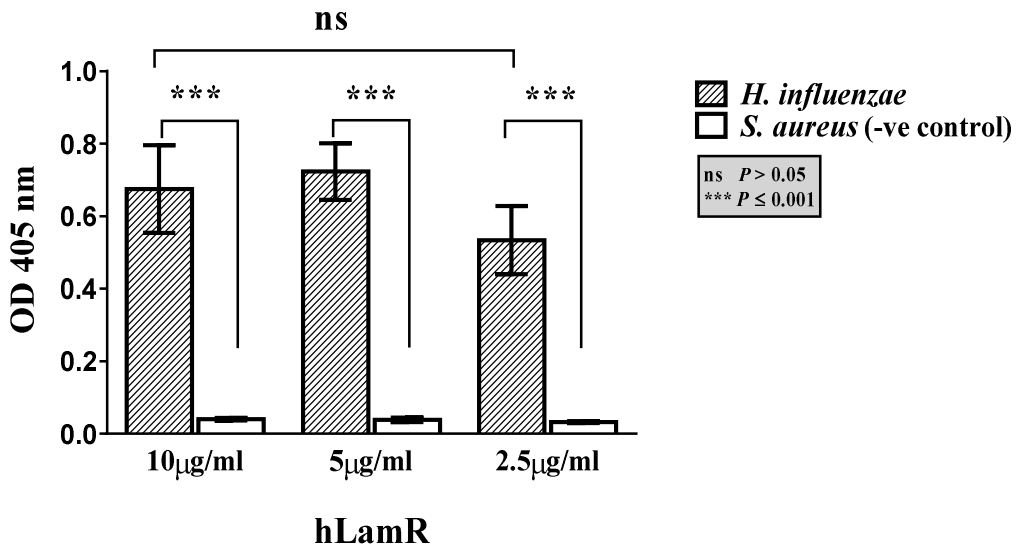


Figure 4-14 Binding of *H. influenzae* and *S. aureus* to various concentrations of rLamR.

Protein A treated wells were coated with various concentration (2.5 μg ml^{-1}; 5μg ml^{-1} and 10μg ml^{-1}) of recombinant hLamR. BSA-coated wells to correct for non-specific background binding. Wells were subsequently incubated with DIG-labelled bacteria followed with anti-DIG antibody. Bound bacteria were detected after 1h of adding AP substrate in an ELISA plate reader. Specific binding of bacteria to rLamR-coated ELISA plates was determined by subtracting the absorbance in BSA-coated wells from that in protein-coated wells. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ns at P >0.05; ***Significant at $P \leq 0.001$.

4.3.7.6 Regions 206-229, 263-282 and Tyrosine 139 of LamR identified as binding sites for *H. influenzae*

To identify the domain(s) of LamR interacting with *H. influenzae*, a DIG-labelled ELISA using the same conditions described earlier (Section 4.3.1) was employed. Amino acids in the regions 206-229 and 263-282, as well as the single amino acid Tyrosine 139 within hLamR were shown to be important for interaction with *H. influenzae* (Figure 4-15). Since the OmpP2/L2 of *H. influenzae* was identified previously as the LamR ligand, the interactions of LamR with both OmpP2 (Figure 4-16) and OmpP2/L2 (Figure 4-17) deficient *H. influenzae* were also

examined. Binding of these strains to wild type hLamR was lower than the wild-type; interestingly amino acids in the regions 206-229 and 263-282, and Tyrosine 139 of LamR were shown to be important for binding irrespective of the presence of OmpP2 or OmpP2/L2. Since mutation of these bacterial ligands did not totally abolish LamR binding, it suggests the presence of other LamR-ligands in *H. influenzae*.

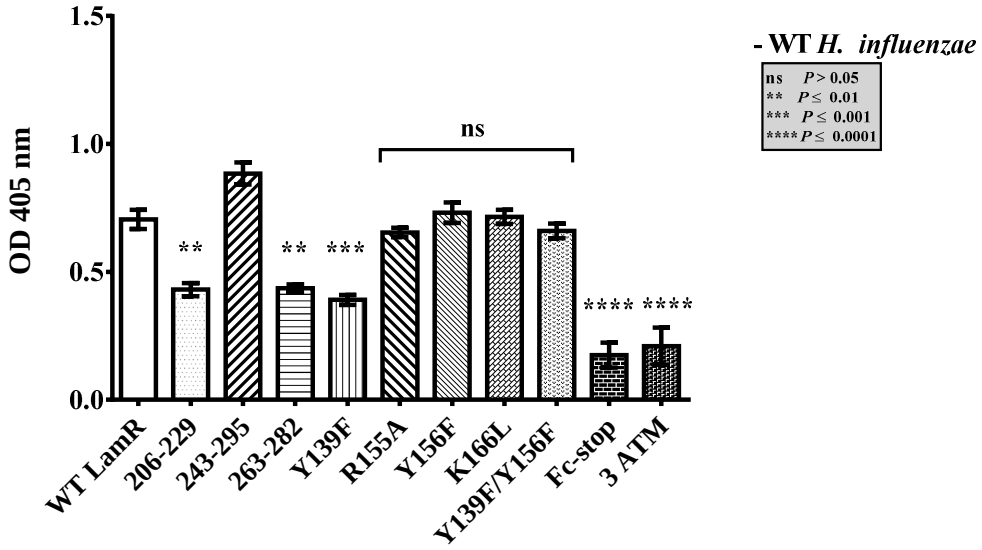


Figure 4-15 Various mutations in hLamR result in a significantly reduced binding to *H. influenzae*.

Protein A-treated wells were coated with hLamR and its mutant derivatives at concentration of 5 μg ml^{-1}. Fc-stop and 3ATM-coated wells were included as negative controls. BSA-coated wells were included to correct for non-specific background binding. Wells were subsequently incubated with DIG-labelled *H. influenzae* followed with anti-DIG antibody. Bound bacteria were detected after 1 h of adding AP substrate in an ELISA plate reader. Specific binding of bacteria to protein-coated ELISA plates was determined by subtracting the absorbance in BSA-coated wells from that in protein-coated wells. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ns at P >0.05; **Significant at $P \leq$ 0.01; *** at $P \leq 0.001$; **** at $P \leq 0.0001$compared to binding to wild type hLamR.

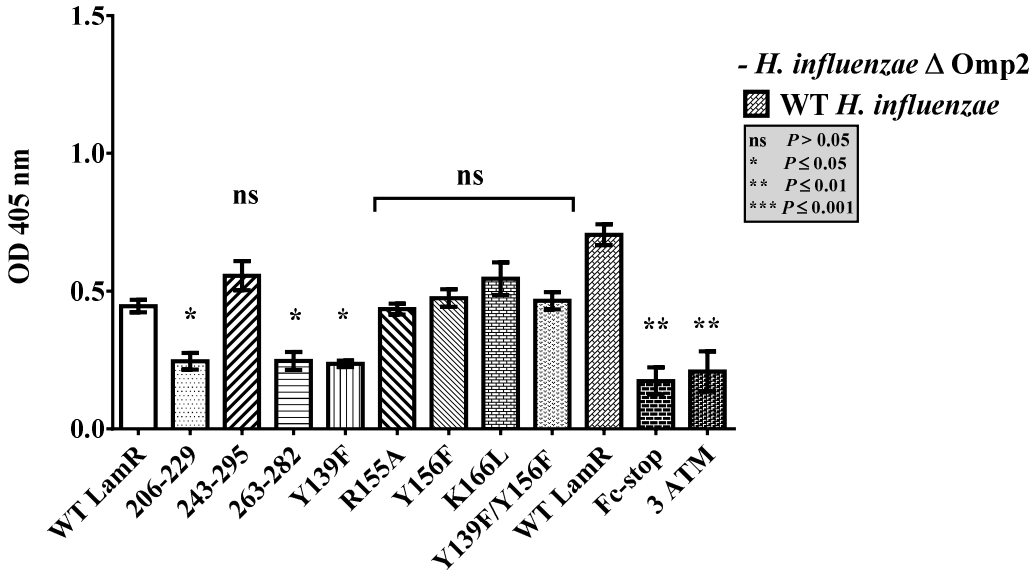


Figure 4-16 LamR regions: 206-229, 263-282 and Tyrosine 139 proposed as binding sites for OmpP2 of *H. influenzae*.

Protein A-treated wells were coated with hLamR and its mutant derivatives at concentration of 5 μg ml^{-1}. Fc-stop and 3ATM-coated wells were included as negative controls. BSA-coated wells were included to correct for non-specific background binding. After coating the wells with hLamR or BSA, the wells were subsequently incubated with DIG-labelled *H. influenzae* lacking OmpP2 followed with anti-DIG antibody. Bound bacteria were detected after 1 h of adding AP substrate in an ELISA plate reader. Specific binding of bacteria to protein-coated ELISA plates was determined by subtracting the absorbance in BSA-coated wells from that in protein-coated wells. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ns at P >0.05; *Significant at $P \leq 0.05$; ** $P \leq 0.01$ compared to binding to wild type hLamR.

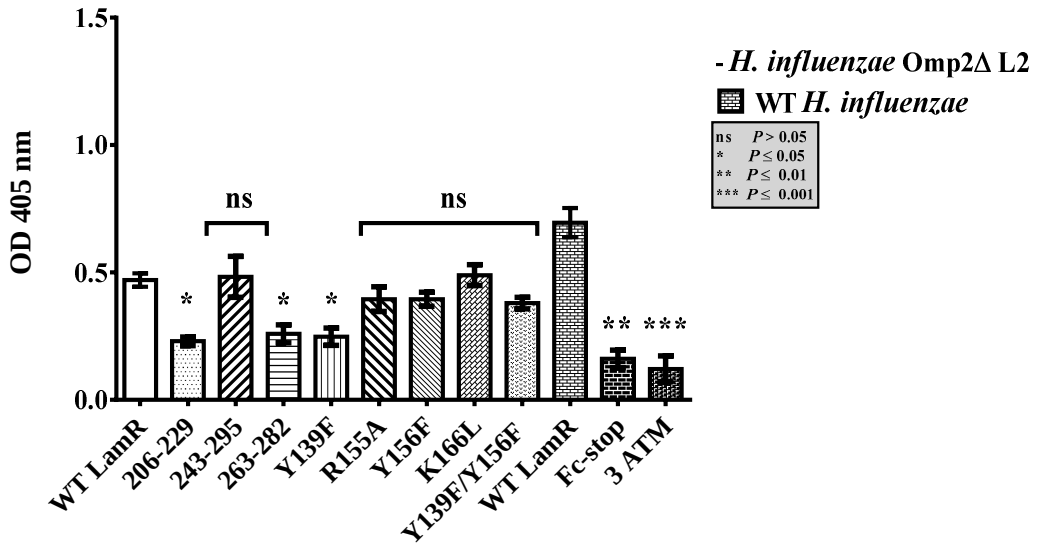


Figure 4-17 Binding of *H. influenzae* lacking L2 of OmpP2 to hLamR confirms the contribution of defined LamR regions in binding with OmpP2/L2 of *H. influenzae.*

Protein A-treated wells were coated with hLamR and its mutant derivatives at concentration of 5 μg ml^{-1}. Fc-stop and 3ATM-coated wells were included as negative controls and BSA-coated wells to correct for non-specific background binding. After coating the wells with hLamR or BSA, the wells were subsequently incubated with DIG-labelled *H. influenzae* lacking L2 of OmpP2 followed with anti-DIG antibody. Bound bacteria were detected after 1 h of adding AP substrate in an ELISA plate reader. Specific binding of bacteria to protein-coated ELISA plates was determined by subtracting the absorbance in BSA-coated wells from that in protein-coated wells. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ns at P >0.05; *Significant at $P \leq 0.05$; ** $P \leq 0.01$; *** at $P \leq 0.001$ compared to binding to wild type hLamR.

4.3.7.7 Mutations in LamR resulted in reduced binding to *S. pneumoniae* and hLamR and bLamR exhibited similar binding behaviour with the *S. pneumoniae*

Earlier investigations revealed that *S. pneumoniae* binds recombinant LamR produced in *E. coli*. The binding behaviour of bLamR with the *S. pneumoniae* was compared with the binding of hLamR with the *S. pneumoniae* using 5μg ml^{-1} of both bLamR and hLamR proteins and the results displayed lower binding ability

of bLamR with *S. pneumoniae* compare with hLamR, but this difference did not reach statistical significance indicating that bLamR and hLamR exhibited similar binding behaviour with *S. pneumonia* (Figure 4-18). Also, using LamR as a solid phase in DIG-labelled ELISA, different concentrations (2.5 µg ml^{-1}; 5µg ml^{-1} and 10µg ml^{-1}) of LamR were used to coat protein A-treated wells, which were then challenged with Dig-labelled *S. pneumoniae* and *S. aureus*. *S. pneumoniae* bound to rLamR expressed in bacteria or human cells at similar levels that were significantly higher than binding to the control preparations (Figure 4 18).

The specificity of binding was demonstrated by comparing binding of *S. pneumoniae* cells with *S. aureus*, which was included as a negative control; binding of *S. aureus* to LamR was negligible and highly significantly lower than binding of *S. pneumonae* cells at all dilutions tested (*p* value <0.001; Figure 4-19).

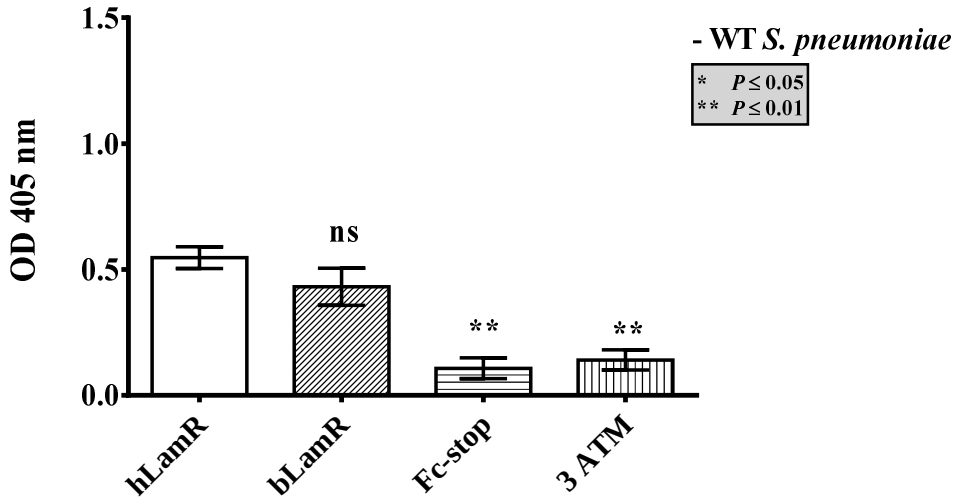


Figure 4-18 Binding of immobilised hLamR and bLamR to *S. pneumoniae*.

Protein A-treated wells were coated with hLamR or bLamR at a concentration of 5 µg ml^{-1}. Fc-stop and 3ATM-coated wells were included as negative controls. BSA-coated wells to correct for non-specific background binding. Wells were subsequently incubated with DIG-labelled *S. pneumoniae* bacteria followed with anti-DIG antibody. Bound bacteria were detected after 1 h of adding AP substrate in an ELISA plate reader. Specific binding of bacteria to protein-coated ELISA plates was determined by subtracting the absorbance in BSA-coated wells from that in protein-coated wells. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ns at P >0.05; *Significant at $P \leq 0.05$; ** $P \leq 0.01$ compared to binding to wild type hLamR.

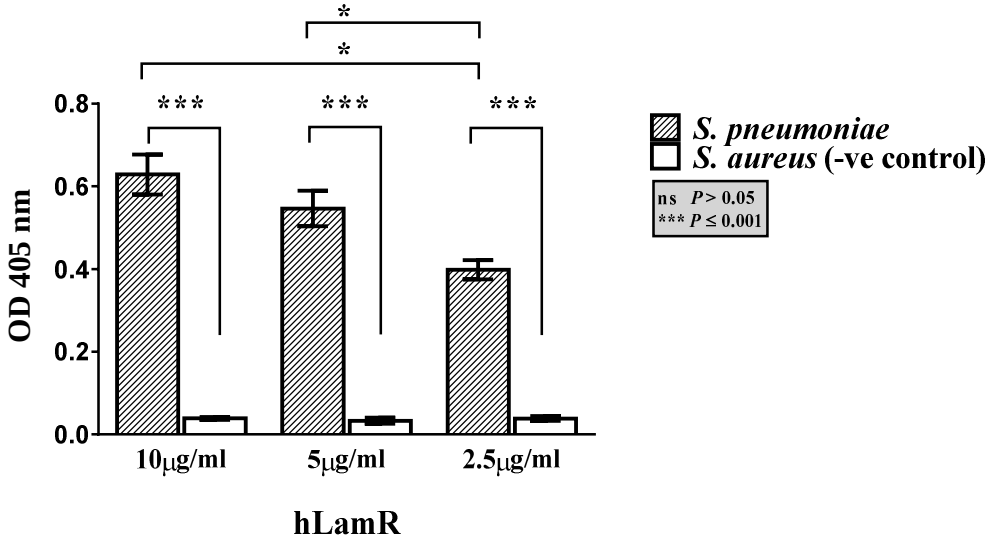


Figure 4-19 Binding of *S. pneumoniae* and *S. aureus* to various concentrations of rLamR.

Protein A-treated wells were coated with various concentration (2.5 μg ml^{-1}; 5μg ml^{-1} and 10μg ml^{-1}) of recombinant hLamR. BSA-coated wells to correct for non-specific background binding. Wells were subsequently incubated with DIG-labelled bacteria followed with anti-DIG antibody. Bound bacteria were detected after 1h of adding AP substrate in an ELISA plate reader. Specific binding of bacteria to rLamR-coated ELISA plates was determined by subtracting the absorbance in BSA-coated wells from that in protein-coated wells. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ns at P >0.05; *Significant at $P \leq 0.05$; ** $P \leq 0.01$.

4.3.7.8 LamR regions: 206-229, 263-282 and Tyrosine 156 were proposed as binding sites for pneumococcal CbpA protein

The pneumococcal adhesin CbpA was reported to be LamR ligand and LamR region, 263-282 was proposed as a binding domain for pneumococcal CbpA (Orihuela et al., 2009), however this LamR has not been further confirmed as LamR binding domain. The current study aimed to identifying the *S. pneumoniae* -binding domain(s) of LamR. Using a similar technique that was employed to identify *N. meningitidi s* and *H. influenzae* binding-domains of LamR, DIG-

labelled ELISA was used to identify *S. pneumoniae*-binding domain(s) within LamR. The interaction level of wild type LamR and wild type *S. pneumoniae* was included as a positive control; 3ATM and Fc-stop included as negative controls. Interestingly, deletion of the two regions: 206-229 and 263-282 of LamR, which were identified previously as binding domains *N. meningitidis* (Section 4.3.3) and *H. influenzae* (Section 4.3.7.6), also resulted in a significant reduction in its ability to bind *S. pneumoniae* (*p* value <0.01; Figure 4-20). Subsequent experiments with CbpA-deficient bacteria confirmed and suggested the strong contribution of these two regions in binding to *S. pneumoniae*. In addition of the defined regions, substitution of 139 Tyrosine in LamR was also resulted in applicable decrease in binding of LamR with both wild type and CbpA lacking pneumococcus (Figure 4-21).

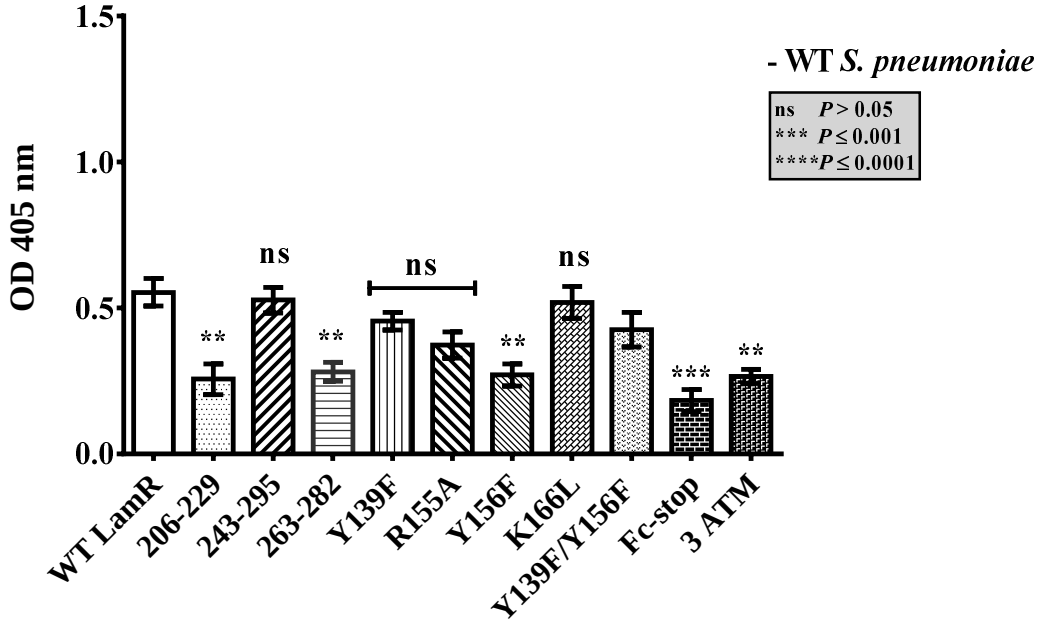


Figure 4-20 Mutations in hLamR resulted in a marked reduction of binding to *S. pneumoniae*.

Protein A-treated wells were coated with hLamR and its mutant derivatives at concentration of 5 µg ml-1. Fc-stop and 3ATM-coated wells were included as negative controls and BSA-coated wells to correct for non-specific background binding. Wells were subsequently incubated with DIG-labelled *S. pneumoniae* bacteria followed with anti-DIG antibody. Bound bacteria were detected after adding AP substrate in an ELISA plate reader. Specific binding of bacteria to protein-coated ELISA plates was determined by subtracting the absorbance in BSA-coated wells from that in protein-coated wells. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ns at P >0.05; **Significant at $P \leq 0.01$; *** at $P \leq 0.001$ compared to binding to wild type hLamR.

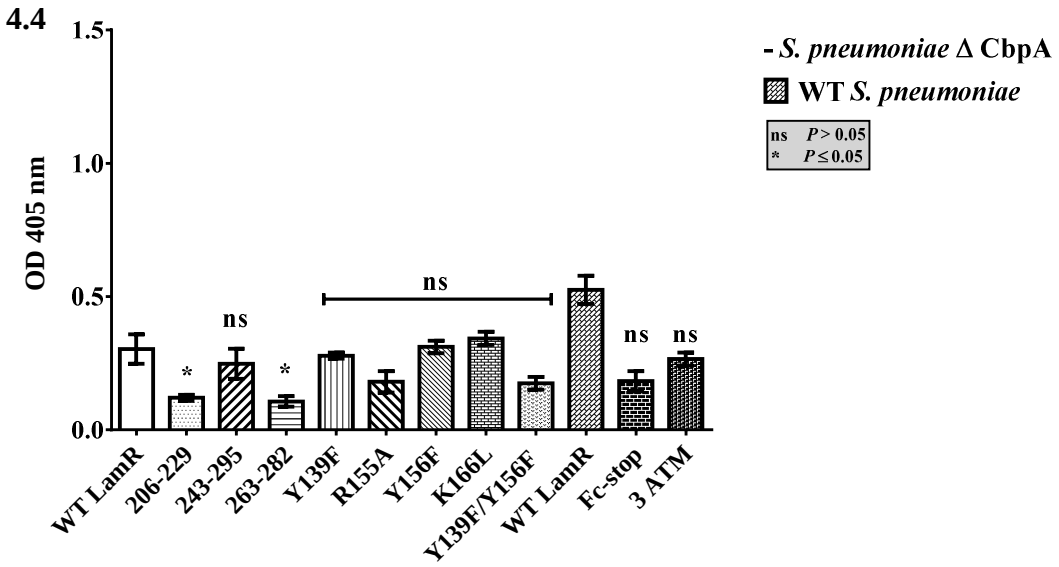


Figure 4-21 LamR regions: 206-229, 263-282 and Tyrosine 156 were proposed as binding sites for pneumococcal CbpA protein.

Protein A-treated wells were coated with hLamR and its derivatives mutants at concentration of 5 μg ml^{-1}. Fc-stop and 3ATM-coated wells were included as negative controls. BSA-coated wells to correct for non-specific background binding. After coating the wells with LamR or BSA, the wells were subsequently incubated with DIG-labelled *S. pneumoniae* lacking CbpA followed with anti-DIG antibody. Bound bacteria were detected after 1 h of adding AP substrate in an ELISA plate reader. Specific binding of bacteria to protein-coated ELISA plates was determined by subtracting the absorbance in BSA-coated wells from that in protein-coated wells. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ns at P >0.05; **Significant at $P \leq 0.01$; *** at $P \leq 0.001$ compared to binding to wild type hLamR.

4.4 Discussion

Microbes interact with their hosts by recruiting several multiple receptors exposed on the host cells (Pan et al., 2014). Laminin receptor (LamR) on the surface of brain microvascular epithelial cells (BMECs) has recently been shown to play an important role in infection by bacterial pathogens capable of causing meningitis by serving as a receptor for specific adhesins on their surfaces and allowing these pathogens to enter host cells (Nelson et al., 2008, Rea et al., 2012). Furthermore, it has been observed that several bacterial meningeal pathogens bind host glycoproteins, such as laminin; collagen; fibrinogen and fibronectin. These glycoproteins may act as bridging molecules between those bacteria and HBMECs (Khan et al., 2007, Tenenbaum et al., 2007, Banerjee et al., 2011). An early study of the mechanism of *E coli* attachment to the HBMECs was reported by Chung and co-workers, who demonstrated that cytotoxic necrotizing factor 1 (CNF1) of *E coli* K1 promotes bacterial internalization into human cells (Chung et al., 2003). CNF1 triggered clustering of LamR on the human brain microvascular endothelial cells (HBMECs) as well as association with focal adhesion kinase and paxillin. This process is likely causing cytoskeletal rearrangement in order to promote bacterial endocytosis (Chung et al., 2003, Kim et al., 2005, Wang and Kim, 2013). In addition to bacteria, a role for LamR in binding and internalization of many viruses and infectious prions into neuronal cells has been demonstrated (Ludwig et al., 1996, Gauczynski et al., 2001, Gauczynski et al., 2006, Scheiman et al., 2010b). Also, in cultured neuronal cells, it has been found that LamR is targeted by the scrapie isoforms of the prion (PrP^{Sc}) receptor at the cell surface (Malygin et al., 2009).

Three bacteria that cause the majority of cases of bacterial meningitis: *N. meningitidis*; *H. influenzae* and *S. pneumoniae* were shown to target LamR (Orihuela et al., 2009). This study was undertaken to characterize regions within LamR which might have role in the bacterial interaction with a particular focus on recently published data which identified meningococcal PilQ and PorA, pneumococcal CbpA, and OmpP2 of *H. influenzae* as being particularly important in adherence of bacteria to HBMECs (Orihuela et al., 2009, Abouseada et al., 2012). Investigation of LamR-bacteria binding sites will provide a broader understanding of the mechanisms underlying this interaction.

Several regions on LamR were hypothesised to contribute in the interaction of LamR with the three bacterial pathogens; those regions were deleted or substituted to test their direct effects on binding of LamR to the bacteria. Importantly, hLamR, expressed in human cells and purified under non-denaturing conditions was used in order to ensure as far as possible that the protein was being presented in its native form including any post-translational modifications (PTMs) that would normally occur in human cells.

In current study and for the first time, the interaction of bLamR and hLamR with three bacteria: *N. meningitidis*; *H. influenzae* and *S. pneumoniae* was compared. bLamR and hLamR exhibited similar binding behaviour with those bacterial pathogens. These results suggested that post translational modifications may not be required for LamR binding with such bacteria since the recombinant bLamR is likely to lack such modifications. However, it is important to note that LamR is a multifunctional protein which is targeted by a number of viruses and other bacteria, as well as laminin and prions, which were not tested in the current study

so the question of whether PTMs are required for the binding of LamR to its other ligands has not yet been addressed.

The results described in this chapter show that *N. meningitidis; S. pneumoniae* and *H. influenzae* bind recombinant hLamR and the specificity of the interaction was confirmed by comparing the binding of these pathogens with *S. aureus* and other negative controls (3ATM and Fc-stop). The mutant bacterial strains: *N. meningitidis* MC58 strain (Δ*porA; ΔporAL2; ΔpilQ* and Δ*porA/pilQ*); *S. pneumoniae* (Δ*cbpA*) and *H. influenzae* (Δ*ompP2* and Δ*ompP2L2*) were also investigated for their ability to bind LamR. In these experiments, the binding reduction reflected the level of ligands contribution in bacterial binding with LamR. Binding of wild type LamR to all mutant strains of the three tested bacteria was typically reduced by half in comparison with their wild type parent strains confirming the critical role of deleted bacterial porins or surface proteins adhesins in LamR binding.

In *N. meningitidis*, since two ligands: PorA and PilQ contributed in LamR-binding, the partial binding was expected in case of single mutation. The PorA/L4 deficient MC58 strain was also tested for its ability to bind LamR and the results confirmed the role of L4 in binding of LamR-PorA and demonstrated that in the absence of L4 PorA did not display any residual LamR binding activity. When both PorA and PilQ were deleted, LamR binding was reduced to levels lower than either deletion alone, confirming the partial redundancy between these two surface proteins with respect to LamR binding.

Attempts were also made to generate double knockout mutants of PilT and PilE in a *porA*-deletion background *N. meningitidis* MC58 by natural transformation to

examine whether having a pilus protruding through PilQ would affect binding to LamR. We hypothesised that, in *N. meningitidis* lacking PorA, any LamR binding will be mediated by PilQ. Also, in *N. meningitidis* lacking both PorA and PilT, the pilus will be extruded and as it cannot be retracted, so a much greater proportion of available PilQ secretin pores will be occupied by a pilus. While in *N. meningitidis* lacking both PorA and PilE, the pilus will not be produced, so all secretin pores should be empty. However, these attempts of natural transformation were unsuccessful and time restrictions prevented further attempts.

In *S. pneumoniae* and *H. influenzae*, only OmpP2 loop2 and CbpA were identified as LamR-ligands. Testing the binding of wild type LamR to mutant strains of these two bacteria showed a signification reduction compared with wild type bacteria. The interaction of the OmpP2/L2-deficient *H. influenzae* mutant strain with LamR confirmed the role of L2 of OmpP2 in its interaction with LamR. Our study complements and confirms the previous studies which showed that the maximal LamR-binding was localized to pneumococcal CbpA and the amino acids containing the surface-exposed loop 2 in OmpP2 of *H. influenzae* (Orihuela et al., 2009, Abouseada et al., 2012).

As mentioned earlier, the main aim of the work described in this chapter was to characterize the bacteria-binding domain(s) within the LamR protein. Several novel sites within LamR that act as bacterial binding domains were revealed. Two specific sites LamR: 206-229 and 263-282 respectively, were proposed to contain binding domains for *N. meningitidis*; *H. influenzae* and *S. pneumoniae* as deletion of these regions resulted in the most prominent reduction in level of interaction with the three bacteria. Furthermore, this study has shown that Tyrosine 156 of

LamR contributed to attachment to *N. meningitidis* and *H. influenzae*. Also, substitution of Tyrosine 139 was observed to reduce LamR-pneumococcal binding. The degree to which binding was reduced in these single-mutant residues was similar to deletion of the defined regions (206-229 and 263-282), suggesting a direct involvement of these residues in bacterial-interactions.

This study has revealed that LamR region from 206-229 was involved in binding to meningococcal PilQ, while regions containing amino acids 206-229 and 263-282, as well as the single amino acid Tyrosine 156, were found to mediate the interaction of meningococcal PorA, in particular its L4 with LamR. The second novel finding was identifying the *H. influenzae*-binding domains on LamR. Similar to the meningococcal ligands, two regions within LamR: 206-229 and 263-282; and single residue: Tyrosine 156, contributed to binding of LamR to *H. influenzae*. Including the mutant strains: Δ*ompP2* and *ompP2:L2*, which are deficient in OmpP2 or the second extracellular loop of that porin, respectively, demonstrated that these defined regions on LamR interact with L2 of OmpP2, but that other unidentified molecules of *H. influenzae* may also contribute to LamR binding. The *S. pneumoniae* interaction domain on LamR was the third novel findings in current study. Two regions within LamR: 206-229 and 263-282; and single residue: Tyrosine 139, were found to be important in binding of LamR to CbpA of *S. pneumoniae*. However, deletion of these ligands did not totally abolish LamR-bacteria binding in those tested mutants suggesting that other unidentified molecules may also contribute to LamR binding in this pathogen as well, however such interactions are likely to be less important than those of the identified ligands in LamR-bacteria binding.

The interaction of two recombinant versions of the LamR protein: hLamR and bLamR, with both rPorA and rPilQ were compared using ELISA. Also, the interaction of recombinant hLamR was tested with either soluble rPorA or immobilised rPorA. Similar to our previous findings, no significant differences were observed in rPorA-binding to either hLamR or bLamR. Further, recombinant hLamR bound at similar levels to soluble and immobilised rPorA protein (Figure 4-11 A&B respectively). The recombinant LamR mutant proteins: $LamR^{\Delta 206-229}$; $LamR^{\Delta 263-282}$, and $LamR^{T156Y}$, also showed higher reductions in their binding activity to rPorA, confirming our previous results. While other mutated LamR proteins bound to rPorA at similar levels to the wild type protein (Figure 4-12). Similar results were observed when rPilQ was used in ELISA: deletion of amino acids 263-282 of LamR resulted in a reduction of binding to PilQ. However, due to time constraints, the experiment was achieved only twice and the observed differences did not reach statistical significance.

Interestingly, LamR region 206-229 has been reported as a binding domain for the β1 chain of laminin-1(Nelson et al., 2008), heparin (Rea et al., 2012) and the prion protein (Hundt et al., 2001). A single study focussed on residues 263-282 of LamR, which were proposed as a binding domain for meningococcal PorA (Orihuela et al., 2009). The work described here confirms and builds on those findings. Our results revealed that deletion of most of the C-terminal portion of LamR (amino acid residues, 243-295) resulted an increase in the binding ability of LamR to all three bacteria. Jamieson., (2011) indicated that deletion of the whole C-terminal portion of LamR did not affected its physiological functions including its ability to bind laminin with similar affinity as full-length LamR and inhibition of Sindbis virus infection *in vitro* (Jamieson et al., 2011), however due to a lack of

information regarding the crystal structure of the C-terminal portion of LamR, the interpretation of such activity remains a challenge. It is possible that deletion of these sites may expose new hidden residues which have similar affinity to binds LamR.

Collectively, our results strongly suggest that *N. meningitidis*; *H. influenzae* and *S. pneumoniae* each target the same LamR domains. Whether these LamR regions contribute collectively or individually to the binding of LamR to the bacterial ligands was not previously considered. Identification of the precise amino acid residues within these regions, either by site-directed substitution of individual amino acids, or using by the synthesis of synthetic peptides corresponding to the defined regions within LamR will answer the question of whether or not these bacteria target the same amino acid residues in LamR protein.

In light of the role of several LamR-bacterial ligands in infection, Alqahtani (2012) reported the involvement of Gal3 in meningitis infection and found that meningococcal PorA indirectly recruits Gal-3 through LamR dimers with Gal-3. The same study reported that meningococcal PilE and PilQ interacted directly with Gal-3, indicating that the identified meningococcal ligands utilized further receptors on human cells, and this may contribute to the bacteria gaining entry into the CNS. Interestingly, treating of HBMECs with meningococcal rPorA, loop 4 of PorA (synthesised as a synthetic peptide) and rPilQ proteins upregulated the expression of LamR on these cells (Alqahtani et al., 2014). Similar results were earlier observed by Orihuela and co-workers using whole bacteria; they revealed the up-regulation of the LamR expression in the brains of mice infected with *N. meningitidis* (Orihuela et al., 2009). Also, Kim et al (2005) observed the up-

regulation of LamR protein in HBMECs in response to CNF1of *E. coli* K1(Kim et al., 2005). Moreover, significant evidence showed that OmpP2 of *H. influenzae* plays a critical role in the pathology of infection; the antibodies against OmpP2 protein are found to be bactericidal in both type b and non-typeable *H. influenzae* strains. In an infant rat model, antibodies against OmpP2 were provided the protection of bacteraemia. Also, OmpP2 mutant of *H. influenzae* in an infant rat model was unable to cause bacteraemia (Cope et al., 1990, Chong et al., 1993, Duim et al., 1996, Yi and Murphy, 1997).

Concluding remarks

From our results, it has been observed that three meningeal pathogens target the same domains on LamR. Further investigation in future studies to map the precise sites using either synthetic peptides or mutations to test whether the amino acids of the defined regions are involved collectively or individually in LamR-binding will be of great value.

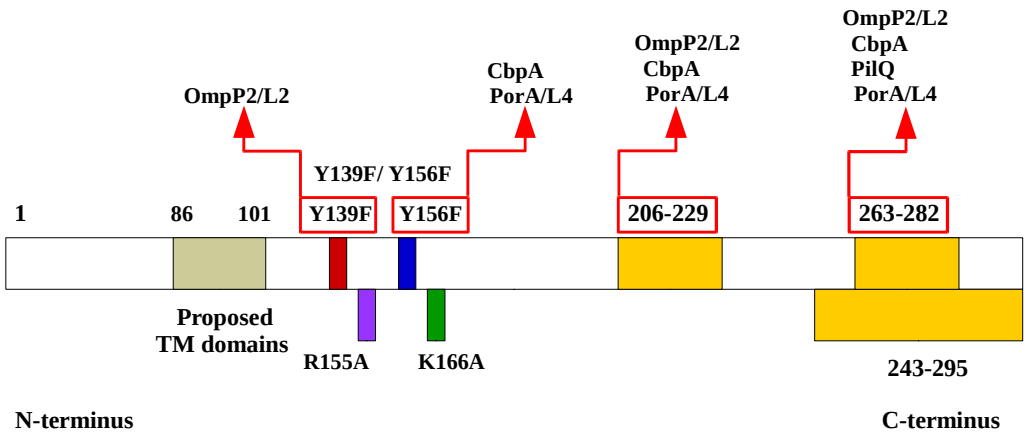


Figure 4-22 Schematic of identified bacteria-binding domains within human LamR protein.

Chapter five

Identification of the interface determinants of 37LRP that participate in both LamR homo and heterodimerisation

CHAPTER FIVE

5 IDENTIFICATION OF THE INTERFACE DETERMINANTS OF 37LRP THAT PARTICIPATE IN BOTH LAMR HOMO AND HETEROFDIMERISATION

5.1 Introduction

The 37/67kDa laminin receptor, LamR, is a multifunctional protein. This protein has been found to associate with the 40S ribosomal subunit and also localizes to the cytosol and the cell membrane where it can interact with the extracellular matrix (DiGiacomo and Meruelo, 2015). Several endothelial cells, including human brain microvascular endothelial cells (HBMECs), have been reported to express LamR. Interestingly, a role for LamR in bacterial meningitis has been established with LamR exhibiting affinity to three meningitis-causing bacteria (Orihuela et al., 2009).

Several studies have revealed the presence of two forms of LamR: the monomer termed as 37kDa LRP, which localizes to the nucleus, cytosol and cell surface; and a cell-surface associated form called 67LR (Nelson et al., 2008, Alqahtani et al., 2014, DiGiacomo and Meruelo, 2015). 67LR has been suggested to result from post-translational modification or alternatively from homodimerisation of two 37LRP molecules (Gauczynski et al., 2006, Nelson et al., 2008), or heterodimerisation of 37LRP with a molecule of a protein called Galectin-3 (Gal-3) (Castronovo et al., 1992, Buto et al., 1998). Gal-3 is a lectin with specificity for β-galactoside-containing carbohydrates; it is a structurally unique member of the galectin family and involved in a variety of normal and pathological biological processes. Its carbohydrate-binding properties constitute the basis for cell-cell and

cell-matrix interactions and cancer progression (Dumic et al., 2006). Galectin-3 is expressed in many cells, including HBMECs. A role for this molecule in meningococcal meningitis has been previously suggested by Quattroni et al., (2012), who showed that Gal3 binds to *N. meningitidis*, that Gal-3 was expressed during meningococcal disease, and that it co-localized with bacterial colonies in infected tissues from patients (Quattroni et al., 2012). More recently, Alqahtani and colleagues identified meningococcal PilQ and PilE as additional Gal-3 ligands (Alqahtani et al., 2014). Indeed, 37LRP and Gal-3 proteins share several common features: they are both multifunctional non-integrin laminin-binding proteins which have meningococcal-ligand binding domain(s); they are both present in cells in both monomeric and dimeric forms; they are also both present in multiple subcellular locations in the cell. These two receptors were differentially expressed in various cancer cells (Castronovo et al., 1992, Nelson et al., 2008, Rea et al., 2012, DiGiacomo and Meruelo, 2015). Further, it was shown by Alqahtani and co-workers that both cell-surface 37LRP and Gal-3 co-localized with 67LR in HBMECs, astrocyte and COS7 cells. In the same study, the cell surface co-localization of Gal-3 and 37LRP was tested either by co-transfecting constructs encoding fluorescently tagging-recombinant versions of 37LRP and Gal-3 into COS7 cells, or by labelling the endogenous LamR and Gal-3 in HBMECs and the immunoblotting results using anti-37 LRP [A7]; anti-67 LR and anti-Gal-3 antibodies of membrane fractions showed co-localization of LamR and Gal-3 (Alqahtani et al., 2014). Although, 37LRP maturation and the direct relationship between 37LRP and Gal-3 remains controversial, and the current literature is often confusing, based on theoretical considerations derived from the intensive investigations of LamR protein, a plausible hypothesis for the role of a LamR

dimer form constituting 67 LR might be required for its interaction with Laminin or other substrates such as elastin or collagen (Rao et al., 1989, Jamieson et al., 2008, Nelson et al., 2008), virus and bacteria (DiGiacomo and Meruelo, 2015). However, the 37LRP monomer has also been found to have laminin-binding properties as well as the ability to bind viruses; bacteria and prion proteins (Jamieson et al., 2008, Zidane et al., 2013, DiGiacomo and Meruelo, 2015). Although an increasing amount of conflicting data and evidence have been reported to support or refute the hypothesis that proposed LamR exists as a homo or heterodimer, this provocative hypothesis has progressively become accepted by several of scientists studying this important change throughout the maturation process from precursor (37LamR) to a mature receptor (67LamR) (DiGiacomo and Meruelo, 2015). A key event in the acceptance of the dimerisation idea of LamR was the findings that showed evidence for both possibilities. Consequently, the recurring question about LamR dimerisation has recently changed from ‘do LamR dimers really exist?’ to ‘why do LamR need to exist as dimers?’

The answer to the latter question is possibly to be that LamR forms such a complex to be mature (Menard et al., 1998, Scheiman et al., 2010b) and for membrane association. However, the function of receptor homo or heterodimers has been more difficult to elucidate, mainly because conclusive experiments to differentiate the potential role of monomers from that of homodimers are not easy to design and/or to perform. In our recent publication, we presented evidence that supports the general roles that both homo and heterodimerisation could have in LamR biology, with particular emphasis on finding the amino acid residues within 37LRP that may responsible on LamR homo and heterodimerisation (Alqahtani et al., 2014).

Characterization of protein-protein interactions (PPI) is essential to the understanding biological processes (Charbonnier et al., 2008, Kar et al., 2009). Several non-invasive techniques have been used to study protein-protein interactions. These include, yeast two-hybrid screens and co-immunoprecipitation assay (Shyu et al., 2006, Kerppola, 2008, Witherow and Carson, 2011). The bimolecular fluorescence complementation (BifC) assay, is a new powerful technique that allows the visualization of either single or dual PPI at the sub-cellular level in live cells (Kerppola, 2013b). The greatest strength of this approach compared with others is that it has higher detection sensitivity, and permits for the detailed characterisation of such interactions in the native biological system. The approach is based on protein fragment complementation by splitting of a fluorescent reporter protein into two inactive non-fluorescent fragments (N- and C-terminus) which are tagged with the target protein(s) to test their dimerisation properties. The interaction between target proteins brings the reporter moieties into close contact, leading to the spontaneous reconstitution of an active reporter (Hu et al., 2002, Kerppola, 2009, Hollender and Liu, 2010, Kerppola, 2013a, Alqahtani et al., 2014). Complemented fluorescent signals can be then visualized by fluorescence-activated cell sorting (FACS assay) (Giese et al., 2005, Watanabe et al., 2008), fluorescence microscopy or confocal microscopy in live cells or in animals (Shyu and Hu, 2008). Several fluorescent proteins such as green fluorescent protein (GFP) of *Aequorea victoria* (Walter et al., 2004, Kerppola, 2006, Shyu and Hu, 2008) or its mutant derivatives such as venus yellow fluorescent protein (vYFP) (Nagai et al., 2002); the red mCherry; and the cyan Cerulean (Rizzo et al., 2004) are usually used for BifC assay. Due to some limitations, including the requirement to pre-incubate GFP fragments at

30°C to be able to detect the BifC signal, new BifC techniques were developed by using GFP variants such as Venus, a known YFP mutant (vYFP) which was truncated at either 155 or 173 residues. The vYFP has been showed a great improvement in the efficiency of BifC complementation and allowed the formation of BifC complementation under physiological conditions in mammalian cells. Also, it is characterised by its fast maturation at 37°C and bright fluorescence (Nagai et al., 2002, Shyu et al., 2006, Rose et al., 2010).

Aim of the work described in this chapter

Work described in this chapter was designed to identify amino acids in 37LRP that are essential for the formation of LamR homodimers and heterodimers with Gal-3. An *in vitro* approach using ELISA was complemented by using site-directed mutagenesis and biomolecular fluorescence complementation (BifC) in living cells.

5.2 Experimental procedure

5.2.1 Molecular modelling and computer simulation for the interaction of galectin-3 (Gal-3) with LamR

Three-dimensional structures of each carbohydrate recognition domain (CRD) of Gal-3 and LamR (LamR^{220}) were obtained the RCSB protein data bank (www.rcsb.org). The wild type crystal structure of Gal-3 (3zsj.pdb: lactose-liganded structure; 3zsm.pdb: non-liganded structure) and wild type LamR (3bch.pdb) were used as templates for the molecular docking to identify predicted binding sites for LamR dimerisation on each protein using a server in zdock.umassmed.edu (Mintseris et al., 2007) based on available knowledge of crystal structures. RASMOL (Sayle and Milnerwhite, 1995) and UCSF CHIMERA (Pettersen et al., 2004) were used to create images of the molecular interactions and the docking sites.

5.2.2 PCR site-directed mutagenesis

Primers were designed using the www.agilent.com/genomics/qcpd tool and listed in chapter two (Table 2-2). Mutagenesis was carried out following the manufacturer's instructions using the Quick Change Lightning Site Directed Mutagenesis Kit (Agilent) as described in chapter two (section 2.2.6.2.3). The mutated samples were sequenced by DNA sequencing to confirm the mutations (Source Bioscience).

5.2.3 Cell line and cell culture condition

COS7 and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic-

antimycotic solution (Gibco) and incubated at 37°C in an atmosphere of 5% CO2. The media was changed every two days and cells were split using trypsin-EDTA (Gibco) when they became 90% confluent. Cells were plated the day before transfection in either 6-well or 12-well plates until they reached a confluency of 50-70 % on the day of transfection. For immunofluorescence assays, cells were grown on 12mm diameter glass coverslips with serum and antibiotic supplemented DMEM media. For the primary human brain microvascular endothelial cells (HBMECs, ScienCell Research Laboratories), they were cultured either in fibronectin-coated 75 cm^2 flasks or 12-well plates (Biocoat, BD-Falcon) and incubated at 37ºC in an atmosphere of 5% CO_2 in endothelial cell medium (ECM, Science Cell Research Laboratories) supplemented with endothelial cell glial supplement (ECGS, Science Cell Research Laboratories), 1% (v/v) antibiotic-antimycotic solution (Gibco) and 10% (v/v) fetal bovine serum (FBS, Science Cell Research Laboratories).

5.2.4 Transfection reagents and procedures

Three types of transfection reagent were used in current study.

5.2.4.1 Lipid-based transfection method using Trans IT®-2020 transfection reagent

For the lipofection procedure, Trans IT®-2020 transfection reagent (Mirus) was used to transfect DNA constructs into COS7 and HBMEC cells. The transfection was performed according to the manufacturers' instructions. In brief, the cells were seeded the day before transfection in 6- or 12-well plate to reach ~ 70% on the day of transfection. The DNA was diluted with TransIT-2020 in serum- and antibiotic-free DMEM or ECM media for COS7 and HBMECs respectively at a

ratio of 2µg DNA to 1µl TransIT-2020 and left for 25 min at room temperature (RT) before 250µl or 100µl of transfection solution (DNA and TransIT-2020 complex) were added dropwise to each well of the 6- or 12-well plates. The plates were incubated for 4h at 37ºC in a 5% CO_2 atmosphere, then the media was changed and the plates were incubated for 30h. After 24h, cells were harvested using RIPA buffer (APPENDIX) and subjected to SDS-PAGE and immnoblot assays to detect protein expression (section 3.3.1.7.3) and 30h post transfection for Fluorescence-activated cell sorting (FACS) analysis to measure transfection efficiency. For BifC signal detection and cell imaging, cells were prepared for confocal microscopy or FACS assay as described in later sections.

5.2.4.2 Lipid-based transfection method using jetPRIM transfection reagent

Cells were transiently transfected with jetPRIME® reagent (Polyplus). For 6-well plates, 200,000 cells were seeded per well in 2ml of cell growth medium the day before transfection to reach 60- 80% confluent at the time of transfection. In order to identify the optimal transfection efficiency, dilution series with different ratios of transfection reagent to plasmid DNA were used. Then, the DNA was diluted in jetPRIME® buffer. After mixing the DNA-buffer complex, the jetPRIME® was added to the complex and vortexed for 10s and incubated for 10min at RT. The transfection mix was then dropwise onto serum-containing media. The plates were incubated for 4h at 37°C in a 5% CO_2 atmosphere, then the transfection media was changed with cell growth media and the plates were incubated for 24h.

5.2.4.3 Chemical-based transfection method using $CaPO_4$ transfection reagent

Transient transfection of COS7; HEK293T and HBMECs cells performed using calcium phosphate coprecipitation method as described previously in chapter three (3.2.3.8.1).

5.2.5 ELISA assay to detect the hetero- and homo-dimerisation of LamR

ELISA was performed to detect the hetero- and homodimerisation of LamR. The details of ELISA steps were described previously in chapter four (section 4.2.1.2). In these experiments, the wild type recombinant human cell produced 37LRP (full molecule and its mutant derivatives) fused with Fc-tag were coated on a solid phase-microplate wells at concentration 5μg ml^{-1} diluted in PBS. After blocking, an equal molarity of wild type recombinant 37LRP produced in *E coli* and fused with His-tag or wild type human rGal-3 full molecule expressed in *E coli* (Millipore) were added and later probed with mouse monoclonal anti-pentahistidine (Qiagen; 1:2000 dilution) or mouse monoclonal anti-galectin-3 (MAB4033, Millipore; 1:8000 dilution) primary antibodies. Then the plates were incubated with the alkaline phosphatase conjugated anti-mouse IgG, produce in Goat (Sigma; 1:20000 dilution) secondary antibody. 100μl of alkaline phosphatase substrate (APPENDIX) were added to each well, and the absorbance was measured at 405nm after 1h using an ELISA plate reader.

5.2.6 Immunoblot analysis

Immunoblot analysis was performed as described in chapter three (section 3.3.1. 7.3). Here membranes were probed with anti-37LRP [A7] mouse monoclonal IgG antibody (Santa Cruz Biotechnology; 1:2000 dilution) or anti-GFP rabbit polyclonal antibody (sc-8334, Santa Cruz; 1:200 dilution) or anti-Galectin-3 mouse monoclonal antibody (MAB4033, Millipore; 1:1000 dilution) primary antibodies. After washing, membranes were then incubated with anti-mouse IgG horseradish peroxidase (HRP)-conjugated (Millipore; 1:125000 dilution) secondary antibody or HRP-conjugated anti-rabbit IgG polyclonal antibody at dilution: 1:20000. Afterwards, membranes were developed with Luminata western HRP substrate (Millipore).

5.2.7 Flow cytometry

Transiently transfected COS7 or HBMECs were analysed by flow cytometry for both protein expression and protein dimerisation. The COS7 or HBMECs were grown in 6-well plates; 30h post transfection, the cells were detached using trypsin (Sigma). Detached cells were collected in FACS tubes and centrifuged at 300 x*g* for 5 min. Supernatants were removed and pellets were washed with PBA buffer (0.5% BSA and 0.5% sodium azide) and resuspended in 0.5% paraformaldehyde in PBS and subjected to flow cytometry (Coulter Altra Flow Cytometer). Data acquisition and analysis were performed with Weasel 2.5 software, cells were gated using forward scatter (FSC) and side scatter (SSC) to exclude dead cells, cellular debris etc. In each case, 50,000 cells were counted in triplicate and used to calculate the average BifC signal intensity ± SEM. Full-length YFP fluorescence signal (vYFLP) was used as the interassay reference for

maximal fluorescence in each experiment, against which the BifC signal intensities were compared.

5.2.8 Immunofluorescence

For immunofluorescence assays, COS7 or HBMECs transfected with required constructs were fixed on cover slips with 1% (w/v) paraformaldehyde (PFA) in PBS for a maximum of 10 min at room temperature and then washed thrice with PBS, followed by blocking with 4% BSA in PBS (blocking solution) for 1h at room temperature. Then, the cover slips were incubated for 1h with primary antibody(s): anti-37LRP [A7] mouse monoclonal IgG primary antibody (Santa Cruz Biotechnology; 1:100 dilution); anti-67LR Mlu5 and Ab711(Abcam;1:100 dilution); anti-GFP rabbit polyclonal antibody (sc-8334, Santa Cruz; 1:100 dilution); mouse monoclonal anti-human galectin-3 antibody (MAB4033, Millipore; 1:100 dilution), and rabbit monoclonal anti-human galectin-3 antibody (Abcam; 1:100 dilution) in 4% BSA in PBS for 1h. Then the primary antibody solution was removed by washing the coverslips 20 times in PBS-T; 20 times in PBS and 20 times in dH_2O. Afterwards, the coverslips were incubated with secondary antibody(s): anti-mouse Alexa Fluor 680 antibody (1:100); anti-rabbit Alexa Fluor 680 antibody (1:100); anti-rabbit Alexa Fluor 488 antibody (1:400); anti-mouse Alexa Fluor 488 (1:400) (all from Molecular Probes), in 4% BSA in PBS for 1h and washed 20 times in PBS-T, 20 times in PBS and 20 times in dH2O. Finally, the coverslips were mounted using Fluoromount (Sigma) mounting media and images obtained by confocal microscope (Zeisis LSM700).

5.2.9 Confocal microscopy

Visualization and image acquisition of BifC signal formation was performed using a Zeiss LSM700 confocal microscope (Carl Zeiss, Ltd., Welwyn, UK) using Plan-Apochromat 63x/1.4 Oil DIC M27. Cells were imaged using following lasers: (i) Hoechst, 405nm; (ii) Alexa 488, 488nm; and (iii) Alexa 680; 639nm. The image size was 1024 × 1024 with 8 averages taken per frame and a pinhole size of 1 Airy Unit. All control images were captured with the same laser power, gains and zoom settings. Images were captured using the Zen 2009 software package and analysed using LSM Image Browser software and Image J.

5.3 Results

In the current study, the aim was to identify the residues that contribute to the stability of LamR homodimers and heterodimers with Galectin3 (Gal-3). Using *in vitro* models (COS7 and HBMECS) enables a better understanding of cell surface LamR dimer forms that have critical roles in binding with different pathogens including the bacterial meningeal pathogens as well as virus; prion protein and extracellular matrix (ECM) components.

To achieve our goal, several 37LRP mutants were examined for their ability to interact with either wild type 37LRP to form homodimer or with wild type Gal-3 to form heterodimer. *In vitro* investigations were performed using ELISA assays; dimerisation was further analysed in living cells using the BifC assay. In order to investigate the influence of different selected 37LRP mutations on dimerisation, initially, the expression pattern of wild type 37LRP and wild type Gal-3 in COS7 and HBMECs was investigated. Further, the dimerisation ability of several LamR mutants with either 37LRP or Gal-3 to form homo- or hetero-dimer respectively in COS7 cells was examined *in vitro* and in living cells.

5.3.1 Computational approaches for modeling LamR dimerisation predicted the involvement of several amino acid residues in both 37LRP and Gal-3 proteins to form LamR dimer form

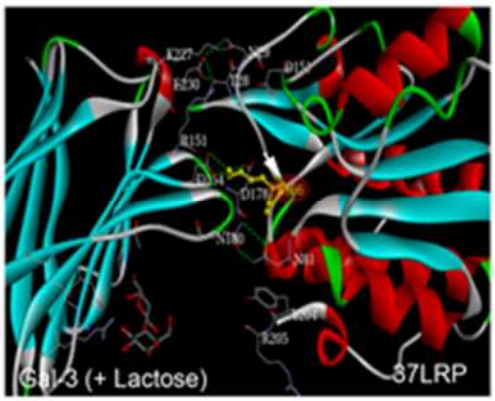
Protein-protein interactions (PPIs) form the core of numerous biological processes such as: cell signalling; protein dimerisation; enzyme inhibition; interaction hot spots among proteins; interface prediction and immune recognition. Jamison et al., (2008) previously reported the crystal structure of human LamR using a truncated LamR (amino acids 1-220; therefore lacking the C-terminal portion). In

Jamison's study, several amino acid residues were speculated to be involved in either laminin-binding (including Phenylalanine32, Glutamic acid35 and Arginine155) or the interface residues for LamR homodimerisation (Alanine114, Phenylalanine116 and Tyrosine139). Within the $LamR^{220}$ crystallographic homodimer, Alanine114 packs into a tight pocket in the symmetry-related molecule and Phenylalanine116 is in van der Waals contact with Tyrosine139 (Jamieson et al., 2008). It should be noted, however, that the LamR crystal structure results from a truncated protein and the suggested structure of the homodimer is speculative; it is possible that the proposed homodimer interface may be an artefact of crystallisation.

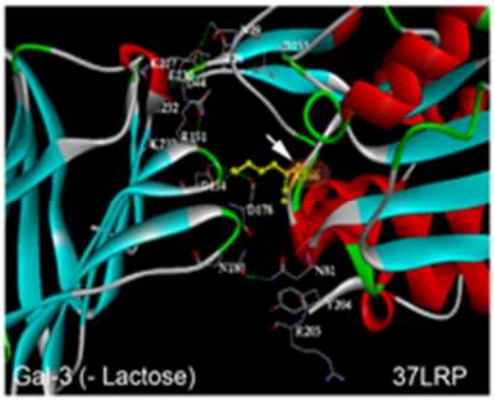
In current study, ZDOCK (zdock.umassmed.edu) was used to run rigid body docking for the two target proteins, wild type $LamR^{220}$ and wild type Gal-3 (Dr. Necmettin Pirinccioglu, University of Dicle). The model revealed a large protein–protein interface between the 37LRP and Gal-3 subunits, which predicted the involvement of several prominent interaction residues within 37LRP in heterodimerisation with Gal-3, mainly through salt bridges and hydrogen bonds (Alqahtani et al., 2014). The predicted interface residues are listed in (Table 5-1) and displayed in (Figure 5-1A & B). Importantly, binding of Gal-3 to the proposed heterodimer interface is incompatible with both LamR homodimer creation (Figure 5-1C) and also with laminin binding to at least one of the four suggested laminin-binding sites of 37LRP, because the indicated residues (Alanine114, Phenylalanine116 and Tyrosine139 for homodimerisation and Phenylalanine32, Glutamic acid35 and Arginine155 for 37LRP-laminin binding)(Jamieson et al., 2008) are either sterically hindered or implicated in the heterodimer interaction. Indeed, our model supports a hypothesis of the mutual

exclusivity of 37LRP homodimerisation to form 67LR and 37LRP/Gal-3 heterodimerisation for a given 37LRP molecule, while allowing for the possibility that both homo- and hetero-dimers could coexist as separate molecules.

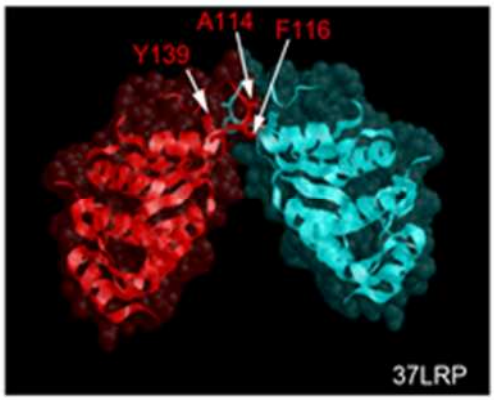
A



B



C



D

37LRP and Lactose-associated Gal-3/salt bridges (Alqahtani et al., 2014)	
1.	K166 of 37LRP with D178 of Gal-3
2.	K166 of 37LRP with D154 of Gal-3
3.	R80 of 37LRP with D178 of Gal-3
4.	D151 of 37LRP with R151 of Gal-3
5.	D151 of 37LRP with K227 of Gal-3
6.	D44 of 37LRP with K233 of Gal-3.
37LRP and non-Lactose-associated Gal-3/hydrogen bonds (Alqahtani et al., 2014)	
1.	N81 of 37LRP and N180 of Gal-3
2.	N29 of 37LRP and K233 and E230 of Gal-3
3.	N29 of 37LRP and K233 and E230 of Gal-3
4.	Y28 of 37LPR and E230 of Gal-3
37LRP and non-liganded Gal-3/hydrogen bonds (Alqahtani et al., 2014)	
1.	K166 of 37LRP and D178 of Gal-3
2.	K166 of 37LRP and D154 of Gal-3
3.	D44 in 37LRP and S233 of Gal-3
37LRP homodimer interface (Jamieson et al., 2008)	
	A114, F116 and Y139 of 37LRP

Figure 5.1 Interaction residues predicted by ZDOCk on each 37LRP and Gal-3 to form LamR heterodimer.

The proposed interface of 37LRP and Gal-3 was examined by means of the ZDOCK server. Panel 1 showed the images obtained from molecular modelling of 37LRP interaction with Galectin-3 displayed ribbon diagram of the heterodimer interaction of 37LRP with (A) Lactose-liganded or (B) Non-lactose-liganded Gal-3. Residues participated in the hypothetical large protein-protein interface are represented with sticks. (C) Ribbon diagram with superimposed molecular surface of the homodimer interaction of 37LRP (red) with a second 37LRP (cyan) as proposed by Jamieson et al. (Jamieson et al., 2008). (D) Proposed amino acid residues which may involved in heterodimerisation and homodimerisation.

5.3.2 Generation of 37 LRP mutants tagged to vYFP constructs in mammalian expression vectors BifC constructs

In order to experimentally confirm the *in silico* predictions of which specific amino residues were involved in facilitating LamR dimerisation, several 37LRP mutant constructs were generated. These constructs were based on three existing plasmids; each encoding wild type 37LRP fused at the C-terminus to whole, C-terminal or N-terminal fragments of vYFP (Alqahtani et al., 2014). Site-directed mutagenesis was used to mutate four specific amino acids (namely, Tyrosine139; Arginie155; Tyrosine156 and Lysine166); as a result 12 mutant constructs were generated and sequence analysis confirmed their correct identity. The mutated 37LRP constructs were listed in (Table 5-1).

Table 5-1 The 37 LRP/BifC mutant constructs generated for BifC assay.
Wild type 37LRP fused at the C-terminus to whole, C-terminal or N-terminal fragments of vYFP were used as templates to generate several 37LRP mutant constucts using site-directed mutagenesis. These constructs were used for BifC assay.

No.	Mutation	37LRP/BifC constructs	No.	Mutation	37LRP/BifC constructs
1.	Y139F	37LRP/Y139F.vYFLP	7.	Y156F	37LRP/Y156F.vYFLP
2.		37LRP/Y139F.vYNL	8.		37LRP/Y156F.vYNL
3.		37LRP/Y139F.vYCL	9.		37LRP/Y156F.vYCL
4.	R155A	37LRP/R155A.vYFLP	10.	K166A	37LRP/K166A.vYFLP
5.		37LRP/R155A.vYNL	11.		37LRP/K166A.vYNL
6.		37LRP/R155A.vYCL	12.		37LRP/K166A.vYCL

5.3.3 *In vitro* investigation of 37LRP homodimerisation and heterodimerisation with Gal-3

5.3.3.1 Wild type recombinant 37LRP and Gal-3 interact *in vitro* to form a heterodimer

The heterodimerisation of wild-type recombinant Gal-3 and Fc-tagged 37LRP was examined *in vitro* by ELISA using anti-Gal-3 antibody. Initially, the experiment was optimized using different concentrations of proteins (37LRP and Gal-3) and primary and secondary antibodies (data not shown). The optimized conditions utilized were tested before confirming the final result. The optimal results were obtained when 5 µg ml^{-1} of each protein was used; a significant level of binding between 37LRP and Gal-3 was observed compared to the negative control ($P \leq 0.0001$), confirming heterodimerisation of 37LRP and Gal-3 (Figure 5-2). Similar results were observed when Gal-3 was immobilized and binding of 37LRP was assessed using anti-LRP [A7] antibody (data not shown).

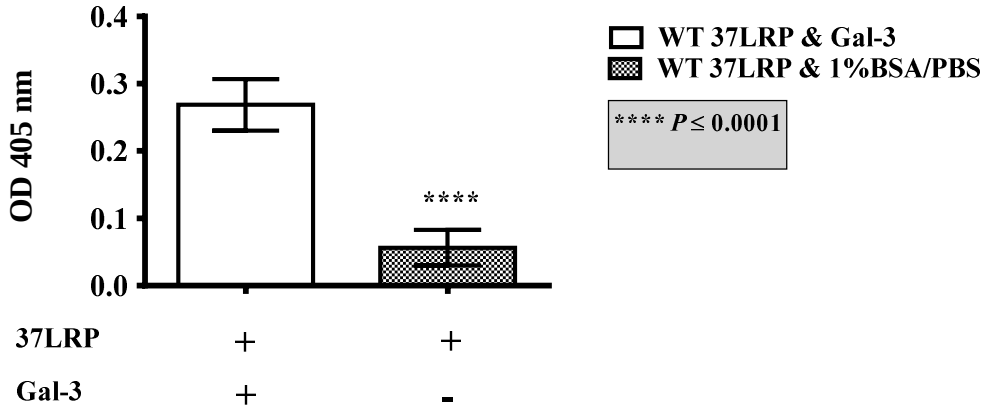


Figure 5-2 37LRP interacts significantly with Gal-3 to form a heterodimer *in vitro*.
Protein A-coated wells were incubated with 37LRP-Fc at a concentration of 5µg ml^{-1}. Wells were subsequently probed with Gal-3 or 1% BSA/PBS which was used as a -ve control. Anti-Gal-3 mouse monoclonal antibody was added as a primary antibody and anti-mouse IgG HRP-conjugated antibody was added as secondary antibody. The interaction was detected 1h after adding alkaline phosphatase (AP) substrate. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ****Significant at $P \leq 0.0001$.

5.3.3.2 Single-amino acid substitutions in 37LRP can significantly influence 37 LRP and Gal-3 heterodimerisation *in vitro*

After confirming the interaction between wild type 37LRP and Gal-3, the impact of single amino acid mutations substitution in 37LRP on Gal-3 binding was examined. Wild type 37LRP-Gal-3 binding was used as a positive control, and wells coated with BSA were included as a negative control; the mean value obtained from the later wells was subtracted from 37LRP-Gal-3 wells. Additionally, Fc-stop coated-wells included to determine the binding of Gal-3 to Fc (as a negative control). A significant reduction in binding of Gal-3 to each of the 37LRP were shown to be most heavily involved in dimerisation of 37LRP and Gal-3; substitution of either of these amino acids resulted in a significant reduction in binding of 37LRP to Gal-3 (p value ≤ 0.001 & 0.001 respectively; Figure 5-3).

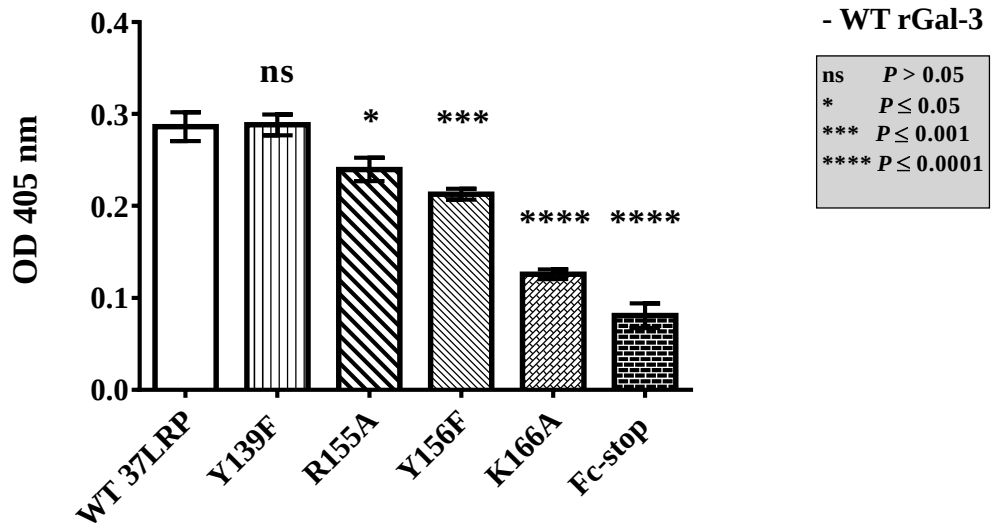


Figure 5-3 Mutation of Arginine 155, Tyrosine156 and Lysine 166 in 37LRP significantly reduce binding to Gal-3 *in vitro*.

Protein A treated wells were coated with WT 37LRP or its derivatives; wells were subsequently incubated with WT Gal-3, followed by mouse monoclonal anti-Gal-3 antibody and alkaline phosphatase-conjugated goat anti-mouse IgG. The interaction was detected 1h after adding AP substrate. The mean value obtained from Gal-3 binding to BSA-coated wells was subtracted from all data points. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ns at $P > 0.05$; *Significant at $P \leq 0.05$; *** at $P \leq 0.001$ and **** at $P \leq 0.0001$.

5.3.3.3 Recombinants 37 LRP interacts *in vitro* to form homodimers

In addition to heterodimerisation, another aim of the work presented in this chapter was to investigate the homodimerisation of 37 LRP. For this purpose two differentially tagged full length wild type recombinant 37LRP proteins: Fc-tagged 37 LRP protein expressed in human cells and His-tagged 37 LRP expressed in *E. coli* were tested for their ability to form dimers *in vitro* in ELISA assays. Wild type Fc-tagged 37LRP was immobilized on ELISA plates and its interaction with His-tagged 37LRP was assessed by comparing it with the binding of His-tagged 37LRP to BSA/PBS-treated negative control wells. The binding of two proteins

was determined using anti-pentahistidine or anti-Fc primary antibodies. Then the plates were incubated with the alkaline phosphatase conjugated anti-mouse IgG, produced in goat as a secondary antibody. The two WT recombinant 37LRP proteins bound significantly (p value ≤ 0.0001) when compared with the -ve control (Figure 5-4). Our result suggested the homodimerisation of two 37LRP

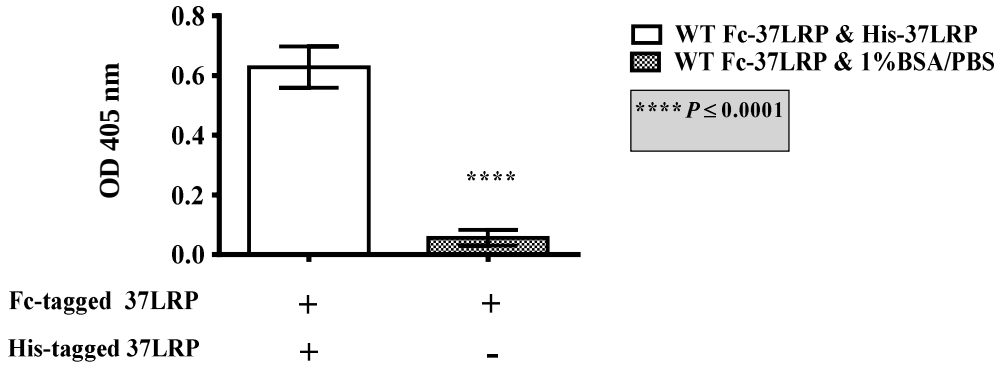


Figure 5-4 Significant self-association of wild type 37LRP to form LamR homodimer *in vitro*.

The Protein A-treated wells were coated with Fc-tagged 37LRP at a concentration of 5 µg ml^{-1}. Wells were subsequently incubated with His-tagged 37LRP or 1% BSA/PBS as a -ve control. The interaction was detected with mouse monoclonal anti-pentahistidine at dilution: 1:2000 as a primary antibody and alkaline phosphatase conjugated anti-mouse IgG, produce in goat at dilution: 1:20000 as secondary antibody. The interaction was detected after 1 h of adding AP substrate in an ELISA plate reader. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ****Significant at $P \leq 0.0001$.

proteins *in vitro*.

5.3.3.4 Single-substitution of 37 LRP significantly influenced the 37 LRP homodimerisation and Arginine 155; Tyrosine 156 and Lysine 166 of 37LRP might involve in homodimerisation *in vitro*

Using ELISA assay, we sought to exam ine the influence of 37LRP mutations in dimerisation ability of 37LRP proteins and determine which part of 37LRP

interacts with Gal-3 to form LamR homodimer and heterodimers, respectively. Several single-substituted Fc-tagged 37LRP proteins were tested for their ability to bind wild type His-tagged 37LRP protein using mouse anti-pentahistidine antibody. The levels of binding of full length wild type 37LRP to wild type and mutant 37LRP proteins were compared. 37LRP-BSA/PBS coated wells were included as a -ve control, and the mean value obtained was subtracted from 37LRP-37LRP coated wells. A significant reduction in binding of LamR mutants: $37LRP^{R155A}$ ($P \leq 0.05$); $37LRP^{Y156F}$ ($P \leq 0.001$) and $37LRP^{L166A}$ ($P \leq 0.0001$) to full length wild type His-tagged 37LRP protein. These results suggest the participation of 37LRP residues: Arginine 155; Tyrosine 156 and Lysine 166 in the homodimerisation of 37LRP proteins *in vitro* (Figure 5-5).

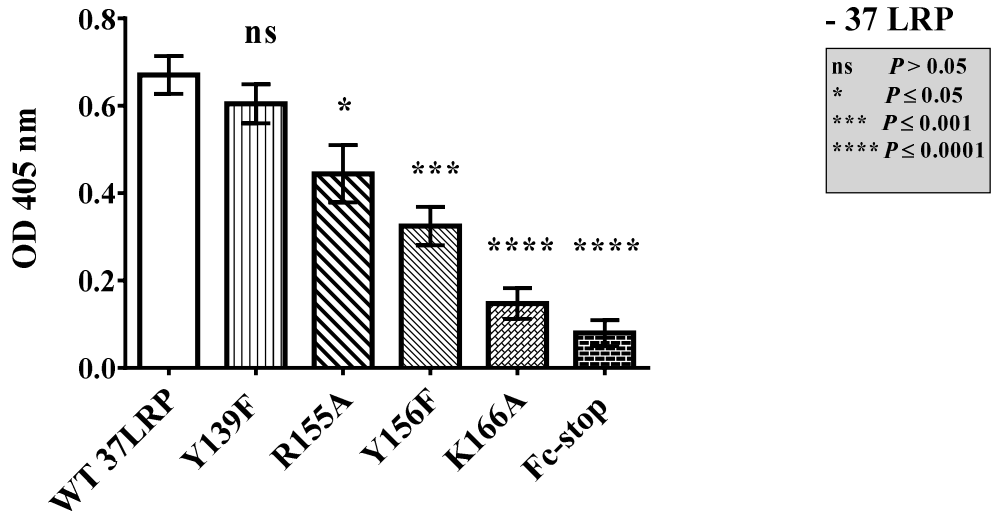


Figure 5-5 The involvement of 37LRP residues Arginine 155; Tyrosine 156 and Lysine 166 in binding of 37LRP and Gal-3 to form LamR homodimer *in vitro*.

Binding of two recombinant 37LRP was tested *in vitro* using ELISA assay. The Protein A treated wells were coated with full length WT Fc-tagged 37LRP or its mutant derivatives at a concentration of 5 μg ml^{-1}. Wells were subsequently incubated with full length WT his-tagged 37LRP. The binding of 37LRP with Fc-stop was used as a -ve control, followed with mouse anti-pentahistidine antibody at dilution 1:2000 and secondary antibody at dilution alkaline phosphatase conjugated anti-mouse IgG, produce in Goat at dilution 1:20000. The interaction was detected after 1 h of adding AP substrate in an ELISA plate reader. The mean value obtained from Gal-3 binding to BSA-coated wells was subtracted from all data points. Data shown are the means of three independent experiments, with each sample tested in triplicate. Error bars indicate± SEM. ns at P >0.05; *Significant at P ≤ 0.05; *** at P ≤ 0.001**** at *P* ≤ 0.0001.

5.3.3.5 Investigating the dimerisation of LamR in living cells

To investigate dimerisation of LamR in living cells biomolecular fluorescence complementation (BifC) was employed. Initially, the experiments performed by Alqahtani were repeated (Alqahtani, 2012) in which fragments of fluorescently labelled wild type 37 LRP and wild type Gal-3 were co-expressed to confirm the homo and heterodimerisation of LamR and/or Gal-3. Briefly, cells were

transfected with the appropriate BifC constructs: either 37LRP-encoding and Gal-3-encoding constructs to detect hetero-dimerisation, or two 37LRP-encoding constructs to investigate homodimerisation. In each case, one construct was fused with amino-terminus of the fluorescent protein (YNL), whilst the other was fused with the carboxyl-terminus of fragment fluorescent protein (YCL). After allowing the transfected cells time to develop fluorescent signals, the protein complex was visualized by measuring the mean fluorescence intensity (MFI) of BifC using flow cytometry, in parallel with immunoblot analysis to confirm the identity and expression levels of the proteins of interest. Fluorescence microscope screening was employe to determine the optimal conditions of signal developments and confocal microscope imaging was performed for further validation of the BifC signal obtained by flow cytometry.

Dimerisation of wild type 37LRP and wild type Gal-3 was tested with several control experiments to ensure that the fluorescence signal generated was not due to background fluorescence. Experiments included non-transfected cells, as well as cells transfected with single constructs to confirm that each N or C-terminus fragment was not individually capable of generating a fluorescence signal. Each combination of the two fragments which listed in Table 5-2 and Figure 5-6 was tested. Proteins fused with full length YFP (termed vYFLP) were employed as positive controls.

Table 5-2 Single and Co-transfections of 37LRP; Gal-3 or both into COS7 cells to test homo and heterodimerisation.

All BifC constructs were transfected either alone or co-transfected considering all the possible combinations between indicated BifC constructs.

Single transfection into COS7 cells used as +ve controls used for BifC assay			
1.	WT 37LRP.vYFLP	**5.**	37LRP/K166A.vYFLP
2.	37LRP/Y139F.vYFLP	**6.**	Gal-3.vYFLP
3.	37LRP/R155A.vYFLP	7.	Gal-3/C173A.vYFLP
4.	37LRP/Y156F.vYFLP		
Single transfection into COS7 cells used as -ve controls used for BifC assay			
1.	WT 37LRP.vYNL	**7.**	37LRP/Y156F.vYNL
2.	WT 37LRP.vYCL	**8.**	37LRP/Y156F.vYCL
3.	37LRP/Y139F.vYNL	**9.**	37LRP/K166A.vYNL
4.	37LRP/Y139F.vYCL	**10.**	37LRP/K166A.vYCL
5.	37LRP/R155A.vYNL	**11.**	WT Gal-3.vYNL
6.	37LRP/R155A.vYCL	**12.**	WT Gal-3.vYCL
Co-transfection into COS7 cells used as -ve controls used for BifC assay			
1.	WT Gal-3.vYNL & WT Gal-3.vYNL	**8.**	37LRP/Y139F.vYCL & WT Gal-3.vYCL
2.	WT Gal-3.vYCL & WT Gal-3.vYCL	**9.**	37LRP/R155A.vYNL & WT Gal-3.vYNL
3.	WT 37LRP.vYNL & WY 37LRP.vYNL	**10.**	37LRP/R155A.vYCL & WT Gal-3.vYCL
4.	WT Gal-3.vYCL & WY 37LRP.vYCL	**11.**	37LRP/Y156F.vYNL & WT Gal-3.vYNL
5.	WT Gal-3.vYNL & WY 37LRP.vYNL	**12.**	37LRP/Y156F.vYCL & WT Gal-3.vYCL
6.	WT Gal-3.vYCL & WY 37LRP.vYCL	**13.**	37LRP/K166A.vYNL & WT Gal-3.vYNL
7.	37LRP/Y139F.vYNL & WT Gal-3.vYNL	**14.**	37LRP/K166A.vYCL & WT Gal-3.vYCL
Co-transfection into COS7 cells to test the Heterodimerisation			
1.	37LRP/Y139F.vYNL & WT Gal-3.vYCL +ve control	**6.**	37LRP/R155A.vYCL & WT Gal-3.vYNL
2.	37LRP/Y139F.vYCL & WT Gal-3.Vynl +ve control	**7.**	37LRP/Y156F.vYNL & WT Gal-3.vYCL
3.	37LRP/Y139F.vYNL & WT Gal-3.vYCL	**8.**	37LRP/Y156F.vYCL & WT Gal-3.vYNL
4.	37LRP/Y139F.vYCL & WT Gal-3.vYNL	**9.**	37LRP/K166A.vYNL & WT Gal-3.vYCL
5.	37LRP/R155A.vYNL & WT Gal-3.vYCL	**10.**	37LRP/K166A.vYCL & WT Gal-3.vYNL

Co-transfection into COS7 cells to test the Homodimerisation			
1.	WT 37LRP.vYNL & WY 37LRP.vYCL +ve control	**6.**	37LRP/Y156F.vYNL & WT 37LRP.vYCL
2.	37LRP/Y139F.vYNL & WT 37LRP.vYCL	**7.**	37LRP/Y156F.vYCL & WT 37LRP.vYNL
3.	37LRP/Y139F.vYCL & WT 37LRP.vYNL	**8.**	37LRP/K166A.vYNL & WT 37LRP.vYCL
4.	37LRP/R155A.vYNL & WT 37LRP.vYCL	**9.**	37LRP/K166A.vYCL & WT 37LRP.vYNL
5.	37LRP/R155A.vYCL & WT 37LRP.vYNL		

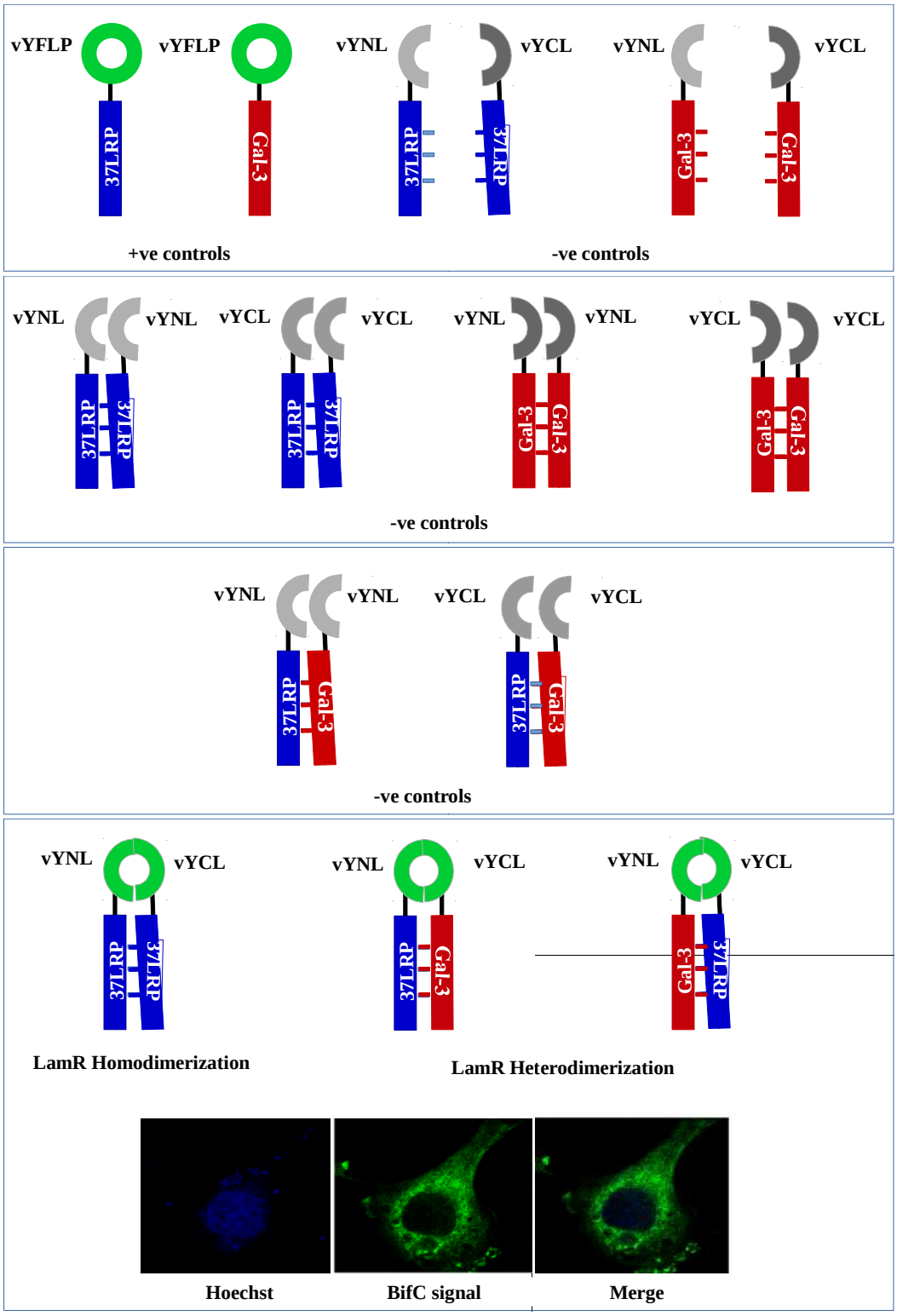


Figure 5-6 Schematic showing the multiple combinations of fusion proteins tested for bimolecular fluorescence complementation (BifC).

Amino- and carboxyl-terminal fusions were used in several combinations of 37LRP and Gal-3 to test LamR homo and heterodimerisation.

5.3.3.5.1 Expression of fluorescently labelled 37 LRP and Gal-3 in COS7 cells

Expression of 37LRP and Gal-3 were initially examined in COS7 cells due to their high transfection efficiency and expression level. The wild type and mutant 37LRP BifC constructs carrying either C or N-terminus of vYFP were transiently co-transfected with wild type Gal-3 into COS7 cells using Trans IT-2020 transfection reagent. For optimisation purposes, the cells were transfected with different amounts of both 37 LRP and Gal-3.vYFLP plasmids (1-4.5µg) or Trans IT®-2020 transfection reagent (0.5-7.5µl), and the transfection efficiencies for each conditions were by fluorescence microscopy, while their statistical significance were measured by flow cytometry at 24; 30 and 48h post transfection. The optimal amount of plasmid DNA was determined to be 2µg per 7.5µl of transfection reagent incubated for 30h in 12 and 6 well plates (data not shown) and these conditions were used in all further experiments. In addition to Trans-IT, co-transfection of 37LRP and Gal-3 was examined using other transfection reagents and methods: $CaPO_4$ and jetPRIME® DNA transfection reagents, no significant differences in efficiency were observed between $CaPO_4$ and Trans-IT TRs which both exhibited higher levels of transfection than jetPRIME®. The Trans IT®-2020 transfection reagent was selected for all further experiments due to its serum compatibility and high efficiency of transfection. Also, as current work was complementary to the previous study of Alqahtanti and co-workers and demonstrated the dimerisation of 37LRP and Gal-3 (Alqahtani et al., 2014), Trans IT®-2020 TR was selected to be consistent with that study. Further, after optimization of transfection conditions in COS7 cells, the same

experiment was repeated in HEK293T to confirm the dimerisation behaviour of LamR in different cells.

COS7 cells were co-transfected using Trans IT®-2020; each experiment was performed in three wells and experiments were repeated three times to confirm the results. Whole cell lysates of COS7 cells transiently transfected with the indicated construct were analysed 30h post-transfection by immunoblotting after SDS-PAGE under reducing conditions. Immunoblotting using anti-GFP, anti-LRP [A7] and anti-Gal-3 antibodies confirmed the expression of each vYFP-tagged 37LRP and Gal-3 proteins. For detection of vYFP-tagged 37LRP, both anti-LamR [A7] and anti-GFP antibodies (Figure 5-7 A& B respectively) revealed bands at the expected size of 67 kDa; 61 kDa and 52 kDa in the cell lysates of cells transfected with 37 LRP.vYFLP; 37 LRP.vYNL and 37 LRP.vYCL respectively. Also, analysis of Gal-3 transfected cell lysates with anti-Gal-3 and anti-GFP antibodies (Figure 5-7 D & E respectively) showed bands at expected size 57 kDa; 50 kDa and 40 kDa for each of Gal-3.vYFLP; Gal-3.vYNL and Gal-3.vYCL respectively. These results indicated that both 37LRP and Gal-3 fusion proteins were expressed successfully in transfected COS7 cells.

After optimizing and confirming the expression of the wild type proteins, the fluorescently labelled 37LRP mutant proteins were transfected into COS7 and expression was examined 24 and 30h post-transfection by fluorescence microscopy, western blot and flow cytometry. Expression of each of the vYFLP-fused 37LRP mutants ($37LRP^{Y139F}$; $37LRP^{R155A}$; $37LRP^{Y156F}$; $37LRP^{K166A}$) was confirmed in COS7 cells by immunoblotting (Figure 5-7 C) and flow cytometry 30h post transfection (Figure 5-8). In immunoblots each mutant protein had an apparent molecular weight that was indistinguishable from that of the wild type

37LRP.vYFP construct (Figure 5-7 C). The flow cytometric analysis revealed similar expression levels of wild type and mutant receptors (data shown separately for each mutant in next sections) indicating that the expression was un-affected by the indicated mutations.

Before examining the effect of several single-substitutions within 37LRP on their capability to form heterodimers with wild type Gal-3, wild type 37LRP was co-transfected with wild type Gal-3 into HBMECs; COS7 and HEK293T cells and the dimerisation between two proteins was examined 30h post-transfection by flow cytometry and fluorescence microscopy. To investigate the homo- and heterodimerisation ability of 37LRP mutants, they were co-transfected into COS7 cells with either wild type Gal-3 or wild type 37LRP and later analysed by immunoblotting, flow cytometry, fluorescence and confocal microscopy.

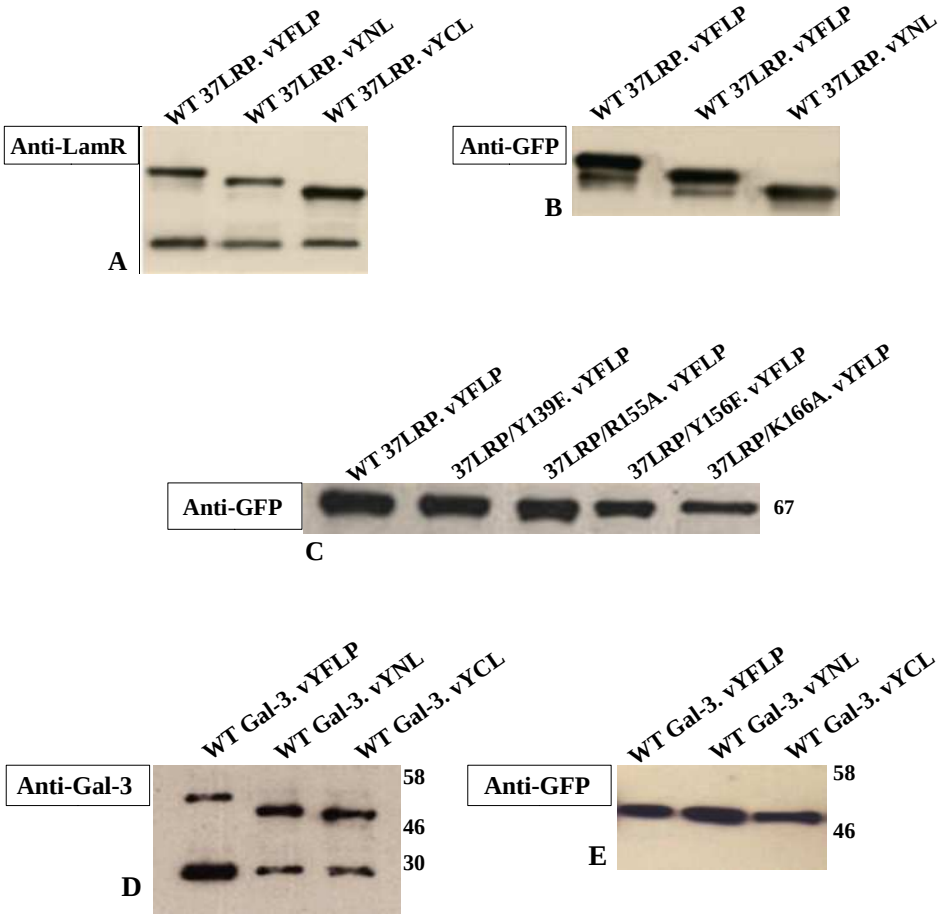


Figure 5.7 Immunoblotting to detect expression of LamR or Gal-3 fusion proteins in COS7 cells 30h post transfection.

All BifC constructs were transiently transfected into COS7 cells and immunoblotting analysis performed under reducing conditions using anti-37LRP [A7] mouse monoclonal IgG primary antibody at dilution 1: 2000 or anti-Gal-3 mouse monoclonal antibody at dilution 1:1000 or anti-GFP rabbit polyclonal antibody at dilution 1:100 as primary antibodies, followed by (1:30000) dilution of anti-rabbit or anti-mouse conjugated to HRP. (A & B) Show expression of 37LRP tagged with vYFP; Lane 1: 37LRP.vYFLP; Lane 2: 37LRP.vYNL; Lane 3: 37LRP.vYCL. In A, the test was performed using anti-37LRP [A7] antibody; the results showed expression of 37LRP with expected size of 38 kDa for e37LRP, and 67 kDa; 61 kDa and 52 kDa for each 37 LRP.vYFLP; 37 LRP.vYNL and 37 LRP.vYCL respectively. In B, the test was performed using anti-GFP antibody; the results showed one band corresponding to r37LRP at the same size detected by anti-LRP [A7] antibody. To indicate the comparable expression of WT and several mutants of LamR, the WCL of COS7 cells transfected with either WT or mutants 37LRP was tested using (C) anti-GFP and the western blot analysis confirmed the expression of

all mutants 37LRP/BifC constructs, these include: Lane 1: WT r37LRP.vYFLP; Lane 2: r37LRP/Y139F.vYFLP; Lane 3: r37LRP/R155A.vYFLP; Lane 4: r37LRP/Y156F.vYFLP; Lane 5: r37LRP/K166A.vYFLP. (D & E) shows the expression of Gal-3 tagged with vYFP. Lane 1: Gal-3.vYFLP; Lane 2: Gal-3.vYNL; Lane 3: Gal-3.vYCL. In D, anti- Gal-3 antibody was used; the results showed eGal-3 with expected size of 28 kDa and a band corresponding to rGal-3.vYFLP; r37Gal-3.vYNL or rGal-3.YCL at size of 57 kDa; 50 kDa and 40kDa, respectively. In E, anti-GFP antibody was used; the results showed one band corresponding to rGal-3 at the same size detected by anti-Gal-3 antibody.

- Binding sites of anti-37LRP [A7] with LamR= 253-289
- e37LRP = endogenous 37LRP
- r37LRP = Recombinant 37LRP

5.3.3.5.2 LamR heterodimerisation confirmed using BifC assay

High intensity vYFP signals (BifC signal) were observed by fluorescence microscopy in cells transfected either with wild type 37LRP and Gal-3 fused with vYFLP or co-transfected with both wild type 37LRP-Gal-3 fused with half portion of vYFP. Afterwards, the dimerisation of the two proteins was visualized by measuring the MFI of BifC signal using flow cytometry; dead cells and cell debris were excluded from analysis using a forward scatter (FSC) and side scatter (SSC) double plot. A fluorescent signal resulted from interaction of wild type 37LRP and wild type Gal-3 compared with the negative controls, indicating the formation of a LamR heterodimer consistent with the previous ELISA results. No significant differences were observed between the levels of fluorescence resulting from co-transfection of wild type 37LRP.vYCL with wild type Gal-3.vYNL or wild type 37LRP.vYNL with wild type Gal-3.vYCL, which provides further confirmation of the stability of the dimerisation. All positive controls (37LRP or Gal-3 fused with full length vYFP; YFLP) yielded high intensity fluorescence signals. The BifC signal detected by fluorescent microscope is shown in Figure 5-8. Flow cytometry is shown in Figure 5-9, and the confocal imaging for endogenous and recombinant 37LRP are presented in Figure 5-10 and Figure 5-11 respectively.

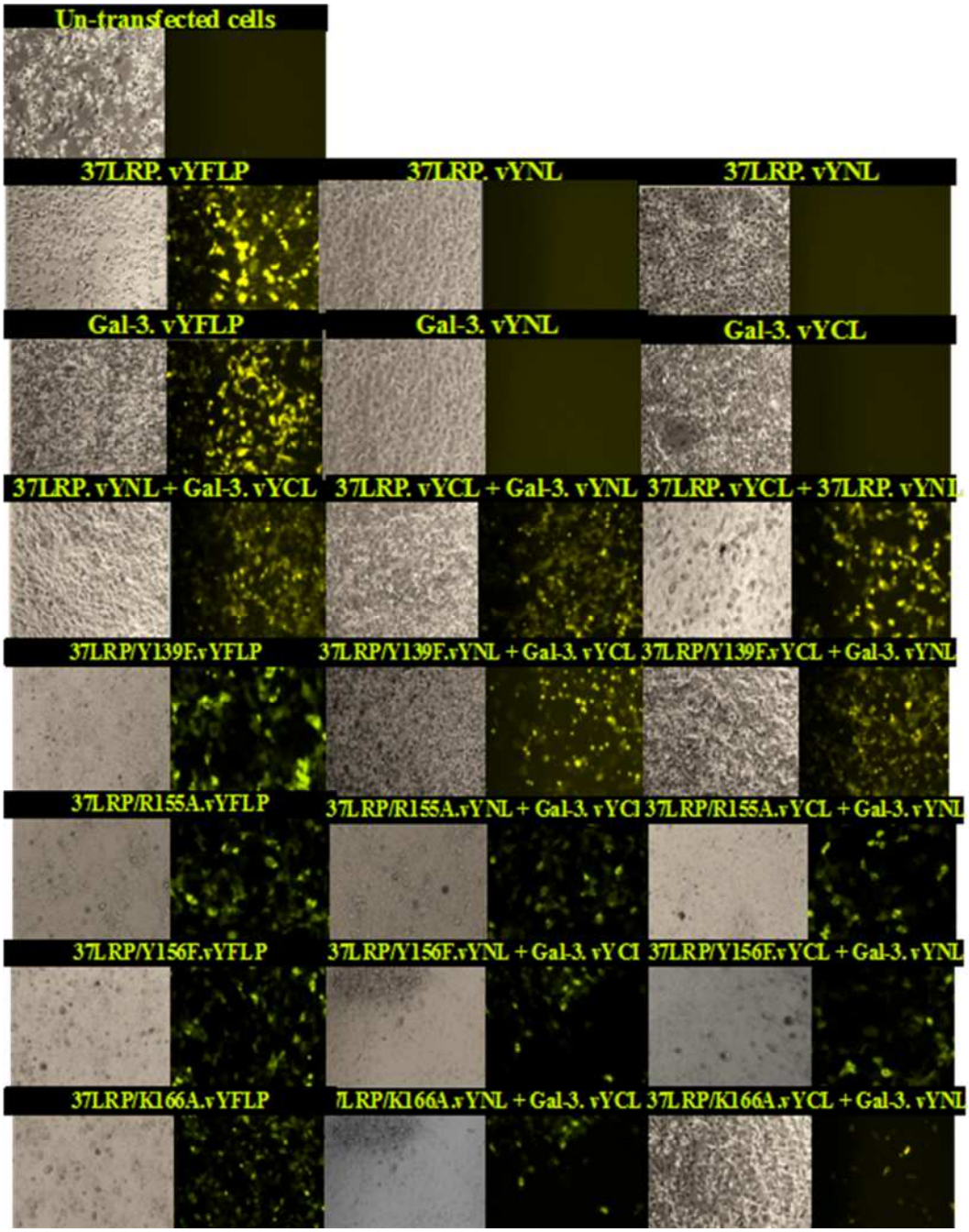


Figure 5-8 Fluorescence microscopy screening of COS7 cells 24h post transfection. The optical (left, gray) and fluorescent (right, green) light imaging of cells. WT 37LRP or WT Gal-3 tagged with vYFP were transfected separately, or co-transfected into COS7 cells, the vYFP +ve cells (green) using fluorescen microscope indicating a successful transfection of the recombinant proteins.

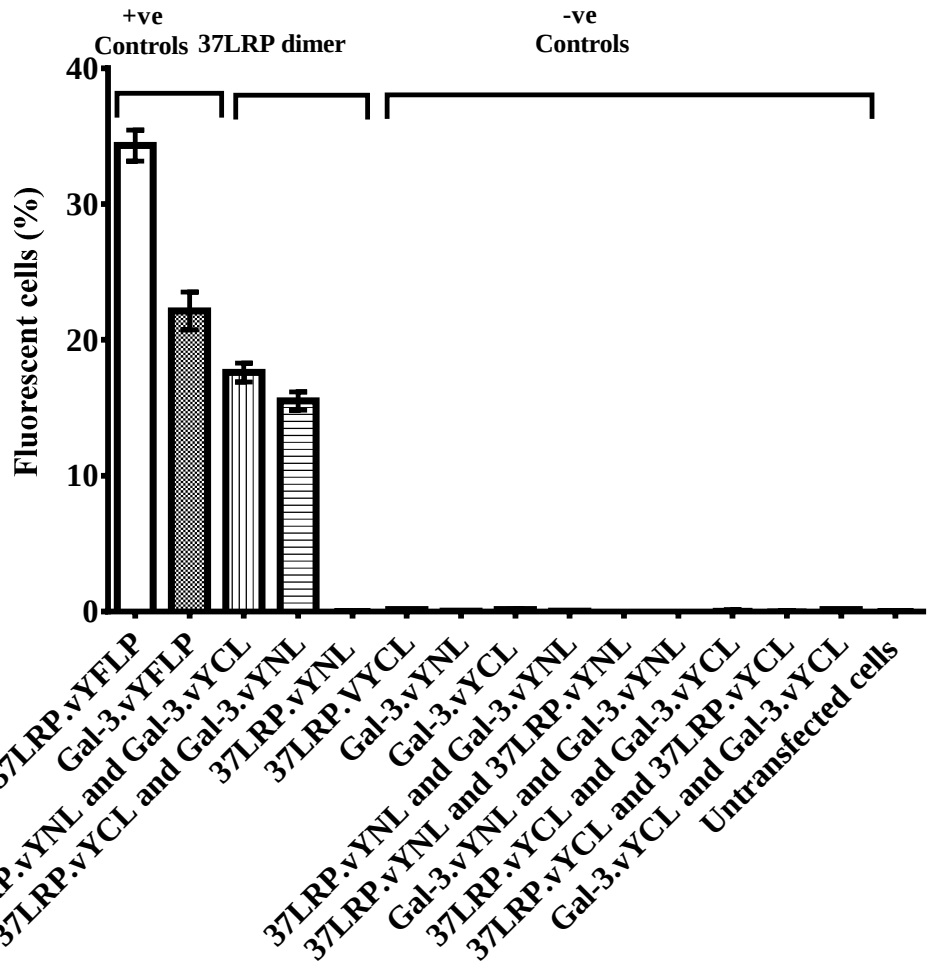


Figure 5-9 Combinations of 37LRP and Gal-3.vYFP fused with Venus fluorescent protein and controls for the BifC assay.

Following co-transfection of COS7 cells, the cells were harvested 30h post-transfection and analysed for BifC signal. Cells were harvested 30h post-transfection and analysed for BifC signal using flow cytometry assay. The fluorescence intensity quantified and expressed as a percentage of gated cells.

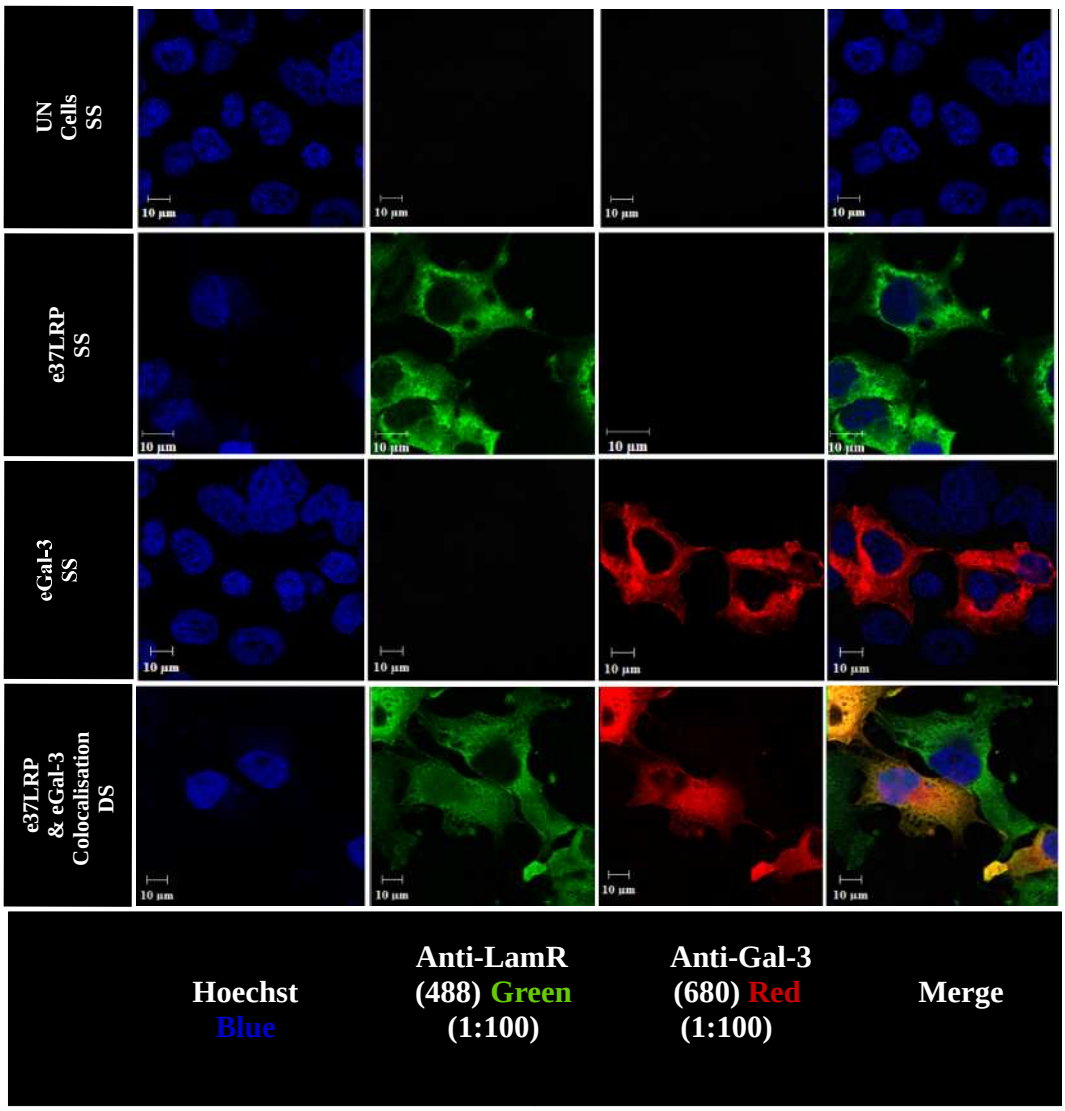


Figure 5.10 Confocal cell imaging shows the colocolisation of endogenous 37LRP with endogenous Gal-3.

The colocalisation of e37LRP with eGal-3. Cells grown on coverslips were fixed with 1% paraformaldehyde and single stained (SS) with anti-37LRP [A7] or anti-Gal-3, or double stained (DS) with anti-37LRP [A7] and anti-Gal-3 antibodies (1:100) using secondary antibodies: Alexa Fluor 680 antibody (Red) or/and Alexa Fluor 488 antibody (Green). Nuclei are stained with Hoechst 33258 (Blue). Coverslips were mounted and images obtained by confocal microscope.

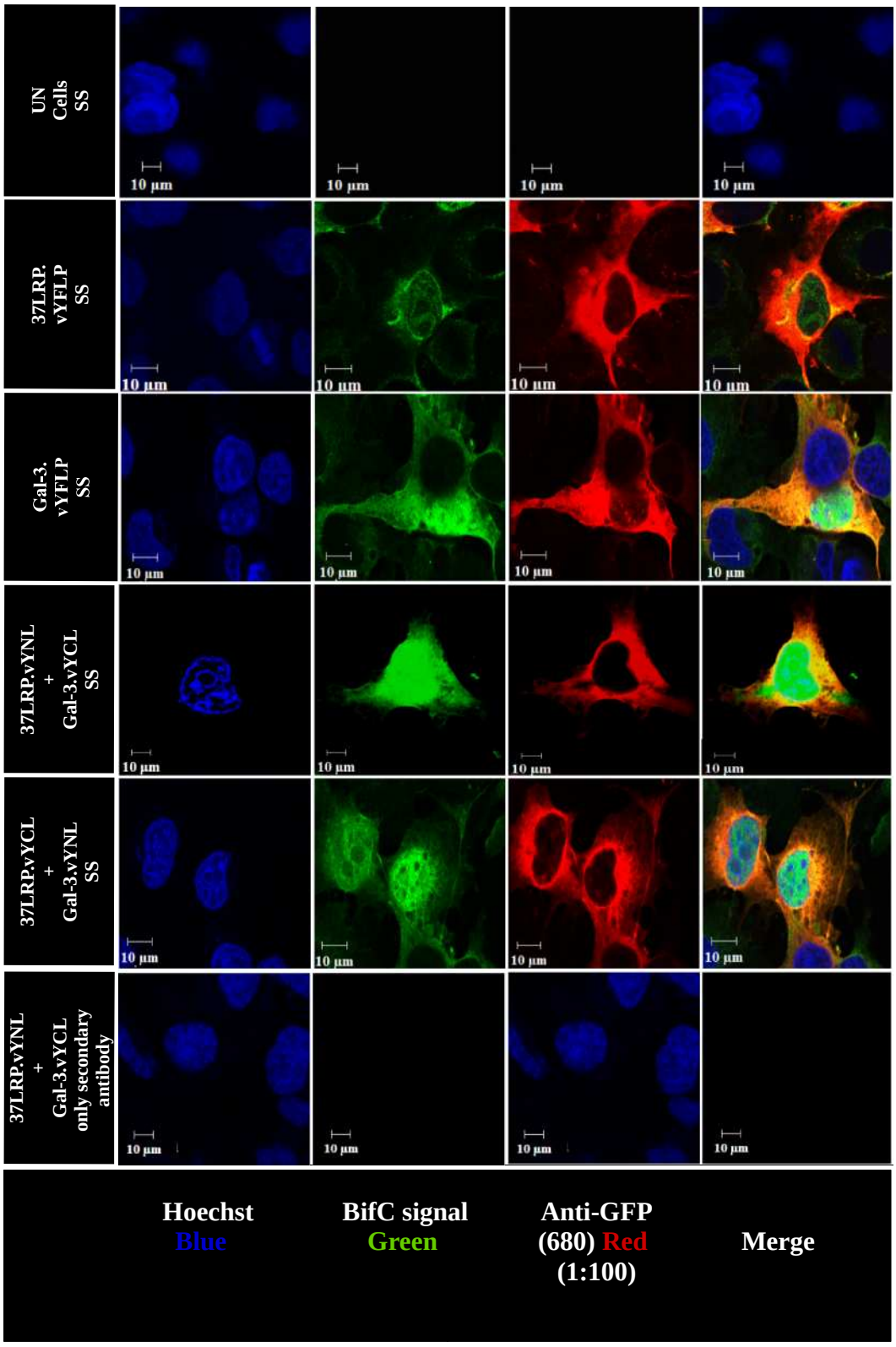


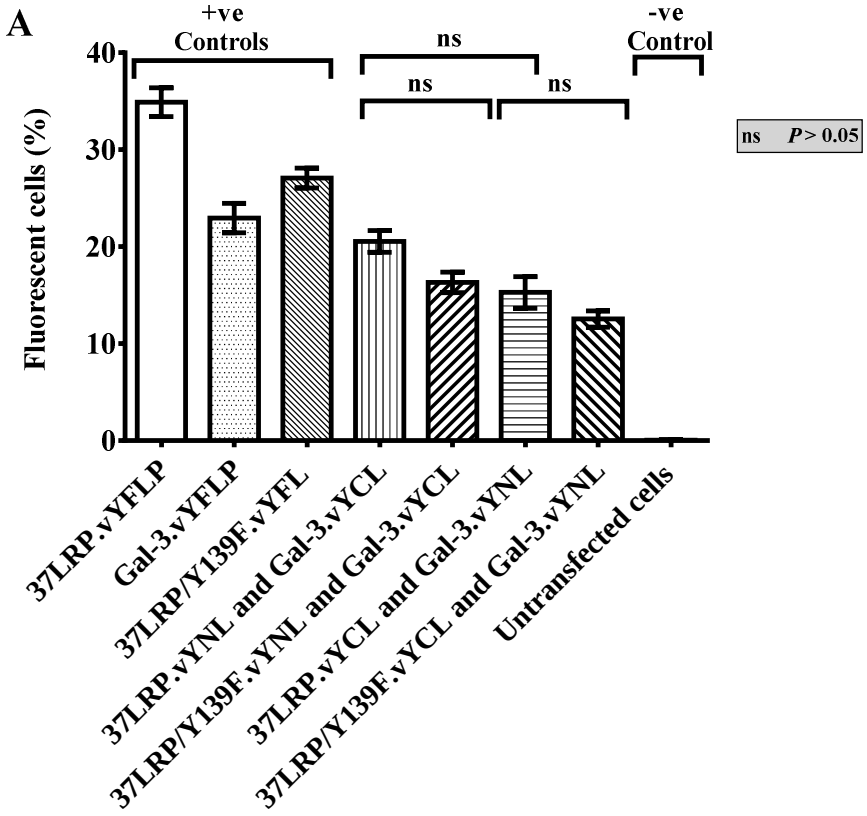
Figure 5-11 Combinations of 37LRP and Gal-3 fused with Venus fluorescent protein and controls for the BifC assay.

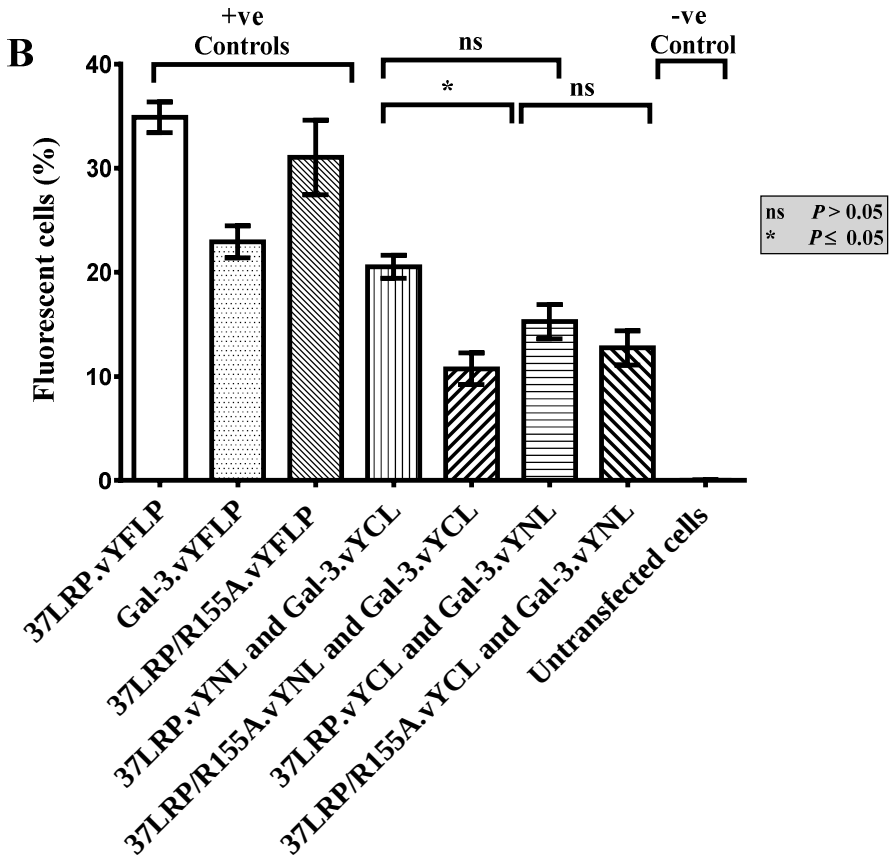
Combinations of single and co-transfections with 37LRP and Gal-3 fusion constructs were tested for heterodimerisation of 37LRP and Gal-3. Cells were harvested 30h post-

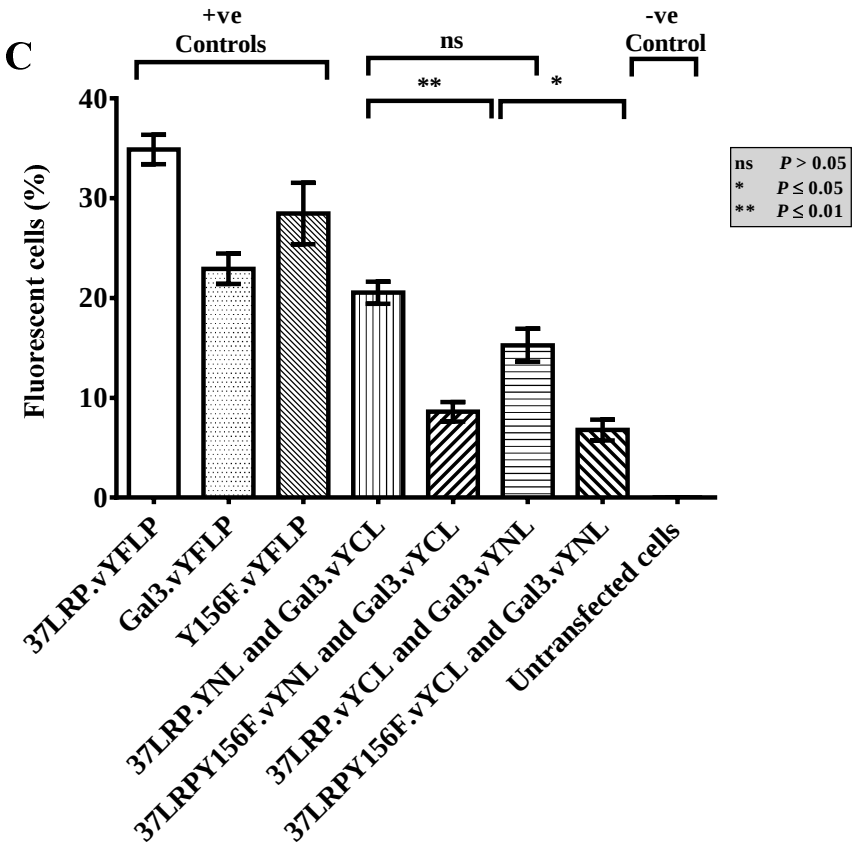
transfection and analysed for BifC signal using confocal cell imaging. The cells grown on coverslips were fixed with 1% paraformaldehyde and single stained (SS) with anti-GFP antibody (1:100) and secondary antibody; Alexa Fluor 680 antibody (Red). Nuclei are stained with Hoechst 33258 (Blue). Coverslips were mounted and images obtained by confocal microscope.

5.3.3.5.3 Tyrosine 156 and Lysine 166 of 37LRP contributed in heterodimerisation of 37LRP with Gal-3 in living cells

After confirming the hetero-dimerisation of wild type 37LRP and Gal-3, the next step was to determine the impact of the 37LRP mutations (37LRPY139F; 37LRPR155A; 37LRPY156F and 37LRPK166A) on heterodimerisation using the same protocol (Figure 5-12A, B, C and D respectively). The effect of the mutations was confirmed using both 37LRP.vYCL and vYNL fragments to confirm specificity of the BifC assay. Overall, the ability of 37LRP to heterodimerise with Gal-3 was significantly decreased by mutation of Tyrosine 156 and Lysine 166 of 37LRP compared with the positive control. In contrast, the BifC signals resulting from the interaction of 37LRPY139F or 37LRPR155A with wild type Gal-3 were not significantly different from wild type 37LRP.


A
+ve Controls
ns
-ve Control
ns
ns
Fluorescent cells (%)
40
30
20
10
0
ns P > 0.05
37LRP.vYFLP
Gal-3.vYFLP
37LRP/Y139F.vYFL
37LRP.vYNL and Gal-3.vYCL
37LRP/Y139F.vYNL and Gal-3.vYCL
37LRP.vYCL and Gal-3.vYNL
37LRP/Y139F.vYCL and Gal-3.vYNL
Untransfected cells


B
+ve Controls
ns
-ve Control
*
ns
Fluorescent cells (%)
40
30
20
10
0
ns P > 0.05
* P ≤ 0.05
37LRP.vYFLP
Gal-3.vYFLP
37LRP/R155A.vYFLP
37LRP.vYNL and Gal-3.vYCL
37LRP/R155A.vYNL and Gal-3.vYCL
37LRP.vYCL and Gal-3.vYNL
37LRP/R155A.vYCL and Gal-3.vYNL
Untransfected cells


C
+ve Controls
ns
-ve Control
**
*
Fluorescent cells (%)
40
30
20
10
0
ns P > 0.05
* P ≤ 0.05
** P ≤ 0.01
37LRP.vYFLP
Gal3.vYFLP
Y156F.vYFLP
37LRP.YNL and Gal3.vYCL
37LRPY156F.vYNL and Gal3.vYCL
37LRP.vYCL and Gal3.vYNL
37LRPY156F.vYCL and Gal3.vYNL
Untransfected cells

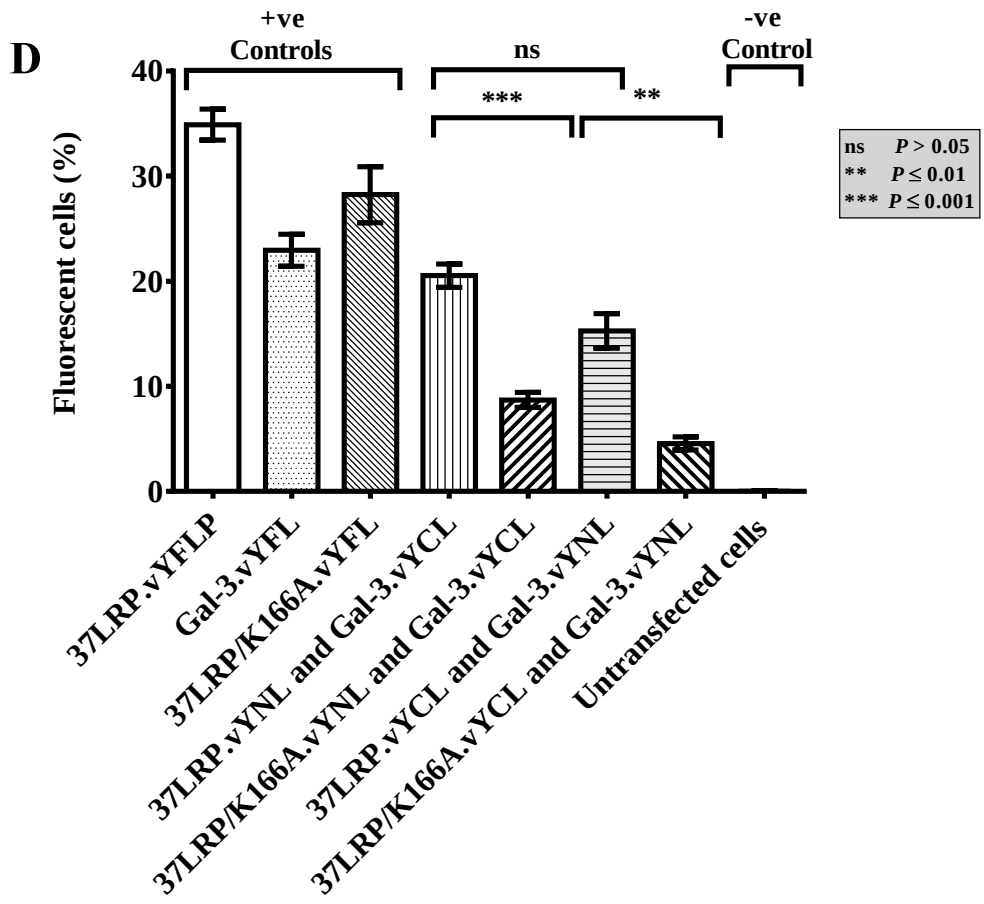


Figure 5-12 The effects of: (a) 37LRP^{Y139F}; (b) 37LRP^{R155A}; (c) 37LRP^{Y156F} and (d) 37LRP^{K166A} mutations within 37LRP on 37LRP and Gal-3 heterodimerisation.

Following co-transfection of COS7 cells, cells were harvested 30h post-transfection and analysed for BifC signal by quantifying the fluorescence intensity and expressing it as a percentage of gated cells. Difference in BifC signal were analysed by one-way ANOVA and Tukey test. ***p , 0.001; **p , 0.01 while a p value >0.05 is considered not significant (ns). Error bars = mean of triplicate values on three occasions ± SEM.

5.3.3.5.4 The homodimerisation of 37 LRP was confirmed in living cells using BifC assay

Flow cytometry was used to quantify the interaction of two wild type 37LRP proteins, one fused to the N terminal and other to the C terminal portion of vYFP. The dimerisation was measuring at 30h post transfection., Flow cytometry showed

a high signal compared to un-transfected cells (Figure 5 13) and all other negative controls (Table 5-3) indicating a significant dimerisation of two 37 LRP proteins

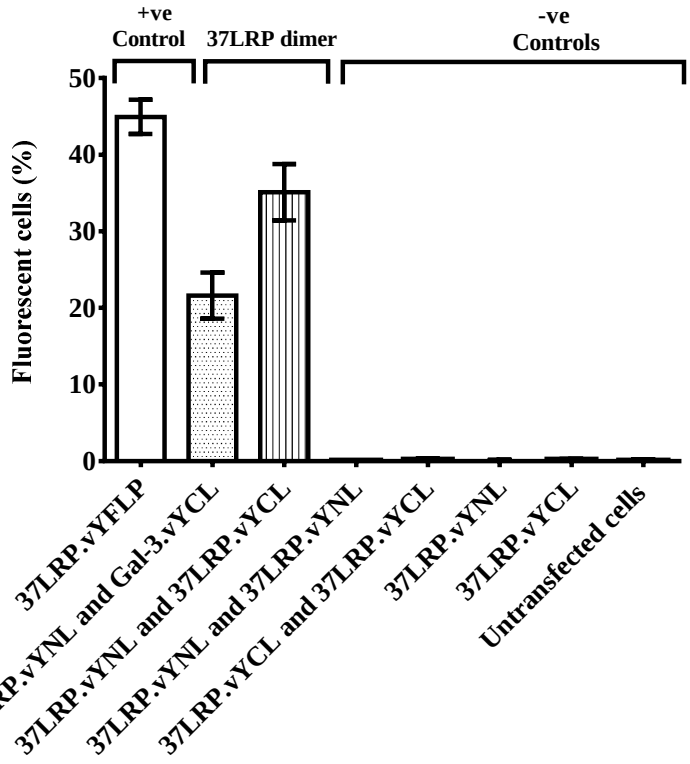


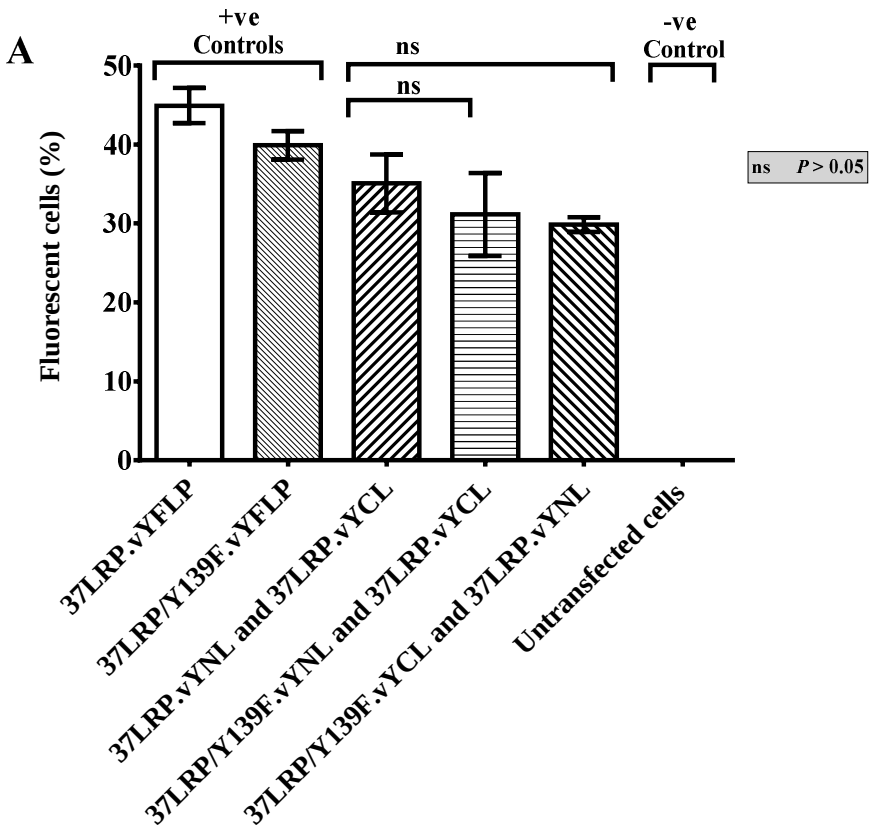
Figure 5-13 LamR homodimerisation confirmed by BifC analysis.
Combinations of 37LRP fused with either full length, N- or C-terminal vYFP were tested. Cells were harvested 30 h post-transfection and analysed for their BifC signal using flow cytometry to quantify the fluorescence intensity and express the data as a percentage of gated cells. Error bars = mean of triplicate values on three occasions ± SEM.

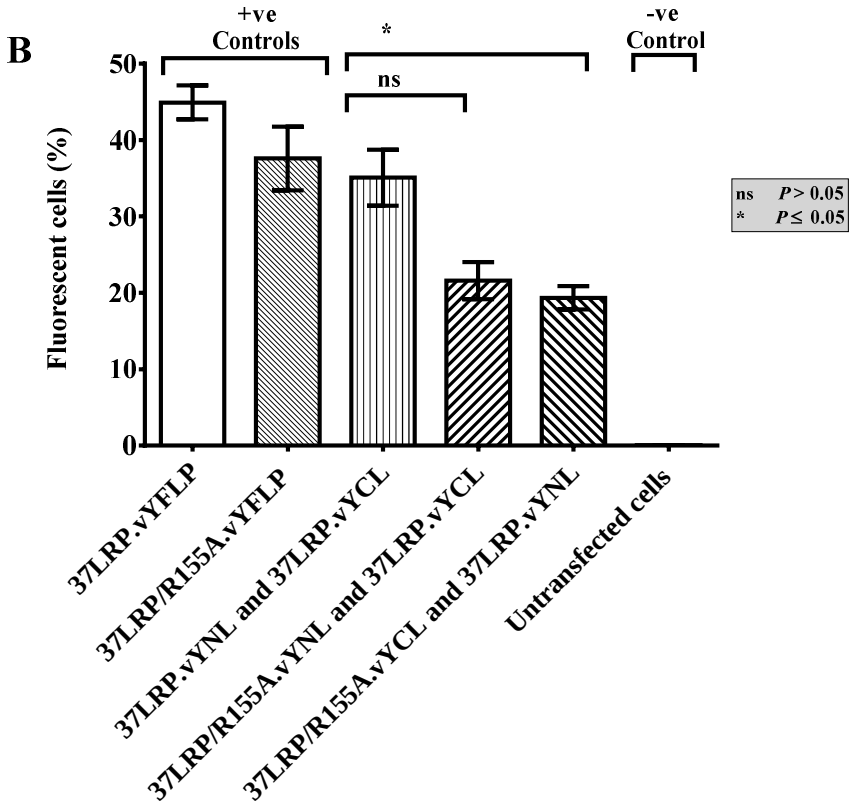
in living cells to create homodimer isoform of LamR.

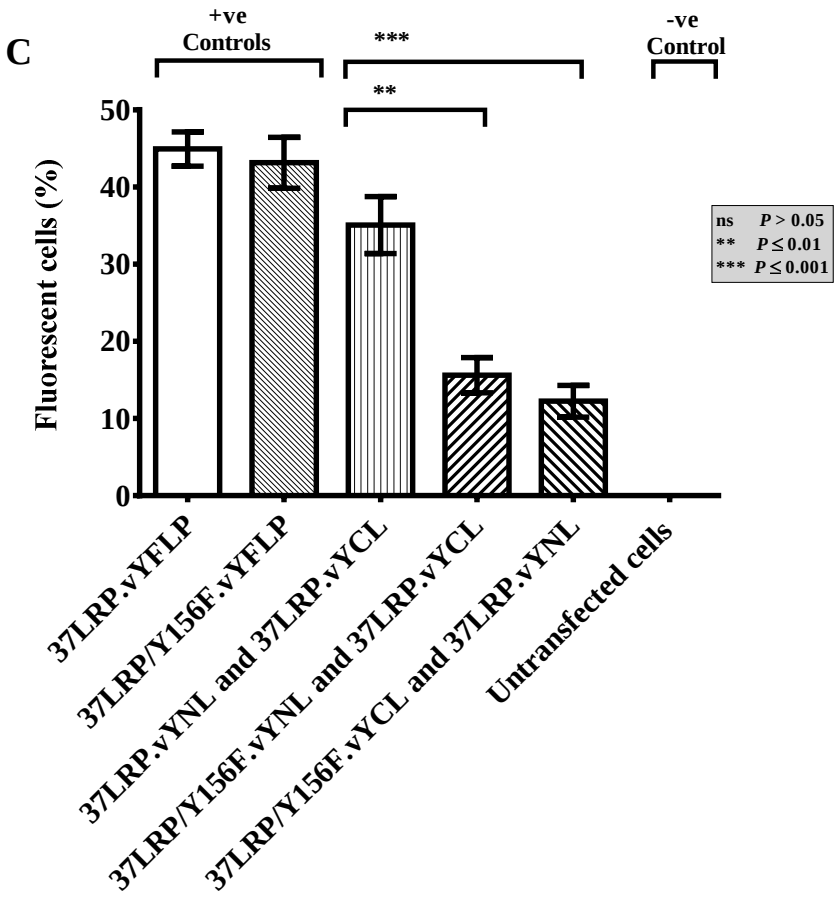
5.3.3.5.5 Tyrosine 156 and Lysine 166 of 37LRP contribute to the homodimerisation of LamR in living cells

After identification of two amino acid residues within 37LRP that have critical roles in the heterodimerisation of 37LRP with Gal-3, the next aim was

identification of residues important for homodimerisation by investigating the influence of several 37LRP mutations on homodimerisation (37LRPY139F; 37LRPR155A; 37LRPY156F and 37LRPK166A). This data is shown in Figures 5.11A, B, C and D respectively). Interestingly, two 37LRP mutants: 7LRPY156F results which also showed a similar effect *in vitro* (Figure 5 14).




B
+ve Controls
*
-ve Control
ns
Fluorescent cells (%)
50
40
30
20
10
0
ns P > 0.05
* P ≤ 0.05
37LRP.vYFLP
37LRP/R155A.vYFLP
37LRP.vYNL and 37LRP.vYCL
37LRP/R155A.vYNL and 37LRP.vYCL
37LRP/R155A.vYCL and 37LRP.vYNL
Untransfected cells


C
+ve Controls
***
-ve Control
**
Fluorescent cells (%)
50
40
30
20
10
0
ns P > 0.05
** P ≤ 0.01
*** P ≤ 0.001
37LRP.vYFLP
37LRP/Y156F.vYFLP
37LRP.vYNL and 37LRP.vYCL
37LRP/Y156F.vYNL and 37LRP.vYCL
37LRP/Y156F.vYCL and 37LRP.vYNL
Untransfected cells

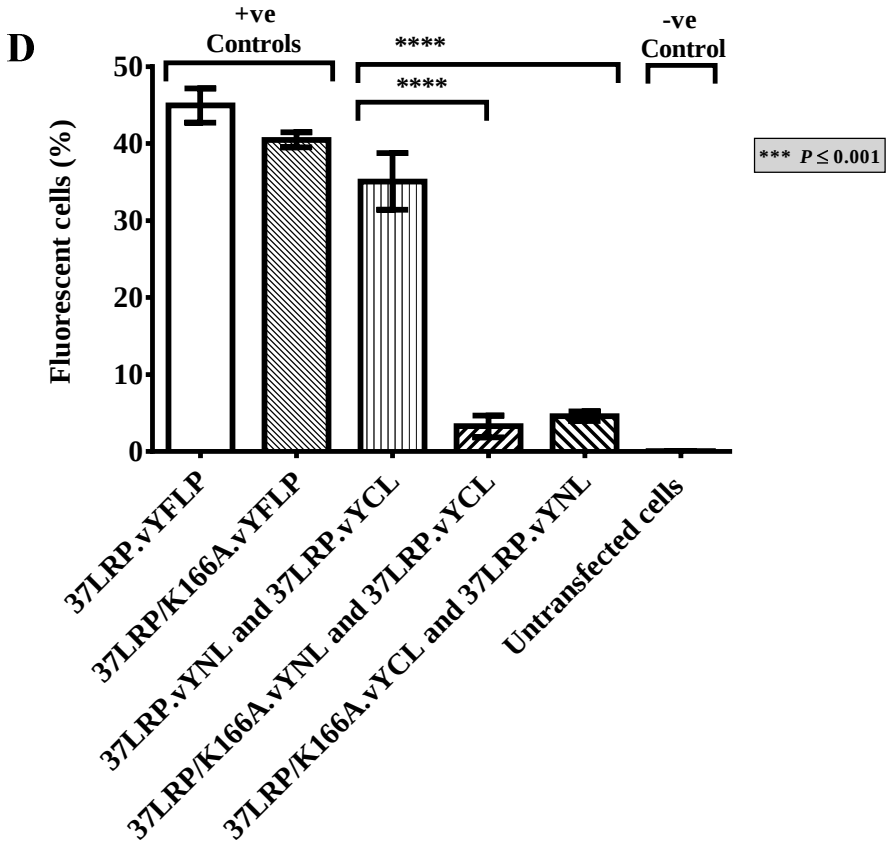


Figure 5-14 The effects of: (A) 37LRP^{Y139F}; (B) 37LRP^{R155A}; (C) 37LRP^{Y156F} and (D) 37LRP^{K166A} mutations on LamR homodimerisation.

Following co-transfection of COS7 cells, cells were harvested 30h post-transfection and analysed for their BifC signal by quantifying the fluorescence intensity and expressing as a percentage of gated cells. Difference in BifC signal was analysed by one-way ANOVA and Tukey test. *Significant at $P \leq 0.05$; ** at ≤ 0.01; *** at $P \leq 0.001$while a p value >0.05 is considered not significant (ns). Error bars = mean of triplicate values on three occasions ± SEM.

5.3.3.5.6 Expression of fluorescently labelled 37 LRP and Gal-3 in HBMEC cells

After confirming both 37LRP and Gal-3 expression in COS7 cells, the expression of both receptors was examined in HBMEC cells, by transient transfection. These cells represent a more physiologically relevant cell line with respect to meningococcal disease, Different transfection reagents were selected for

transfection of 37LRP and Gal-3 in HBMEC cells. Initially, the proteins were expressed using Trans IT®-2020 and $CaPO_4$ transfection reagents, which previously resulted in a successful expression of protein in each of COS7 and HEK293T cells with high efficiency. However, the expression level of 37LRP and Gal-3 in HBMEC cells using these methods was very low compared with COS7 and HEK293T cells. Based on the literature, transfection into HBMEC cells is challenging as modulation of gene function via plasmid DNA in HBMEC very difficult to achieve (Gresch and Altrogge, 2012, Slanina et al., 2012). JetPRIME® DNA transfection reagent is claimed by the manufacturer to provide higher transfection efficiencies with cell lines that are hard to transfect by other methods http://www.polyplus-transfection.com. For this reason, jetPRIME® DNA TR was selected to express both 37LRP and Gal-3 to examine the homo and hetero-dimerisation of both proteins in HBMECs cells. Several volumes of jetPRIME® reagent (2-6µl), of TR; plasmid DNA (0.5; 0.7; 2 and 3 µg/ml), and different incubation times (24; 48 and 72h) were tested to select the optimal transfection conditions. However, the highest transfection efficiency obtained was *ca.* 10% for LamR heterodimerisation and *ca.* 12% for homodimerisation using 3µg/ml plasmid DNA, 4 µl of TR and incubation time of 48 h, which was still very low compared with our previous results using COS7 and HEK293T.

Immunoblotting of total cell extracts of primary HBMECs were analysed using anti-37 LRP [A7] and anti-Gal-3 antibodies. HBMECs transiently transfected to express recombinant r37LRP fused with different fragments of YFP were assessed by immunoblotting using anti-GFP antibody; bands were detected at the expected sizes of 67kDa; 61kDa and 52kDa, corresponding the recombinant proteins 37LRP.vYFLP; 37LRP.vYNL and 37LRP.vYCL, respectively. Expression of

rGal-3 in HBMECs was tested using anti-GFP antibody, which recognized bands at with apparent molecular weights of 67kDa, 58kDa and 47kDa corresponding to recombinant proteins Gal-3.vYFLP; Gal-3.vYNL and Gal-3.vYCL, respectively, which are slightly higher than their predicted sizes of 60kDa, 50kDa and 40kDa, respectively. These results have shown that primary HBMECs can express the recombinant proteins 37 LRP and Gal-3 (Figure 5-15).

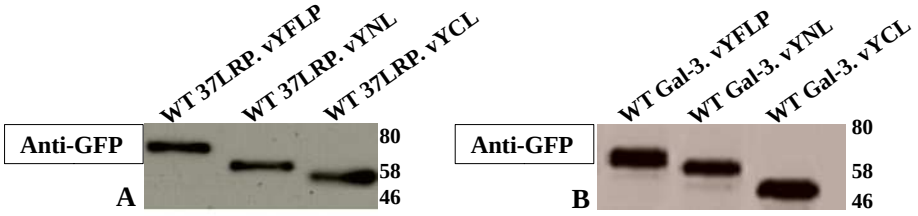


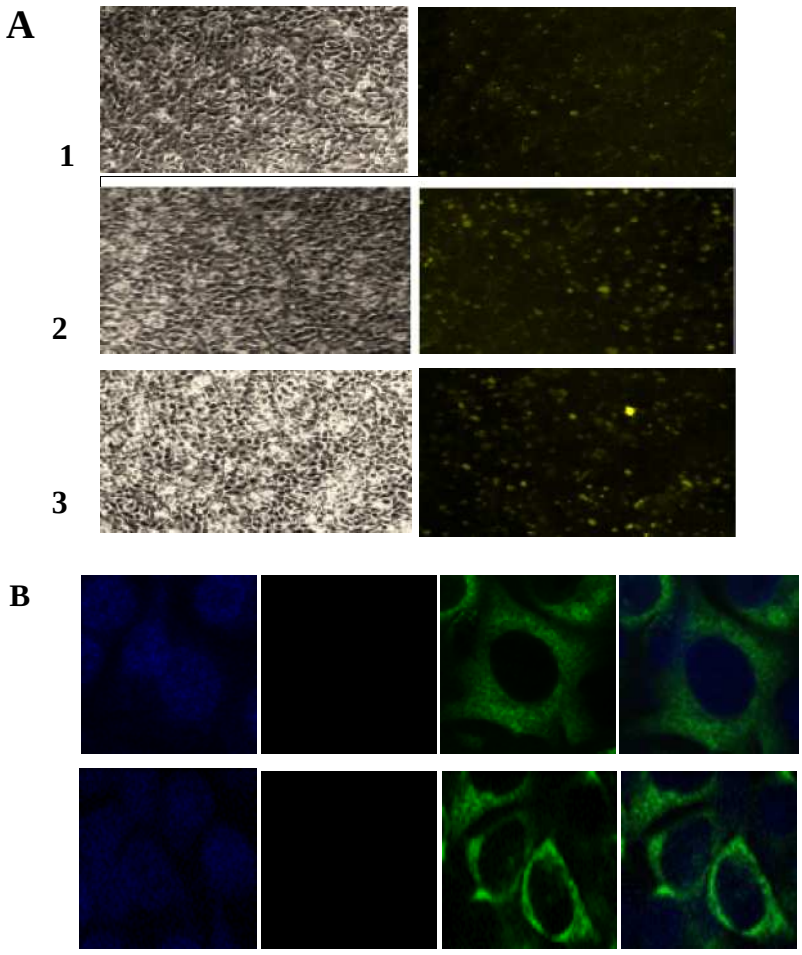
Figure 5-15 Immunoblotting analysis for the presence of BifC constructs in HBMECs cells 24h post transfection.

All BifC constructs were transiently transfected into HBMECs cells and their expression were tested by immunoblotting analysis which performed under reducing conditions using anti-GFP antibody rabbit polyclonal antibody at dilution 1:100 as primary antibodies and followed by (1:30000) dilution of anti-rabbit conjugated to HRP. Then the membranes were developed with luminata western HRP substrate. (A) Showed the expression of 37LRP/BifC constructs tagged with vYFP at sizes of 67kDa; 61kDa and 52kDa displayed in Lane 1: 37LRP.vYFLP; Lane 2: 37LRP.vYNL; Lane 3: 37LRP.vYCL respectively, were detected by testing the whole cell lysates (WCL) of HBMECs cells for expression of recombinants 37LRP. (B) Showed the expression of Gal-3/BifC constructs tagged with vYFP at sizes 60kDa; 50kDa and 40kDa for Lane 1: Gal-3.vYFLP; Lane 2: Gal-3.vYNL; Lane 3: Gal-3.vYCL were detected by testing the whole cell lysates (WCL) of HBMECs cells using anti-GFP antibody and the results confirmed the expression of all indicated recombinants rGal-3.

5.3.3.6 The dimerisation of wild type recombinant proteins 37LRP and Gal-3 was confirmed in HBMECs cells using BifC assay

To examine the LamR dimerisation properties in HBMECs, co-transfection of recombinant 37LRP proteins with 37LRP or with Gal-3 were performed to

provide further evidence of homodimerisation and heterodimerisation, respectively, in HBMECs. Using fluorescence microscopy, the vYFP +ve cells were checked 30h post transfection (Figure 5-14A). Attempts were made to investigate the homo and heterodimerisation of LamR in HBMECs as performed in COS7, however, because several attempts were made to optimize the expression of both 37LRP and Gal-3 using different TRs and the available time did not allow examining the effect of all 37LRP mutants in LamR dimerisation. For that, we only examined the homo- and heterodimerisation of wild type 37LRP and Gal-3 fused with vYFP using BifC assay. Expression of each wild type 37LRP; wild type Gal-3; $37LRP^{Y139F}$ and $37LRP^{K166A}$ fused with vYFLP were used as a positive controls as its shows the fluorescent intensity of full length YFP protein. The flow cytometry results for co-transfected wild type 37LRP with each wild type 37LRP or wild type Gal-3 was clearly confirmed the homo- and hetero-dimerisation of LamR respectively in HBMECs (Figure 5-16C) and also provides evidence that the dimerisation demonstrated in COS7 and HEK293T cells could also occur in the more physiologically relevant HBMEC cells. Further, the BifC signals (green) were also observed in non-staining HBMECs cells, which were co-transfected with 37LRP and Gal-3, or with 37LRP (5.16B).


A
1
2
3
B

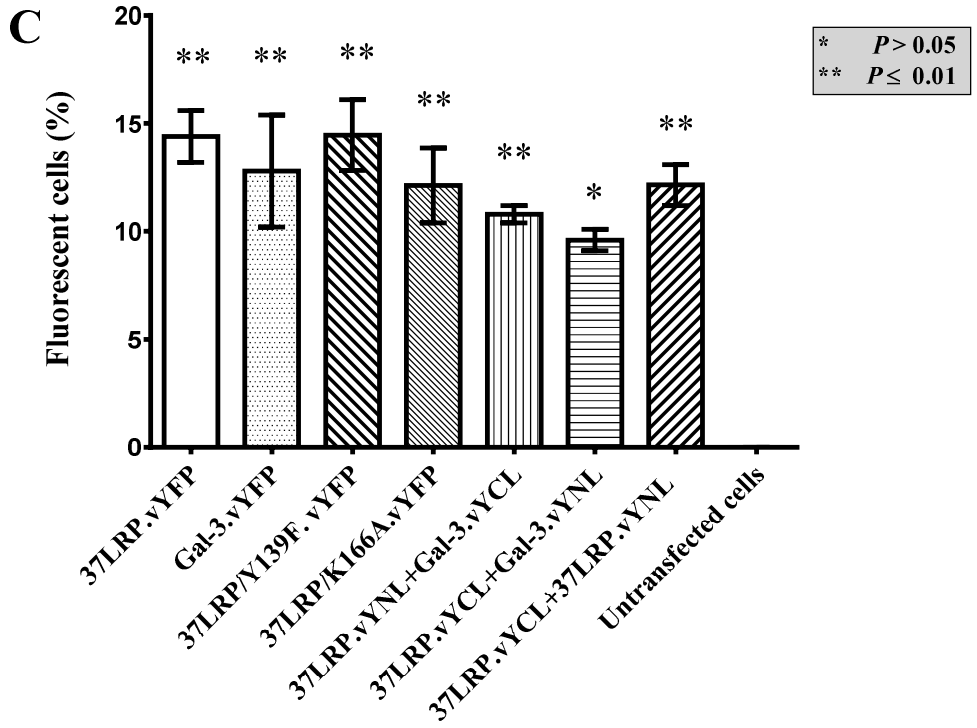


Figure 5-16 Homo and heterodimerisation of recombinants 37LRP and Gal-3 in HBMECs.

Following 30h post transfection of HBMECs with WT 37LRP using (A) Fluorescence microscopy screening of HBMECs. The optical (left, gray) and fluorescent (right, green) light imaging of cells. (1) Transfection of HBMECs with WT 37LRP tagged with vYFP (+ve control), (2) Co-transfection of HBMECs with WT 37LRP and WT Gal-3 tagged with vYCL and vYNL resepectively, (3) Co-transfection of HBMECs with WT 37LRP.vYCL and WT 37LRP.vYNL, vYFP +ve cells (green) using fluorescen microscope indicating a successful transfection of the recombinant proteins. (B) Confocal imaging showing the BifC signal for heterodimerisation of WT 37LRP and WT Gal-3 (upper image), and self-association of 37LRP (lower image). (C) Flow cytometry of WT 37LRP and WT Gal-3 fused with vYFP (+ve controls); heterodimerisation of WT 37LRP and WT Gal-3; and homodimerisation of WT 37LRP. Error bars = mean of triplicate values on three occasions ± SEM.

5.4 Discussion

The 37kDa LRP is thought to be the precursor of the 67kDa LR (DiGiacomo and Meruelo, 2015). Extensive attempts have been made by several investigators to understand the molecular structure of LamR and its cell surface-association and dimerisation properties and several conflicting hypotheses has been suggested to explain the formation of 67LR dimer. However, the exact mechanism(s) by which the 37kDa precursor forms the mature 67kDa isoform is still elusive as evidence supports both a homo and a heterodimer (Rea et al., 2012). It has been suggested that the formation of 67LR is due to a post-translational covalent modification in which the 67LR mature form involves 37LRP acylation or the formation of very tightly associated homo- or hetero-dimers; this post-translation modification was found to be a prerequisite for homodimerisation (Landowski et al., 1995a) or heterodimerisation (Buto et al., 1998). Indeed, several separate studies support these two hypotheses. Immunoblot analysis showed a cross reactivity of two anti-37LRP peptide antibodies with 67-kDa LR and peptides with identical amino acid sequences were found in both 67LR and 37LRP (Landowski et al., 1995a) Acylation (or another post translational modification), which is thought to be a prerequisite for dimerisation, does not happen in yeast cells. On the other hand, other researchers have been supported the hypothesis which proposed 67LR as a heterodimer. Buto et al suggested that 37LRP can associate with another protein carrying epitopes from galectin-3 (Buto et al., 1998). The hypothesis of heterodimerisation was supported by the fact that lectin epitopes are present in 67 LR, but not in 37LRP, that laminin recognition by 67-kDa LR is lactose-dependent, and that 67-kDa LR can be eluted from laminin-affinity chromatography columns by lactose (Castronovo et al., 1992). However,

conflicting results were shown by Gauczynski et al, in which both 37LRP and 67-kDa LR were present on the plasma membrane of N2a cells, which do not express galectin-3, suggesting that dimerisation of Gal-3 with 37LRP is not necessarily required for membrane-association or formation of 67LR, at least, in this cell type (Nelson et al., 2008). A previous study in our research group showed the evidence for both homo and heterodimerisation of LamR (Alqahtani et al., 2014). In the same study, Gal-3 exhibited binding affinity for the LamR-bacterial ligand, meningococcal PilQ, indicating that Gal-3 has one or more meningococcal-binding domain(s).

These considerations prompted us to further investigate the LamR dimerisation properties (Alqahtani et al., 2014). For that, the involvement of several putative amino acid residues within 37LRP in LamR dimerisation was hypothesised and the work presented in this chapter was aimed to investigate both homo and heterodimerisation of LamR to identify the residues that may contribute to the stability of LamR homodimers and heterodimers with Gal-3.

In the context of LamR homo and heterodimerisation, the only other study to focus on determining the amino acid residues on LamR important for heterodimerisation was reported by Alqahtanti, 2014 which showed the critical role of cysteine 173 within Gal-3 in the formation of the LamR heterodimer. The same study also examined the impact of two 37LRP residues (C148 and C163) on heterodimerisation and suggested that neither was involved in heterodimerisation (Alqahtani et al., 2014). Here, the results obtained from computer modeling of LamR and Gal-3interactions were used to identify potential candidate residues. The modeling was performed kindly performed by Dr. Necmettin Pirinccioglu, University of Dicle, using ZDOCK software which is an effective and convenient

simulation software proven to provide a fast and highly accurate estimation of protein-protein complexes (Pierce et al., 2011). The modelling suggested a large protein–protein interface between the 37LRP and Gal-3 subunits, which was predicted to involve several prominent 37LRP residues including lysine166 (Alqahtani et al., 2014). On the other hand, a high-resolution crystal structure of the N-terminal portion of LamR, residues 1 through 220 (abbreviated $LamR^{220}$) predicted the involvement of tyrosine 139 of 37LRP in the homodimerisation process (Jamieson et al., 2008).

Based on this information as well as several published papers that studied or reviewed the laminin-binding domains (Jamieson et al., 2008, Nelson et al., 2008, Jamieson et al., 2011), we attempted to generate several substitutions mutations in 37LRP-Fc constructs including: Tyrosine 139; Arginine 155; Tyrosine 156 and Lysine 166. We utilized these in ELISAs and our results revealed a significant contribution of Tyrosine 156 and Lysine 166 to both hetero and homodimerisation. Arginine 155 of 37LRP also appeared to contribute to dimerisation to a lesser extent. However, ELISA results required further confirmation to test these properties in living cells. Therefore, the same point mutations were generated in BifC constructs encoding 37LRP tagged with fragments of venus fluorescent protein (full length, N-terminal or C-terminal) to study their impacts on LamR dimerisation in living cells. Then the wild type or mutant 37LRP proteins were then co-expressed either with wild type 37LRP or wild type Gal-3 to test their ability to form homo- or heterodimer respectively. Co-expression of two proteins bearing N-terminal and C-terminal fragments (complementing fragments) of YFP resulted in their association and detection of dimerisation by fluorescence microscopy, flow cytometry and live cell imaging

(confocal). Point mutations in 37LRP which may disturb homo or heterodimer formation with Gal-3 were tested for variations in the magnitude of fluorescence signal.

To test the homodimerisation and heterodimerisation of the wild type and mutated 37LRP with Gal-3, BiFC assay was conducted using three cell lines, including the physiologically relevant HBMEC cell line, and the immunoblotting using anti-LamR, anti-Gal-3 and anti-GFP antibodies confirmed their expression. Interestingly, transient expression of recombinant Gal-3 in HBMECs resulted in expression of rGal-3 at a higher than expected molecular weight compared with COS7 and HEK293T cells; this corroborates previously published data (Alqahtani, 2012). This anomaly may relate to the presence of different post-translation modifications in the primary HBMECs, but this remains to be determined.

Interestingly, double staining of non-transfected cells with anti-37LRP [A7] and anti-Gal-3 antibody revealed co-localization of both endogenous 37LRP and Gal-3. However, double-staining of co-transfected cells with both 37LRP and Gal-3, each fused with half fragment of YFP stained with both: anti-Gal-3 and anti-67LR antibody called Mluc5 which recognizes just mature the 67LR, and not the monomeric 37LRP, didn't show the presence of 67LR. Thus, it could be that either staining with Mluc5 needs further optimization or the formation of 67 LR required further factors in addition to 37LRP and Gal-3 interaction to be involved.

In the current study, the effect of several single-substituted 37LRP was tested for their ability to form LamR homo or heterodimer in COS7 cells using flow cytometry, fluorescence and confocal microscopy. Interestingly, among the 37LRP mutants tested, FACS data revealed a significant reduction in dimerisation

of wild type 37LRP with only $37LRP^{Y156F}$ and $37LRP^{K166A}$, indicating their critical role in formation of LamR homodimers. Fluorescence and confocal microscopy also provided confirmatory evidence for the effect of the indicated 37LRP amino acid residues. Similarly, the same 37LRP mutants were tested for their ability to form LamR heterodimers with Gal-3. A significant reduction was observed when wild type Gal-3 co-transfected with $37LRP^{Y156F}$ and $37LRP^{K166A}$, suggesting an important role of these residues in LamR heterodimerisation process on the cell surfaces. In short, our model supports a hypothesis of the mutual exclusivity of 37LRP homodimerisation to form 67LR and 37LRP/Gal-3 heterodimerisation for a given 37LRP molecule, while allowing for the possibility that both homo- and heterodimers could coexist as separate molecules. We also looked at a possible role for tyrosine 139 and Arginine 155 in dimerisation. Mutation of tyrosine 139 did not affect the LamR heterodimerisation and homodimerisation. Although, the mutation of 37LRP Arginine in position 155 slightly affect the homodimerisation and heterodimerisation, its impact was statistically not significant. Considering all presented data, we identified for the first time homodimerisation and heterodimerisation interface determinants within 37LRP, comprising residues Tyrosine 156 and Lysine 166, which are highly conserved among mammalian species. The results corroborated our computing model which predicted the role of Lysine 166 in heterodimerisation with 37LRP. Indeed, the role of 37LRP Arginine 155 and Lysine 166 were studied by Jamison et al., (2011) based on the crystal structure of $LamR^{220}$ (Jamieson et al., 2011); their results suggested that Arginine 155 but not Lysine 166 was essential in binding of LamR with laminin. The results presented here showed that Tyrosine 139 is not essential for LamR homodimerisation in contrast to the findings of

Jamison and colleagues, who predicted that Tyrosine 139 may be involved in LamR homodimerisation based on the crystal structure of $LamR^{220}$ (Jamieson et al., 2011). However, in the same study, they were not able to demonstrate LamR homodimer using recombinant LamR220. It should be noted, however, that the crystal structure used in this study was based on a truncated LamR protein ($LamR^{220}$)(Jamieson et al., 2011) , hence it is expected that these prediction may not conclusively explain the full biological features of LamR including dimerisation in living cells, it is possible that the proposed dimer interface may be an artefact of crystallisation. The findings that Lysine 166 and Arginine 155 are implicated in both homodimerisation and heterodimerisation with Galectin-3 contrasts with the findings presented in Chapter 4, in which these residues were shown not to have any role in binding to the bacterial ligands PorA, OmpP2 and CpbA of *N. meningitidis*, *H. influenzae* and *S. pneumoniae*, respectively.

The localization of both 37LRP and Gal-3 on the cell surface of the HBMECs (Fukushi et al., 2004, Markowska et al., 2011, Saint-Lu et al., 2012), and their ability to bind same group of bacteria that cause meningitis, as well as the presence of binding epitopes on each 37LRP and Gal-3 for their dimerisation, all support a hypothesis in which the heterodimerisation of 37LRP and Gal-3 to form LamR heterodimer is important for pathogenesis at the blood-brain barrier. While homo- and heterodimerisation could be demonstrated in the more physiologically relevant HBMEC cells, in addition to the more tractable COS7 and HEK293T cells, it was not possible to dissect the contribution of individual amino acid residues in the dimerisation of mutated 37LRP proteins in these cells due to difficulties in transfection, low transfection efficiency and limited time. However,

it is likely that the same residues would be involved in the dimerisation process in each cell type.

In line with our results, in a study in which various transfection methods were used to transfect HBMECs each of eleven method tested, including $CaPO_4$ precipitation; lipid-based methods such as jetPRIME®; and electroporation with the Amxa Nucleofector II and Neon Transfection system. Only low transfection efficiencies were obtained with most methods, while using the Amxa Nucleofector II and Neon Transfection system these workers able to transfect these cells with 40% and 80% transfection efficiencies, respectively Slanina et al., (2012).

In the current study, tagging of 37LRP and Gal-3 with vYFP protein, and also the amino acid substitutions tested, had little effect on their level of expression. This is similar to our findings reported by Alqahtanti, 2012, in which BiFC constructs with alanine substitution for the two cysteines (C148 and C163) of 37LRP's C173 of Gal-3's cysteines were expressed at levels that were similar to the wild type proteins (Alqahtani, 2012).

Dimerisation of LamR was investigated using bimolecular fluorescence complementation, a protein-protein interaction technique that relies on the capability of the molecular fragments of vYFP (N- and C-terminus) to re-associate and refold into a fluorescent structure. In the current study, a vYFP isoform was employed as its BiFC fragments have the ability to refold at 37°C with fast folding kinetics (Nagai et al., 2002, Kerppola, 2008, Robida and Kerppola, 2009, Kilpatrick et al., 2010, Kodama and Hu, 2010). For homo and heterodimerisation experiment, 37 LRP and Gal-3, fused with either the N- or C-terminus of YFP, were transfected alone into COS7, HBMECs and HEK293T

cells, as well as non-transfected cells and co-transfection of several possible combined of vYFP fragments were used as negative controls (Figure 5-6). As expected, the negative controls did not exhibit any fluorescence, validating the specificity of the BifC assay. Wild type and mutated LamR and Gal-3 fused with full length YFP were used as positive controls. Compared with wild type 37LRP, all substituted-mutant 37LRPs did not exhibite any significant differences in their level of expression or transfection efficiency, indicating that the point-mutations did not affect their expression capability.

All negative and positive controls behaved as expected in terms of their expression and fluorescence in indicated cells. LamR dimerisation was demonstrated in each of three cell types: COS7; HBMECs; and HEK293T, cells using the BifC assay and flow cytometry. Dimerisation of two recombinant wild type 37LRP proteins to form LamR homodimer, as well as dimerisation of wild type 37LRP with wild type Gal-3 was demonstrated by FACS in all three cells types, suggesting that dimerisation of LamR is consistent property in most of cells. To further validate the results of flow cytometry, live cell imaging by confocal microscopy fixed, un-permeabilised cells also demonstrated LamR dimerisation in COS7 by single-cell staining with anti-GFP antibody or double staining with either anti-GFP and anti-LRP [A7] or anti-GFP and anti-Gal-3 antibodies. In all cases, the presence of fluorescence in staining and non-staining transfected cells indicated the interaction of the two targeted proteins in living cells. Also, double staining of non-transfected cells with anti-37LRP [A7] and anti-Gal-3 antibody revealed co-localization of both endogenous 37LRP and Gal-3. However, double-staining of co-transfected cells with both 37LRP and Gal-3, each fused with a half fragment of YFP and stained with either anti-37LRP [A7]

or anti-67LR antibody (Mluc5, which recognizes mature 67LR but not 37LRP), or double-stained with anti-Gal-3 and anti-67LR (Mluc5), detected only 37LRP or Gal-3, but not 67LR. Thus, it could be that either staining with Mluc5 needs further optimization, or the formation of 67 LR requires further factors in addition to dimerisation of 37LRP and Gal-3.

Among the 37LRP mutants tested, a highly significant reduction in dimerisation of wild type 37LRP when either $37LRP^{Y156F}$ or $37LRP^{K166A}$ substitutions were made, indicating critical roles for these amino acid residues in the formation of the LamR homodimer. These findings using FACS provided quantitative data that showed a statistically significant difference in interaction between the subunits, while qualitative data using fluorescence and confocal microcopy corroborated these findings. The 37LRP mutants tested for their ability to form heterodimers with Gal-3 also showed a significant reduction when wild type Gal-3 was co-transfected with $37LRP^{Y156F}$ and $37LRP^{K166A}$, suggesting important roles of these residues in LamR heterodimerisation process on the cell surfaces. In addition to Tyrosine 156 and Lysine 166, Tyrosine 139 and Arginine 155 were also included to test their contribution in LamR dimerisation. Mutation of tyrosine 139 did not affect the LamR dimerisation while mutation of 37LRP Arginine in position 155 had a small effect that was not statistically significant. Considering all presented data, we identified for the first time homodimerisation and heterodimerisation interface determnants within 37LRP, comprising residues Tyrosine 156 and Lysine 166. These residues are highly conserved among mammalian species.

The findings described here corroborate the computer model which predicted a role for Lysine 166 in heterodimerisation with 37LRP.

Concluding remarks

In vitro and *in vivo* experiments have identified, for the first time, several novel amino acids residues within 37LRP which are required for optimal LamR homo and heterodimerisation with Gal-3. Interestingly, Tyrosine 156 and Lysine 166 are required for both LamR homo and heterodimerisation. This has implications for the dimerisation of these proteins as the evidence provided here indicates that the same amino acids are required for both dimerisation events, and thus the two events are mutually exclusive. This provides insight into LamR functionality by enhancing our understanding of the molecular mechanisms of LamR dimer formation.

CHAPTER SIX

General Discussion and future directions

CHAPTER SIX

6 GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1 GENERAL DISCUSION

Central nervous system (CNS) infections including bacterial meningitis continue to be serious and sometimes fatal (Lewis et al., 2013). Inadequate knowledge of the pathogenesis associated with bacterial entry into the central nervous system contributes to the considerable mortality and morbidity associated with bacterial meningitis (Drevets et al., 2004). In recent years, significant progress has been made in understanding the molecular and cellular events that are involved in the development of bacterial meningitis. The three major pathogens causing bacterial meningitis are *N. meningitidis*, *S. pneumoniae* and *H. influenzae* type b (Hib), which have been found to be responsible for the majority of bacterial meningitis in England and Wales (Corless et al., 2001). Normally, these three bacteria inhabit the nasopharynx as commensal flora. However, under certain circumstances, these bacteria migrate to other niches within the human body due to their ability to gain entry into bloodstream, where they may cause bacteremia and/or traverse the blood-brain barrier (BBB) into the cerebrospinal fluid, where they may cause meningitis. To cross the BBB, bacterial adherence and invasion of microvascular endothelial cells is a prerequisite for successful penetration into the CNS (Corless et al., 2001, Kim, 2006). The molecular mechanisms involved in bacterial penetration of the BBB are complex, and may include a variety of mechanisms. Among the most common themes that illustrate these mechanisms are bacterial ligand-receptor interactions, which have been described for several meningitis-causing bacteria including *E coli*, *N. meningitidis*, *S. pneumoniae* and *H.*

influenzae (Kim, 2003, Wang and Kim, 2013). In *E. coli* K1 strains, two major determinants: Type 1 fimbriae and OmpA were found to contribute to binding of bacteria to HBMECs (Khan et al., 2003, Teng et al., 2005) through interactions with CD48 (Khan et al., 2007) and *N*-acetylglucosamine (GlcNAc) residues on HBMEC surface glycoproteins, respectively (Prasadarao et al., 2003, Khan et al., 2003). Besides, Kim and co-workers studied the role of the bacterial virulence factors, including the *E. coli* K1 toxin cytotoxic necrotizing factor 1 (CNF1), as adhesin determinants specific for the BBB capillary endothelium. They found that CNF1 of *E coli* K1 can recognize a receptor on the HBMECs (LamR), which subsequently contributes to invasion of the BBB *in vitro* (Kim et al., 2005) and traversal of the BBB *in vivo*; these events were dependent on RhoA activation (Khan et al., 2002).

On the other hand, the mechanism by which pneumococcus traverse from the circulation across the BBB into the subarachnoid has been demonstrated by Cundell *et al*., and Ring *et al*., in 1995 and 1998 respectively, who observed that the interaction of bacterial phosphoryl-choline to the host PAFr enhanced bacterial adherence, and thus invasion of endothelial, epithelial and PAF-receptor-transfected cell (Cundell et al., 1995, Ring et al., 1998). Of interest, PAFr has been identified as a common portal of CNS entry for *N. meningitidis* and *H. influenzae* (Virji et al., 1993, Orihuela et al., 2009). The pneumococcal invasion of human endothelial cells was found to be promoted by cytokine activation, which increases the amount of surface-expressed PAFr, which in turn binds the phosphorylcholine component of the pneumococcal cell wall (Mook-Kanamori et al., 2011). In addition, Unkmeir and colleagues provided the first evidence for the internalization of meningococci by HBMECs; they showed that uptake of

meningococci by HBMECs correlated with the expression of meningococcal Opc protein, which binds human fibronectin, as the uptake-promoting serum factor by using two different meningococcal hypervirulent lineages: ET-5, which expresses Opc; and ET-37, which lacks the gene encoding the Opc outer membrane protein (Unkmeir et al., 2002).

In 2009, researchers in our group and collaborators investigated the role of the laminin receptor (LamR) in the pathogenesis of three bacteria: *N. meningitidis*; *S. pneumoniae*; and *H. influenzae*. Using *in vitro* and *in vivo* models of the BBB, affinity chromatography, co-immunoprecipitation, re-tagging and *in vivo* imaging approaches, Orihuela et al. revealed that LamR on the surface of BBB acts as the common receptor for the adhesion determinants of three examined neuroinvasive bacteria, which thus revealed the fascinating capability of LamR to modulate the neurotropism of meningitis-causing bacteria. After testing and confirming the binding of the three bacteria with immobilized recombinant LamR using ELISA assays, their adherence was then modelled using blood microvascular endothelial cells (BMECs) from mice, rats and humans.

The mutagenesis approach was used to identify the meningococcal pilus secretin protein PilQ and the class 1 porin PorA; the pneumococcal choline-binding protein A (CbpA), and the outer membrane protein OmpP2 of *H. influenzae* as the corresponding bacterial LamR-binding adhesins (Orihuela et al., 2009, Abouseada et al., 2012). Also, by raising a polyclonal monospecific antiserum against residues 263-282 of LamR, Orihuela and co-workers showed that binding of LamR to the ligands of *N. meningitidis*, *S. pneumoniae* and *H. influenzae* apparently differs from its binding with *E. coli* K1 CNF1, most viruses and prions, in that those bacteria appear to target the C-terminus of LamR. The study

suggested that C-terminus of LamR is likely to be a common targeting site for multiple pathogens (Kim et al., 2005, Orihuela et al., 2009). These findings confirmed for the first time the important roles of LamR in the pathogenesis of bacterial meningitis caused by *N. meningitidis*, *S. pneumoniae* and *H. influenzae* and prompted the current study to further investigate LamR in terms of its interaction with the bacteria.

In the present study, we aimed to identify the LamR-binding domain(s) corresponding to the LamR-binding surface proteins of *N. meningitidis*, *S. pneumoniae* and *H. influenzae*. In this context, we hypothesised that both the NTD and CTD may responsible for binding of LamR to PilQ and PorA of *N. meningitidis*, CbpA of *S. pneumoniae* and OmpP2 of *H. influenzae*.

Initially, the crystal structure of human LamR ($LamR^{220}$)(Jamieson et al., 2011) was studied in detail to identify possible interaction sites for the bacteria; we took into consideration all identified binding sites for laminin, viruses, prion proteins and bacterial toxins which act as LamR ligands. Taking this information into consideration, three regions and five single amino acid residues of LamR were selected for deletion and point mutation, respectively.

In the current study, although the wild type and mutant versions of LamR could be expressed and purified under denaturing conditions successfully, we expressed recombinant human LamR in mammalian cells and purified under non-denaturing conditions. We hypothesised that the resulting LamR would be more representative biologically since it was likely to include all of the normal human cell post translation modifications, thus the protein was more likely to fold properly and behave as the native protein. After extensive attempts, successful protocols were optimized to express both full-length wild type human LamR

protein and mutant forms in HEK293T cells; these were utilized as they share many similarities and features with neuronal cells.

Due to its roles in various physiological and pathological processes, and the wide range of ligands to which it binds, human LamR has attracted considerable attention from researchers in the fields of cell and cancer biology, as well as those interested in the pathogenesis of prion and viral diseases. In the majority of these studies, full-length or truncated human LamR was produced in bacteria or mammalian cells and purified under denaturing conditions (Fatehullah et al., 2010, Jamieson et al., 2011). To the best of our knowledge, this is the first study utilising the full-length wild type and mutated human LamR protein expressed in mammalian cells and purified in a large scale under native conditions. Thus, this work will potentially contribute not only to studies on the role of LamR in meningitis, but also to studies of its other pathological roles in viral infection, prion disease and cancer. In the present study, DIG-based ELISA assays confirmed the affinity of rLamR for the three examined bacteria and showed that the binding ability of human-produced rLamR and bacteria-produced rLamR with the bacterial ligands was similar; this finding suggests that post-translation modifications (PTMs) such as acetylation, which are lacking in the bacterial system, are not essential for binding of LamR to the three defined bacteria *in vivo*. Whether or not binding of LamR with the other ligands such as laminin, viruses and prions requires particular PTMs is not clear yet and needs further investigation.

Furthermore, examining the ability of LamR to bind with mutant bacterial strains confirmed the roles of meningococcal PorA and PilQ, OmpP2 of *H. influenzae* and CbpA of *S. pneumoniae*, in LamR binding, as well as the fourth and second

extracellular loops of meningococcal PorA and OmpP2 of *H. influenzae*, respectively. These results are in agreement with previous studies (Orihuela et al., 2009, Abouseada et al., 2012). In addition, the synergistic nature of meningococcal PorA and PilQ in LamR binding was demonstrated for the first time.

It has been suggested by Orihuela et al., that *N. meningitidis* targets the C-terminal domain of LamR protein since blocking of the C-terminal domain by anti-LamR antibody, which binds amino acids 263-282 of LamR, caused a remarkably decrease in the binding of the bacteria to LamR (Orihuela et al., 2009, Abouseada et al., 2012). To identify which region(s) of LamR are responsible on binding with *N. meningitidis*, the interaction of the wild type with several mutant forms of LamR protein was investigated. The binding of ΔPilQ strain of *N. meningitidis* to LamR mutants resulted in the identification of a region including amino acids 263-229 as a novel PilQ-binding region. Further investigation using ΔPorA and PorAΔL4 strains resulted in the identification of three regions: amino acids 206-229 and 263-282 for meningococcal PorA, and more specifically for L4 of PorA; these sites were located on CTD of LamR. Conversely, Tyrosine 156 was identified as playing a role in the binding of PorA; this residue is located in the NTD, supporting our hypothesis that PorA targets both NTD and CTD.

In *S. pneumoniae*, CbpA has been reported to interact with a region comprising amino acids within the CTD of LamR. The LamR-binding region in CbpA was found to correspond to a highly conserved surface-exposed loop (residues ^{391}EPRNEEK^{397}) linking the second and third anti-parallel α-helices of the R2 domain (Orihuela et al., 2009). Here, using wild type and a CbpA mutant *S. pneumoniae* in ELISA assays deletion of amino acids 263-282 of LamR resulted

in a large reduction in the binding to *S. pneumoniae* confirming previous results. In addition, regions containing amino acids 206-229 and 263-282 as well as Tyrosine were shown to be important for binding of CbpA. The same regions were also shown to be important for the binding of OmpP2 of *H. influenzae* via its second extracellular loop (amino acids ASENGSDNF).

This study demonstrates that three neuroinvasive bacteria: *N. meningitidis*, *S. pneumoniae* and *H. influenzae*, target similar domains on LamR. These results have implications for the design of preventive and therapeutic interventions which might target the interaction of the three bacteria with LamR. For example, synthetic peptide mimietics might be designed to interfere with the binding of the identified bacterial ligands. Administration of polypeptides corresponding the identified meningococcal ligands for LamR have been investigated as a strategy to develop meningococcus vaccines (St. Jude Children's Research Hospital, 2008; University of Nottingham, 2009) by inhibiting the interaction of the bacteria with LamR. It is well established that CNF1 of *E coli* K1 strains is significantly involved in bacterial internalization into HBMECs, an essential step required for development of *E. coli* meningitis by interacting with LamR. Recently, Zhu and co-workers showed a novel approach to prevent the bacterial meningitis caused by *E coli* through treatment with etoposide, which subsequently lead to a remarkable reduction in expression of 67LR on HBMECs and inhibited *E. coli* invasion of HBMECs (Zhu et al., 2010).

Like 37LRP, 67LR has attracted considerable interest due to its involvement in a wide range of pathological and physiological process in the cell. Indeed, 67LR was discovered firstly as laminin-binding protein in 1983 (Lesot et al., 1983, Malinoff and Wicha, 1983, Rao et al., 1983). However, the isolation of the full-

length cDNA of 67LR in 1989 by Rao and co-workers showed that this cDNA encoded a protein with an apparent molecular mass of only 37-kDa (Rao et al., 1989). This anomaly was explained in 1991 by Castronovo and colleagues as a precursor-product relationship (Castronovo et al., 1991a), although the mechanism by which the 37LRP polypeptide gives rise to its mature form is still unknown. Several hypotheses have been proposed to account for the maturation of LamR including dimerisation of 37LRP to form 67LR (Nelson et al., 2008) or post translation modification such as acylation (Landowski et al., 1995a, Buto et al., 1998). Extensive attempts were undertaken to understand the molecular nature of 67LR. There is evidence that the 67LR heterodimer is formed by dimerisation between 37 LRP and the β-galactoside binding lectin known as Galectin-3 (Gal-3) (Castronovo et al., 1991a, Castronovo, 1993, Buto et al., 1998) or self-association of 37LRP (Landowski et al., 1995a, Gauczynski et al., 2001, Nelson et al., 2008). Most of these studies used methods including detergent cell lysis which may affect native receptor-receptor interactions. The first direct evidence for the existence of both homo and heterodimers of LamR was published recently by our group (Alqahtani, 2012). This evidence points to a 3-way interaction between LamR, Gal-3 and meningococci that is likely to be important for the pathogenesis of this organism. Another aim of the current study was to investigate the involvement of several amino acid residues located on the NTD of LamR for their possible role in dimerisation of LamR and Gal-3. These residues were predicted by computer modelling of the crystal structure of human LamR ($LamR^{220}$) and human Gal-3 to be potential sites of interaction. Three lines of evidence were obtained including ELISA, flow cytometry and confocal imaging of live-cells to demonstrate the role of several amino acid residues in both homodimerisation and

heterodimerisation of 67LR. Initially, ELISA assays confirmed the interaction of the two wild type recombinant proteins: human LamR and human Gal-3. Evidence was provided for a possible role for Lysine 166, Tyrosine 156 and Arginine155 in the interaction of LamR with Gal-3. The BifC complementation assay proved to be a powerful approach for directly visualizing the interaction of LamR and Gal-3 and this approach suggested a role for Lysine 166 and Tyrosine 156 and, to a lesser extent, Arginine 155, in heterodimerisation, supporting the validity of our model of heterodimerisation (Alqahtani, 2014). In a study conducted by Jamieson and colleagues to identify laminin-binding domains on LamR, it was observed that substitution of Lysine166 to Alanine in truncated LamR ($LamR^{220}$) did not affect laminin binding. By contrast, the same study revealed a significant contribution of Arginine 155 of LamR in binding to laminin (Jamieson et al., 2011). Whether Lysine 166 and Arginine 155 are involved in hetero of LamR cooperatively or separately has not yet been assessed and it will be interesting in future studies to investigate the ability of a Lysine166/Tyrosine155 double mutant LamR to form heterodimers. Using the same approach we also investigated the influence of cysteine 173 of Gal-3 on homo- and heterodimerisation. Substitution of this residue resulted in a significant reduction in both Gal-3 homodimerisation and heterodimerisation of Gal-3 with LamR (Alqahtani et al., 2014). However, these interactions were not abolished, indicating the presence of other residues contributing to the binding of these molecules. The roles of both 37LRP and Gal-3 in meningococcal infection have been reported in several previous studies. Using animal models, upregulation of the both Gal-3 and 37 LRP has been observed in the brains of CD46-expressing mice and also in rats in response pneumococcal infection (Bellac et al., 2007) and

meningococcal infection (Quattroni et al., 2012), respectively. Similarly, a remarkable increase in the level of LamR expression was observed in the lungs and brains of infected mice in a pneumococcal meningitis model, which lead to significant enhancement of bacterial adhesion in *in vitro* (Orihuela et al., 2009). LamR and Gal-3 are both localized to the nucleus, cytoplasm and the cell surface and are found as in which found as monomers and dimers. On the cell surface, both proteins participate in adhesion to extracellular matrix components including such as laminin and fibronectin and both are associated with many types of cancers (Krzeslak and Lippinska, 2004, Nelson et al., 2008, Newlaczyl and Yu, 2011, DiGiacomo and Meruelo, 2015). It will be of interest to investigate the impact of mutations affecting homo- and heterodimerisation of LamR and Gal-3 on downstream signalling events resulting from bacterial infection of model cells expressing LamR and Gal-3.

Substitution of alanine for either Lysine 166, Tyrosine 156 or, to a lesser extent, Arginine 155 in LamR significantly reduced homodimerisation. The role of Tyrosine 139 was also investigated as a role in homodimerisation was predicted by Jamieson et al., in 2012 (Jamieson et al., 2011Jamieson et al., 2011). It is worth noting that the dimerisation of either endogenous LamR and Gal-3 or self-association of endogenous LamR as well as recombinants LamR and Gal-3 or self-association of two recombinants LamR were tested in three different mammalian cells: HEK203T; COS7; and HBMECs. These data revealed that homodimerisation and heterodimerisation of LamR is a consistent property in a variety of cells.

In conclusion, this study has established reproducible and efficient protocols for expression of full-length and truncated LamR in a human cell system on a large-

scale as well as its purification under native conditions. Furthermore, several novel sites have been identified on LamR that are responsible on binding with ligands of *N. meningitidis*, *S. pneumoniae* and *H. influenzae*. It has been demonstrated that the three meningeal pathogens target the same domains on LamR for their initial attachment to human cells. Thus, blocking these sites could be a promising strategy to prevent bacterial adhesion/invasion and subsequently meningitis infection.

6.2 Future directions

The findings of the current study regarding the interaction between LamR, Gal-3 and meningeal pathogens have been obtained in *in vitro* systems. *In vivo* confirmation using peptides, peptide mimetics or transgenic animals is required to demonstrate the role of the identified regions in reduction of adhesion and/or invasion of bacteria that cause meningitis. This could be applied by raising antibodies that block the identified regions. Although this study has confirmed the central role of PorA and PilQ of the meningococcus, CbpA of the pneumococcus and OmpP2 of *H. influenzae* in LamR-binding, knocking out these proteins from the corresponding bacteria did not completely inhibit the LamR binding activity. This may suggest there is another binding surface molecules with LamR-binding activity. Such molecules could be identified using methods such as cross-linking or immunoprecipitation. Future work should also aim to further examine whether the disruption of dimerisation of LamR to form homodimer and heterodimer using the LamRL166A and LamRY156F will decrease the adhesion/invasion of bacteria to HBMECs.

BIBLIOGRAPHY

ABOUSEADA, N. M., ASSAFI, M. S., MAHDAVI, J., OLDFIELD, N. J., WHELDON, L. M., WOOLDRIDGE, K. G. & ALA'ALDEEN, D. A. 2012. Mapping the laminin receptor binding domains of *Neisseria meningitidis* PorA and *Haemophilus influenzae* OmpP2. *PLoS One,* 7**,** e46233.

ADAMOU, J. E., HEINRICHS, J. H., ERWIN, A. L., WALSH, W., GAYLE, T., DORMITZER, M., DAGAN, R., BREWAH, Y. A., BARREN, P., LATHIGRA, R., LANGERMANN, S., KOENIG, S. & JOHNSON, S. 2001. Identification and characterization of a novel family of pneumococcal proteins that are protective against sepsis. *Infect Immun,* 69**,** 949-58.

ADAMSON, A. D., JACKSON, D. & DAVIS, J. R. 2011. Novel approaches to in vitro transgenesis. *J Endocrinol,* 208**,** 193-206.

AGRAWAL, A. & MURPHY, T. F. 2011. *Haemophilus influenzae* infections in the *H. influenzae* type b conjugate vaccine era. *J Clin Microbiol,* 49**,** 3728-32.

AHMAD, N., GABIUS, H. J., ANDRE, S., KALTNER, H., SABESAN, S., ROY, R., LIU, B. C., MACALUSO, F. & BREWER, C. F. 2004. Galectin-3 precipitates as a pentamer with synthetic multivalent carbohydrates and forms heterogeneous cross-linked complexes. *Journal of Biological Chemistry,* 279**,** 10841-10847.

AHO, E. L., DEMPSEY, J. A., HOBBS, M. M., KLAPPER, D. G. & CANNON, J. G. 1991. Characterization of the opa (class 5) gene family of *Neisseria meningitidis*. *Mol Microbiol,* 5**,** 1429-37.

AKACHE, B., GRIMM, D., PANDEY, K., YANT, S. R., XU, H. & KAY, M. A. 2006. The 37/67-kilodalton laminin receptor is a receptor for adeno-associated virus serotypes 8, 2, 3, and 9. *Journal of Virology,* 80**,** 9831-9836.

AKBARZADEH, S., WHELDON, L. M., SWEET, S. M. M., TALMA, S., MARDAKHEH, F. K. & HEATH, J. K. 2008. The Deleted in Brachydactyly B Domain of ROR2 Is Required for Receptor Activation by Recruitment of Src. *Plos One,* 3.

ALA'ALDEEN, D. A., NEAL, K. R., AIT-TAHAR, K., NGUYEN-VAN-TAM, J. S., ENGLISH, A., FALLA, T. J., HAWKEY, P. M. & SLACK, R. C. 2000. Dynamics of meningococcal long-term carriage among university students and their implications for mass vaccination. *J Clin Microbiol,* 38**,** 2311-6.

ALA'ALDEEN, D. A. & TURNER, D. P. 2006. *Neisseria meningitidis*. *In Principles and Practice of Clinical Bacteriology (Second Edition),* pp. 205-220**,** Edited by P. M. H. Stephen H. Gillespie

ALBIGER, B., JOHANSSON, L. & JONSSON, A. B. 2003. Lipooligosaccharide-deficient *Neisseria meningitidis* shows altered pilus-associated characteristics. *Infect Immun,* 71**,** 155-62.

ALQAHTANI, F. 2012. Investigation of Galectin-3 interaction with *N. meningitidis* and its dimerization with laminin receptor. *Doctor of Philosophy PhD Thesis. The Universirty of Nottingham, pp*.

ALQAHTANI, F., MAHDAVI, J., WHELDON, L. M., VASSEY, M., PIRINCCIOGLU, N., ROYER, P. J., QARANI, S. M., MORROLL, S., STOOF, J., HOLLIDAY, N. D., TEO, S. Y., OLDFIELD, N. J., WOOLDRIDGE, K. G. & ALA'ALDEEN, D. A. A. 2014. Deciphering the complex three-way interaction between the non-integrin laminin receptor, galectin-3 and *Neisseria meningitidis*. *Open Biology,* 4.

ANDERSEN, C., MAIER, E., KEMMER, G., BLASS, J., HILPERT, A. K., BENZ, R. & REIDL, J. 2003. Porin OmpP2 of *Haemophilus influenzae* shows specificity for nicotinamide-derived nucleotide substrates. *Journal of Biological Chemistry,* 278**,** 24269-24276.

ANDERSON, N. E. & WILLOUGHBY, E. W. 1987. Chronic Meningitis without Predisposing Illness - a Review of 83 Cases. *Quarterly Journal of Medicine,* 63**,** 283-295.

ANDERSSON, M., MICHEL, L., LLULL, J. B. & PIPKORN, U. 1994. Complement activation on the nasal mucosal surface--a feature of the immediate allergic reaction in the nose. *Allergy,* 49**,** 242-5.

ANDISI, V. F., HINOJOSA, C. A., DE JONG, A., KUIPERS, O. P., ORIHUELA, C. J. & BIJLSMA, J. J. 2012. Pneumococcal gene complex involved in resistance to extracellular oxidative stress. *Infect Immun,* 80**,** 1037-49.

ANGER, A. M., ARMACHE, J. P., BERNINGHAUSEN, O., HABECK, M., SUBKLEWE, M., WILSON, D. N. & BECKMANN, R. 2013. Structures of the human and Drosophila 80S ribosome. *Nature,* 497**,** 80-5.

ARBING, M. A., HANRAHAN, J. W. & COULTON, J. W. 2001. Mutagenesis identifies amino acid residues in extracellular loops and within the barrel lumen that determine voltage gating of porin from *Haemophilus influenzae* type b. *Biochemistry,* 40**,** 14621-14628.

ARDINI, E., PESOLE, G., TAGLIABUE, E., MAGNIFICO, A., CASTRONOVO, V., SOBEL, M. E., COLNAGHI, M. I. & MENARD, S. 1998. The 67-kDa laminin receptor originated from a ribosomal protein that acquired a dual function during evolution. *Molecular Biology and Evolution,* 15**,** 1017-1025.

AUTH, D. & BRAWERMAN, G. 1992. A 33-kDa polypeptide with homology to the laminin receptor: component of translation machinery. *Proc Natl Acad Sci U S A,* 89**,** 4368-72.

AVERY, O. T., MACLEOD, C. M. & MCCARTY, M. 1944. Studies on the Chemical Nature of the Substance Inducing Transformation of Pneumococcal Types : Induction of Transformation by a Desoxyribonucleic Acid Fraction Isolated from Pneumococcus Type Iii. *J Exp Med,* 79**,** 137-58.

BADGER, J., SAUDER, J. M., ADAMS, J. M., ANTONYSAMY, S., BAIN, K., BERGSEID, M. G., BUCHANAN, S. G., BUCHANAN, M. D., BATIYENKO, Y., CHRISTOPHER, J. A., EMTAGE, S., EROSHKINA, A., FEIL, I., FURLONG, E. B., GAJIWALA, K. S., GAO, X., HE, D., HENDLE, J., HUBER, A., HODA, K., KEARINS, P., KISSINGER, C., LAUBERT, B., LEWIS, H. A., LIN, J., LOOMIS, K., LORIMER, D., LOUIE, G., MALETIC, M., MARSH, C. D., MILLER, I., MOLINARI, J., MULLER-DIECKMANN, H. J., NEWMAN, J. M., NOLAND, B. W., PAGARIGAN, B., PARK, F., PEAT, T. S., POST, K. W., RADOJICIC, S., RAMOS, A., ROMERO, R., RUTTER, M. E., SANDERSON, W. E., SCHWINN, K. D., TRESSER, J., WINHOVEN, J., WRIGHT, T. A., WU, L., XU, J. & HARRIS, T. J. 2005. Structural analysis of a set of proteins resulting from a bacterial genomics project. *Proteins,* 60**,** 787-96.

BAGNOLI, F., MOSCHIONI, M., DONATI, C., DIMITROVSKA, V., FERLENGHI, I., FACCIOTTI, C., MUZZI, A., GIUSTI, F., EMOLO, C., SINISI, A., HILLERINGMANN, M., PANSEGRAU, W., CENSINI, S., RAPPUOLI, R., COVACCI, A., MASIGNANI, V. & BAROCCHI, M. A. 2008. A second pilus type in *Streptococcus pneumoniae* is prevalent in emerging serotypes and mediates adhesion to host cells. *J Bacteriol,* 190**,** 5480-92.

BAKEMA, J. E. & VAN EGMOND, M. 2011. The human immunoglobulin A Fc receptor FcalphaRI: a multifaceted regulator of mucosal immunity. *Mucosal Immunol,* 4**,** 612-24.

BALASINGHAM, S. V., COLLINS, R. F., ASSALKHOU, R., HOMBERSET, H., FRYE, S. A., DERRICK, J. P. & TONJUM, T. 2007. Interactions between the lipoprotein PilP and the secretin PilQ in *Neisseria meningitidis*. *J Bacteriol,* 189**,** 5716-27.

BAMBERGER, D. M. 2010. Diagnosis, initial management, and prevention of meningitis. *Am Fam Physician,* 82**,** 1491-8.

BANDYOPADHYAY, K., KARMAKAR, S., BISWAS, A. & DAS, P. K. 2003. Membrane orientation of laminin binding protein. *Eur J Biochem,* 270**,** 3806-13.

BANERJEE, A., KIM, B. J., CARMONA, E. M., CUTTING, A. S., GURNEY, M. A., CARLOS, C., FEUER, R., PRASADARAO, N. V. & DORAN, K. S. 2011. Bacterial Pili exploit integrin machinery to promote immune activation and efficient blood-brain barrier penetration. *Nature Communications,* 2.

BANEYX, F. & MUJACIC, M. 2004. Recombinant protein folding and misfolding in *Escherichia coli*. *Nat Biotechnol,* 22**,** 1399-408.

BARDY, S. L., NG, S. Y. & JARRELL, K. F. 2003. Prokaryotic motility structures. *Microbiology,* 149**,** 295-304.

BARENKAMP, S. J. & ST GEME, J. W., 3RD 1996. Identification of a second family of high-molecular-weight adhesion proteins expressed by non-typable *Haemophilus influenzae*. *Mol Microbiol,* 19**,** 1215-23.

BAROCCHI, M. A., RIES, J., ZOGAJ, X., HEMSLEY, C., ALBIGER, B., KANTH, A., DAHLBERG, S., FERNEBRO, J., MOSCHIONI, M., MASIGNANI, V., HULTENBY, K., TADDEI, A. R., BEITER, K., WARTHA, F., VON EULER, A., COVACCI, A., HOLDEN, D. W., NORMARK, S., RAPPUOLI, R. & HENRIQUES-NORMARK, B. 2006. A pneumococcal pilus influences virulence and host inflammatory responses. *Proc Natl Acad Sci U S A,* 103**,** 2857-62.

BARONDES, S. H., CASTRONOVO, V., COOPER, D. N., CUMMINGS, R. D., DRICKAMER, K., FEIZI, T., GITT, M. A., HIRABAYASHI, J., HUGHES, C., KASAI, K. & ET AL. 1994a. Galectins: a family of animal beta-galactoside-binding lectins. *Cell,* 76**,** 597-8.

BARONDES, S. H., COOPER, D. N., GITT, M. A. & LEFFLER, H. 1994b. Galectins. Structure and function of a large family of animal lectins. *J Biol Chem,* 269**,** 20807-10.

BARSOUM, A. L., LIU, B., ROHRER, J. W., COGGIN, J. H., JR., TUCKER, J. A., PANNELL, L. K. & SCHWARZENBERGER, P. O. 2009. Production, safety and antitumor efficacy of recombinant Oncofetal Antigen/immature laminin receptor protein. *Biomaterials,* 30**,** 3091-9.

BASSET, A., TRZCINSKI, K., HERMOS, C., O'BRIEN, K. L., REID, R., SANTOSHAM, M., MCADAM, A. J., LIPSITCH, M. & MALLEY, R. 2007. Association of the pneumococcal pilus with certain capsular serotypes but not with increased virulence. *J Clin Microbiol,* 45**,** 1684-9.

BAYAN, N., GUILVOUT, I. & PUGSLEY, A. P. 2006. Secretins take shape. *Mol Microbiol,* 60**,** 1-4.

BELAY, E. D. 1999. Transmissible spongiform encephalopathies in humans. *Annu Rev Microbiol,* 53**,** 283-314.

BELLAC, C. L., COIMBRA, R. S., SIMON, F., IMBODEN, H. & LEIB, S. L. 2007. Gene and protein expression of galectin-3 and galectin-9 in experimental pneumococcal meningitis. *Neurobiol Dis,* 28**,** 175-83.

BERGMANN, S. & HAMMERSCHMIDT, S. 2006. Versatility of pneumococcal surface proteins. *Microbiology,* 152**,** 295-303.

BERRINGTON, A. W., TAN, Y. C., SRIKHANTA, Y., KUIPERS, B., VAN DER LEY, P., PEAK, I. R. & JENNINGS, M. P. 2002. Phase variation in meningococcal lipooligosaccharide biosynthesis genes. *FEMS Immunol Med Microbiol,* 34**,** 267-75.

BERRY, J. L., PHELAN, M. M., COLLINS, R. F., ADOMAVICIUS, T., TONJUM, T., FRYE, S. A., BIRD, L., OWENS, R., FORD, R. C., LIAN, L. Y. & DERRICK, J. P. 2012. Structure and Assembly of a Trans-Periplasmic Channel for Type IV Pili in *Neisseria meningitidis*. *Plos Pathogens,* 8.

BETHE, G., NAU, R., WELLMER, A., HAKENBECK, R., REINERT, R. R., HEINZ, H. P. & ZYSK, G. 2001. The cell wall-associated serine protease PrtA: a highly conserved virulence factor of *Streptococcus pneumoniae*. *FEMS Microbiol Lett,* 205**,** 99-104.

BILLE, E., ZAHAR, J. R., PERRIN, A., MORELLE, S., KRIZ, P., JOLLEY, K. A., MAIDEN, M. C., DERVIN, C., NASSIF, X. & TINSLEY, C. R. 2005. A chromosomally integrated bacteriophage in invasive meningococci. *J Exp Med,* 201**,** 1905-13.

BILUKHA, O. O., ROSENSTEIN, N., NATIONAL CENTER FOR INFECTIOUS DISEASES, C. F. D. C. & PREVENTION 2005. Prevention and control of meningococcal disease. Recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep,* 54**,** 1-21.

BLACK, S., SHINEFIELD, H., FIREMAN, B., LEWIS, E., RAY, P., HANSEN, J. R., ELVIN, L., ENSOR, K. M., HACKELL, J., SIBER, G., MALINOSKI, F., MADORE, D., CHANG, I., KOHBERGER, R., WATSON, W., AUSTRIAN, R. & EDWARDS, K. 2000. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. *Pediatr Infect Dis J,* 19**,** 187-95.

BOGACHEK, M. V., PROTOPOPOVA, E. V., LOKTEV, V. B., ZAITSEV, B. N., FAVRE, M., SEKATSKII, S. K. & DIETLER, G. 2008. Immunochemical and single molecule force spectroscopy studies of specific interaction between the laminin binding protein and the West Nile virus surface glycoprotein E domain II. *J Mol Recognit,* 21**,** 55-62.

BOOS, C., DANESHVAR, C., HINTON, A. & DAWES, M. 2004. An unusual case of chronic meningitis. *BMC Fam Pract,* 5**,** 21.

BORNHORST, J. A. & FALKE, J. J. 2000. Purification of proteins using polyhistidine affinity tags. *Applications of Chimeric Genes and Hybrid Proteins, Pt A,* 326**,** 245-254.

BORROW, R., FOX, A. J., RICHMOND, P. C., CLARK, S., SADLER, F., FINDLOW, J., MORRIS, R., BEGG, N. T. & CARTWRIGHT, K. A. 2000. Induction of immunological memory in UK infants by a meningococcal A/C conjugate vaccine. *Epidemiol Infect,* 124**,** 427-32.

BOULNOIS, G. J. 1992. Pneumococcal proteins and the pathogenesis of disease caused by *Streptococcus pneumoniae*. *J Gen Microbiol,* 138**,** 249-59.

BRANDTZAEG, P. 1992. Humoral immune response patterns of human mucosae: induction and relation to bacterial respiratory tract infections. *J Infect Dis,* 165 Suppl 1**,** S167-76.

BRANDTZAEG, P., BJERRE, A., OVSTEBO, R., BRUSLETTO, B., JOO, G. B. & KIERULF, P. 2001. *Neisseria meningitidis* lipopolysaccharides in human pathology. *J Endotoxin Res,* 7**,** 401-20.

BRANDTZAEG, P., KIERULF, P., GAUSTAD, P., SKULBERG, A., BRUUN, J. N., HALVORSEN, S. & SORENSEN, E. 1989. Plasma endotoxin as a predictor of multiple organ failure and death in systemic meningococcal disease. *J Infect Dis,* 159**,** 195-204.

BRAUN, J. S., SUBLETT, J. E., FREYER, D., MITCHELL, T. J., CLEVELAND, J. L., TUOMANEN, E. I. & WEBER, J. R. 2002. Pneumococcal pneumolysin and H(2)O(2) mediate brain cell apoptosis during meningitis. *J Clin Invest,* 109**,** 19-27.

BRENDISH, N. J. & READ, R. C. 2015. *Neisseria meningitidis* serogroup B bivalent factor H binding protein vaccine. *Expert Rev Vaccines,* 14**,** 493-503.

BRILES, D. E., FORMAN, C., HOROWITZ, J. C., VOLANAKIS, J. E., BENJAMIN, W. H., JR., MCDANIEL, L. S., ELDRIDGE, J. & BROOKS, J. 1989. Antipneumococcal effects of C-reactive protein and monoclonal antibodies to pneumococcal cell wall and capsular antigens. *Infect Immun,* 57**,** 1457-64.

BRILES, D. E., HOLLINGSHEAD, S. K., NABORS, G. S., PATON, J. C. & BROOKS-WALTER, A. 2000. The potential for using protein vaccines to protect against otitis media caused by *Streptococcus pneumoniae*. *Vaccine,* 19**,** S87-S95.

BRILES, D. E., KING, J. D., GRAY, M. A., MCDANIEL, L. S., SWIATLO, E. & BENTON, K. A. 1996. PspA, a protection-eliciting pneumococcal protein: Immunogenicity of isolated native PspA in mice. *Vaccine,* 14**,** 858-867.

BRISSAC, T., MIKATY, G., DUMENIL, G., COUREUIL, M. & NASSIF, X. 2012. The meningococcal minor pilin PilX is responsible for type IV pilus conformational changes associated with signaling to endothelial cells. *Infect Immun,* 80**,** 3297-306.

BROKER, M. & FANTONI, S. 2007. Meningococcal disease: a review on available vaccines and vaccines in development. *Minerva Med,* 98**,** 575-89.

BROOKS-WALTER, A., BRILES, D. E. & HOLLINGSHEAD, S. K. 1999. The pspC gene of *Streptococcus pneumoniae* encodes a polymorphic protein, PspC, which elicits cross-reactive antibodies to PspA and provides immunity to pneumococcal bacteremia. *Infection and Immunity,* 67**,** 6533-6542.

BROUWER, M. C., TUNKEL, A. R. & VAN DE BEEK, D. 2010. Epidemiology, diagnosis, and antimicrobial treatment of acute bacterial meningitis. *Clin Microbiol Rev,* 23**,** 467-92.

BROWN, J. S., GILLILAND, S. M. & HOLDEN, D. W. 2001. A *Streptococcus pneumoniae* pathogenicity island encoding an ABC transporter involved in iron uptake and virulence. *Mol Microbiol,* 40**,** 572-85.

BURRAGE, M., ROBINSON, A., BORROW, R., ANDREWS, N., SOUTHERN, J., FINDLOW, J., MARTIN, S., THORNTON, C., GOLDBLATT, D., CORBEL, M., SESARDIC, D., CARTWIGHT, K., RICHMOND, P. & MILLER, E. 2002. Effect of vaccination with carrier protein on response to meningococcal C conjugate vaccines and value of different immunoassays as predictors of protection. *Infect Immun,* 70**,** 4946-54.

BUTO, S., TAGLIABUE, E., ARDINI, E., MAGNIFICO, A., GHIRELLI, C., VAN DEN BRULE, F., CASTRONOVO, V., COLNAGHI, M. I., SOBEL, M. E. & MENARD, S. 1998. Formation of the 67-kDa laminin receptor by acylation of the precursor. *Journal of Cellular Biochemistry,* 69**,** 244-251.

BYINGTON, C. L., SAMORE, M. H., STODDARD, G. J., BARLOW, S., DALY, J., KORGENSKI, K., FIRTH, S., GLOVER, D., JENSEN, J., MASON, E. O., SHUTT, C. K. & PAVIA, A. T. 2005. Temporal trends of invasive disease due to *Streptococcus pneumoniae* among children in the intermountain west: emergence of nonvaccine serogroups. *Clin Infect Dis,* 41**,** 21-9.

CAMPAGNE, G., GARBA, A., FABRE, P., SCHUCHAT, A., RYALL, R., BOULANGER, D., BYBEL, M., CARLONE, G., BRIANTAIS, P., IVANOFF, B., XERRI, B. & CHIPPAUX, J. P. 2000. Safety and immunogenicity of three doses of a *Neisseria meningitidis* A + C diphtheria conjugate vaccine in infants from Niger. *Pediatr Infect Dis J,* 19**,** 144-50.

CANTISANI, M., VITIELLO, M., FALANGA, A., FINAMORE, E., GALDIERO, M. & GALDIERO, S. 2012. Peptides complementary to the active loop of porin P2 from *Haemophilus influenzae* modulate its activity. *Int J Nanomedicine,* 7**,** 2361-71.

CANVIN, J. R., MARVIN, A. P., SIVAKUMARAN, M., PATON, J. C., BOULNOIS, G. J., ANDREW, P. W. & MITCHELL, T. J. 1995. The Role of Pneumolysin and Autolysin in the Pathology of Pneumonia and Septicemia in Mice Infected with a Type-2 Pneumococcus. *Journal of Infectious Diseases,* 172**,** 119-123.

CARBONNELLE, E. 2009. Laboratory diagnosis of bacterial meningitis: Usefulness of various tests for the determination of the etiological agent. *Medecine Et Maladies Infectieuses,* 39**,** 581-605.

CARBONNELLE, E., HELAINE, S., NASSIF, X. & PELICIC, V. 2006. A systematic genetic analysis in *Neisseria meningitidis* defines the Pil proteins required for assembly, functionality, stabilization and export of type IV pili. *Mol Microbiol,* 61**,** 1510-22.

CARBONNELLE, E., HELAINE, S., PROUVENSIER, L., NASSIF, X. & PELICIC, V. 2005. Type IV pilus biogenesis in *Neisseria meningitidis*: PilW is involved in a step occurring after pilus assembly, essential for fibre stability and function. *Mol Microbiol,* 55**,** 54-64.

CARBONNELLE, E., HILL, D. J., MORAND, P., GRIFFITHS, N. J., BOURDOULOUS, S., MURILLO, I., NASSIF, X. & VIRJI, M. 2009. Meningococcal interactions with the host. *Vaccine,* 27 Suppl 2**,** B78-89.

CARDOSO, F. L., BRITES, D. & BRITO, M. A. 2010. Looking at the blood-brain barrier: molecular anatomy and possible investigation approaches. *Brain Res Rev,* 64**,** 328-63.

CARLSEN, B. D., KAWANA, M., KAWANA, C., TOMASZ, A. & GIEBINK, G. S. 1992. Role of the bacterial cell wall in middle ear inflammation caused by *Streptococcus pneumoniae*. *Infect Immun,* 60**,** 2850-4.

CARTWRIGHT, K. A., STUART, J. M., JONES, D. M. & NOAH, N. D. 1987. The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica*. *Epidemiol Infect,* 99**,** 591-601.

CASTRONOVO, V. 1993. Laminin receptors and laminin-binding proteins during tumor invasion and metastasis. *Invasion Metastasis,* 13**,** 1-30.

CASTRONOVO, V., CLAYSMITH, A. P., BARKER, K. T., CIOCE, V., KRUTZSCH, H. C. & SOBEL, M. E. 1991a. Biosynthesis of the 67 kDa high affinity laminin receptor. *Biochem Biophys Res Commun,* 177**,** 177-83.

CASTRONOVO, V., LUYTEN, F., VANDENBRULE, F. & SOBEL, M. E. 1992. Identification of a 14-Kda Laminin Binding-Protein (Hlbp14) in Human-Melanoma Cells That Is Identical to the 14-Kda Galactoside Binding Lectin. *Archives of Biochemistry and Biophysics,* 297**,** 132-138.

CASTRONOVO, V., TARABOLETTI, G. & SOBEL, M. E. 1991b. Functional domains of the 67-kDa laminin receptor precursor. *J Biol Chem,* 266**,** 20440-6.

CATLIN, B. W. 1973. Nutritional profiles of *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and *Neisseria lactamica* in chemically defined media and the use of growth requirements for gonococcal typing. *J Infect Dis,* 128**,** 178-94.

CATTERALL, J. R. 1999. *Streptococcus pneumoniae*. *Thorax,* 54**,** 929-937.

CAUGANT, D. A. 1998. Population genetics and molecular epidemiology of *Neisseria meningitidis*. *APMIS,* 106**,** 505-25.

CERLIANI, J. P., STOWELL, S. R., MASCANFRONI, I. D., ARTHUR, C. M., CUMMINGS, R. D. & RABINOVICH, G. A. 2011. Expanding the universe of cytokines and pattern recognition receptors: galectins and glycans in innate immunity. *J Clin Immunol,* 31**,** 10-21.

CERRA, R. F., GITT, M. A. & BARONDES, S. H. 1985. Three soluble rat beta-galactoside-binding lectins. *J Biol Chem,* 260**,** 10474-7.

CHANG, L., WANG, Y. H., LIU, Y. Y., JIANG, Z., QIAO, B. X., XIAO, J., GUO, H. L. & WANG, Z. R. 2010. Key region of laminin receptor 1 for interaction with human period 1. *African Journal of Biotechnology,* 9**,** 6401-6407.

CHANG, Y. C., STINS, M. F., MCCAFFERY, M. J., MILLER, G. F., PARE, D. R., DAM, T., PAUL-SATYASEELA, M., KIM, K. S. & KWON-CHUNG, K. J. 2004. Cryptococcal yeast cells invade the central nervous system via transcellular penetration of the blood-brain barrier. *Infect Immun,* 72**,** 4985-95.

CHARBONNIER, S., GALLEGO, O. & GAVIN, A. C. 2008. The social network of a cell: recent advances in interactome mapping. *Biotechnol Annu Rev,* 14**,** 1-28.

CHEN, T. & GOTSCHLICH, E. C. 1996. CGM1a antigen of neutrophils, a receptor of gonococcal opacity proteins. *Proc Natl Acad Sci U S A,* 93**,** 14851-6.

CHENG, Q., FINKEL, D. & HOSTETTER, M. K. 2000. Novel purification scheme and functions for a C3-binding protein from *Streptococcus pneumoniae*. *Biochemistry,* 39**,** 5450-7.

CHIBA, N., MURAYAMA, S. Y., MOROZUMI, M., NAKAYAMA, E., OKADA, T., IWATA, S., SUNAKAWA, K. & UBUKATA, K. 2009. Rapid detection of eight causative pathogens for the diagnosis of bacterial meningitis by real-time PCR. *J Infect Chemother,* 15**,** 92-8.

CHOI, J., COX, A. D., LI, J., MCCREADY, W. & ULANOVA, M. 2014. Activation of innate immune responses by *Haemophilus influenzae* lipooligosaccharide. *Clin Vaccine Immunol,* 21**,** 769-76.

CHONG, P., YANG, Y. P., FAHIM, R., MCVERRY, P., SIA, C. & KLEIN, M. 1993. Immunogenicity of overlapping synthetic peptides covering the entire sequence of *Haemophilus influenzae* type b outer membrane protein P2. *Infect Immun,* 61**,** 2653-61.

CHUNG, J. W., HONG, S. J., KIM, K. J., GOTI, D., STINS, M. F., SHIN, S., DAWSON, V. L., DAWSON, T. M. & KIM, K. S. 2003. 37-kDa laminin receptor precursor modulates cytotoxic necrotizing factor 1-mediated RhoA activation and bacterial uptake. *J Biol Chem,* 278**,** 16857-62.

CIOCE, V., CASTRONOVO, V., SHMOOKLER, B. M., GARBISA, S., GRIGIONI, W. F., LIOTTA, L. A. & SOBEL, M. E. 1991. Increased expression of the laminin receptor in human colon cancer. *J Natl Cancer Inst,* 83**,** 29-36.

CLAASSEN, I., MEYLIS, J., VAN DER LEY, P., PEETERS, C., BRONS, H., ROBERT, J., BORSBOOM, D., VAN DER ARK, A., VAN STRAATEN, I., ROHOLL, P., KUIPERS, B. & POOLMAN, J. 1996. Production, characterization and control of a *Neisseria meningitidis* hexavalent class 1 outer membrane protein containing vesicle vaccine. *Vaccine,* 14**,** 1001-8.

CLAUS, H., MAIDEN, M. C., MAAG, R., FROSCH, M. & VOGEL, U. 2002. Many carried meningococci lack the genes required for capsule synthesis and transport. *Microbiology,* 148**,** 1813-9.

CLEMANS, D. L., MARRS, C. F., BAUER, R. J., PATEL, M. & GILSDORF, J. R. 2001. Analysis of pilus adhesins from *Haemophilus influenzae* biotype IV strains. *Infect Immun,* 69**,** 7010-9.

COLLINS, R. F., DAVIDSEN, L., DERRICK, J. P., FORD, R. C. & TONJUM, T. 2001. Analysis of the PilQ secretin from *Neisseria meningitidis* by transmission electron microscopy reveals a dodecameric quaternary structure. *J Bacteriol,* 183**,** 3825-32.

COLLINS, R. F., FRYE, S. A., BALASINGHAM, S., FORD, R. C., TONJUM, T. & DERRICK, J. P. 2005. Interaction with type IV pili induces structural changes in the bacterial outer membrane secretin PilQ. *J Biol Chem,* 280**,** 18923-30.

COONROD, J. D. 1983. Urine as an Antigen Reservoir for Diagnosis of Infectious-Diseases. *American Journal of Medicine,* 75**,** 85-92.

COOPER, D. N. 2002. Galectinomics: finding themes in complexity. *Biochim Biophys Acta,* 1572**,** 209-31.

COPE, L. D., PELZEL, S. E., LATIMER, J. L. & HANSEN, E. J. 1990. Characterization of a mutant of *Haemophilus influenzae* type b lacking the P2 major outer membrane protein. *Infect Immun,* 58**,** 3312-8.

CORLESS, C. E., GUIVER, M., BORROW, R., EDWARDS-JONES, V., FOX, A. J. & KACZMARSKI, E. B. 2001. Simultaneous detection of *Neisseria meningitidis, Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. *J Clin Microbiol,* 39**,** 1553-8.

COSTA, I., PAJON, R. & GRANOFF, D. M. 2014. Human factor H (FH) impairs protective meningococcal anti-FHbp antibody responses and the antibodies enhance FH binding. *MBio,* 5**,** e01625-14.

COULTON, J. W., CHIN, A. C. & VACHON, V. 1992. Recombinant porin of *Haemophilus influenzae* type b. *J Infect Dis,* 165 Suppl 1**,** S188-91.

CRAIG, L., VOLKMANN, N., ARVAI, A. S., PIQUE, M. E., YEAGER, M., EGELMAN, E. H. & TAINER, J. A. 2006. Type IV pilus structure by cryo-electron microscopy and crystallography: implications for pilus assembly and functions. *Mol Cell,* 23**,** 651-62.

CRAIN, M. J., WALTMAN, W. D., TURNER, J. S., YOTHER, J., TALKINGTON, D. F., MCDANIEL, L. S., GRAY, B. M. & BRILES, D. E. 1990. Pneumococcal Surface Protein-a (Pspa) Is Serologically Highly Variable and Is Expressed by All Clinically Important Capsular Serotypes of *Streptococcus-Pneumoniae*. *Infection and Immunity,* 58**,** 3293-3299.

CUNDELL, D. R., GERARD, N. P., GERARD, C., IDANPAAN-HEIKKILA, I. & TUOMANEN, E. I. 1995. *Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor. *Nature,* 377**,** 435-8.

DAGHER, S. F., WANG, J. L. & PATTERSON, R. J. 1995. Identification of galectin-3 as a factor in pre-mRNA splicing. *Proc Natl Acad Sci U S A,* 92**,** 1213-7.

DALY, J. A., GOOCH, W. M., 3RD & MATSEN, J. M. 1985. Evaluation of the Wayson variation of a methylene blue staining procedure for the detection of microorganisms in cerebrospinal fluid. *J Clin Microbiol,* 21**,** 919-21.

DANIELS, C. C., COAN, P., KING, J., HALE, J., BENTON, K. A., BRILES, D. E. & HOLLINGSHEAD, S. K. 2010. The Proline-Rich Region of Pneumococcal Surface Proteins A and C Contains Surface-Accessible Epitopes Common to All Pneumococci and Elicits Antibody-Mediated Protection against Sepsis. *Infection and Immunity,* 78**,** 2163-2172.

DAVE, S., BROOKS-WALTER, A., PANGBURN, M. K. & MCDANIEL, L. S. 2001. PspC, a pneumococcal surface protein, binds human factor H. *Infect Immun,* 69**,** 3435-7.

DE JONGE, M. I., HAMSTRA, H. J., VAN ALPHEN, L., DANKERT, J. & VAN DER LEY, P. 2003. Mapping the binding domains on meningococcal Opa proteins for CEACAM1 and CEA receptors. *Mol Microbiol,* 50**,** 1005-15.

DE MANZONI, G., GUGLIELMI, A., VERLATO, G., TOMEZZOLI, A., PELOSI, G., SCHIAVON, I. & CORDIANO, C. 1998. Prognostic significance of 67-kDa laminin receptor expression in advanced gastric cancer. *Oncology,* 55**,** 456-60.

DEGHMANE, A. E., GIORGINI, D., LARRIBE, M., ALONSO, J. M. & TAHA, M. K. 2002. Down-regulation of pili and capsule of *Neisseria meningitidis* upon contact with epithelial cells is mediated by CrgA regulatory protein. *Mol Microbiol,* 43**,** 1555-64.

DELBAZ, S. A., SIADAT, S. D., AGHASADEGHI, M. R., PIRYAIE, M., PEERAYEH, S. N., MOUSAVI, S. F., MOSHIRI, A., SADAT, S. M., ZANGENEH, M., NEJATI, M., KASHANIZADEH, N. & BOUZARI, S. 2013. Biological and Immunological Evaluation of *Neisseria meningitidis* Serogroup A Outer Membrane Vesicle as Vaccine Candidates. *Jundishapur Journal of Microbiology,* 6.

DEMIANOVA, M., FORMOSA, T. G. & ELLIS, S. R. 1996. Yeast proteins related to the p40/laminin receptor precursor are essential components of the 40 S ribosomal subunit. *J Biol Chem,* 271**,** 11383-91.

DENNEHY, P. H. 2001. Active immunization in the United States: developments over the past decade. *Clin Microbiol Rev,* 14**,** 872-908, table of contents.

DEVOY, A. F., DYET, K. H. & MARTIN, D. R. 2005. Stability of PorA during a meningococcal disease epidemic. *J Clin Microbiol,* 43**,** 832-7.

DIAZ ROMERO, J. & OUTSCHOORN, I. M. 1994. Current status of meningococcal group B vaccine candidates: capsular or noncapsular? *Clin Microbiol Rev,* 7**,** 559-75.

DIGIACOMO, V. & MERUELO, D. 2015. Looking into laminin receptor: critical discussion regarding the non-integrin 37/67-kDa laminin receptor/RPSA protein. *Biol Rev Camb Philos Soc*.

DORAN, K. S., LIU, G. Y. & NIZET, V. 2003. Group B streptococcal beta-hemolysin/cytolysin activates neutrophil signaling pathways in brain endothelium and contributes to development of meningitis. *J Clin Invest,* 112**,** 736-44.

DRAKE, S. L., SANDSTEDT, S. A. & KOOMEY, M. 1997. PilP, a pilus biogenesis lipoprotein in *Neisseria gonorrhoeae*, affects expression of PilQ as a high-molecular-mass multimer. *Mol Microbiol,* 23**,** 657-68.

DREVETS, D. A., LEENEN, P. J. M. & GREENFIELD, R. A. 2004. Invasion of the central nervous system by intracellular bacteria. *Clinical Microbiology Reviews,* 17**,** 323-+.

DUIM, B., VOGEL, L., PUIJK, W., JANSEN, H. M., MELOEN, R. H., DANKERT, J. & VANALPHEN, L. 1996. Fine mapping of outer membrane protein P2 antigenic sites which vary during persistent infection by *Haemophilus influenzae*. *Infection and Immunity,* 64**,** 4673-4679.

DUMIC, J., DABELIC, S. & FLOGEL, M. 2006. Galectin-3: an open-ended story. *Biochim Biophys Acta,* 1760**,** 616-35.

DUNN, K. L., VIRJI, M. & MOXON, E. R. 1995. Investigations into the molecular basis of meningococcal toxicity for human endothelial and epithelial cells: the synergistic effect of LPS and pili. *Microb Pathog,* 18**,** 81-96.

DURAND, M. L., CALDERWOOD, S. B., WEBER, D. J., MILLER, S. I., SOUTHWICK, F. S., CAVINESS, V. S., JR. & SWARTZ, M. N. 1993. Acute bacterial meningitis in adults. A review of 493 episodes. *N Engl J Med,* 328**,** 21-8.

ELM, C., BRAATHEN, R., BERGMANN, S., FRANK, R., VAERMAN, J. P., KAETZEL, C. S., CHHATWAL, G. S., JOHANSEN, F. E. & HAMMERSCHMIDT, S. 2004. Ectodomains 3 and 4 of human polymeric immunoglobulin receptor (hpIgR) mediate invasion of *Streptococcus pneumoniae* into the epithelium. *Journal of Biological Chemistry,* 279**,** 6296-6304.

ERIKSSON, J., ERIKSSON, O. S., MAUDSDOTTER, L., PALM, O., ENGMAN, J., SARKISSIAN, T., ARO, H., WALLIN, M. & JONSSON, A. B. 2015. Characterization of motility and piliation in pathogenic Neisseria. *BMC Microbiol,* 15**,** 92.

ERLANDER, S. R. 1995. The solution to the seven mysteries of AIDS: the 'Trojan horse'. *Med Hypotheses,* 44**,** 1-9.

ESKOLA, J., PELTOLA, H., TAKALA, A. K., KAYHTY, H., HAKULINEN, M., KARANKO, V., KELA, E., REKOLA, P., RONNBERG, P. R., SAMUELSON, J. S. & ET AL. 1987. Efficacy of *Haemophilus influenzae* type b polysaccharide-diphtheria toxoid conjugate vaccine in infancy. *N Engl J Med,* 317**,** 717-22.

FAKRUDDIN, M., MOHAMMAD MAZUMDAR, R., BIN MANNAN, K. S., CHOWDHURY, A. & HOSSAIN, M. N. 2013. Critical Factors Affecting the Success of Cloning, Expression, and Mass Production of Enzymes by Recombinant E. coli. *ISRN Biotechnol,* 2013**,** 590587.

FATEHULLAH, A., DOHERTY, C., PIVATO, G., ALLEN, G., DEVINE, L., NELSON, J. & TIMSON, D. J. 2010. Interactions of the 67 kDa laminin receptor and its precursor with laminin. *Biosci Rep,* 30**,** 73-9.

FELDMAN, C. A. 2014. Cost of dental education, student indebtedness, and our social contract. *J Dent Educ,* 78**,** 1479-80.

FENG, S., TIAN, E., ZHANG, L., WANG, Q. & DENG, H. 2012. Development of the Fc-III tagged protein expression system for protein purification and detection. *PLoS One,* 7**,** e44208.

FERMINO, M. L., POLLI, C. D., TOLEDO, K. A., LIU, F. T., HSU, D. K., ROQUE-BARREIRA, M. C., PEREIRA-DA-SILVA, G., BERNARDES, E. S. & HALBWACHS-MECARELLI, L. 2011. LPS-induced galectin-3 oligomerization results in enhancement of neutrophil activation. *PLoS One,* 6**,** e26004.

FERNAAYS, M. M., LESSE, A. J., CAI, X. & MURPHY, T. F. 2006. Characterization of igaB, a second immunoglobulin A1 protease gene in nontypeable *Haemophilus influenzae*. *Infect Immun,* 74**,** 5860-70.

FINDLER, G. & HOFFMAN, H. J. 1981. Chronic meningococcal meningitis in an infant. *Childs Brain,* 8**,** 390-4.

FINK, D. L., BUSCHER, A. Z., GREEN, B., FERNSTEN, P. & ST GEME, J. W., 3RD 2003. The *Haemophilus influenzae* Hap autotransporter mediates microcolony formation and adherence to epithelial cells and extracellular matrix via binding regions in the C-terminal end of the passenger domain. *Cell Microbiol,* 5**,** 175-86.

FINK, D. L., GREEN, B. A. & ST GEME, J. W., 3RD 2002. The *Haemophilus influenzae* Hap autotransporter binds to fibronectin, laminin, and collagen IV. *Infect Immun,* 70**,** 4902-7.

FINNE, J., BITTER-SUERMANN, D., GORIDIS, C. & FINNE, U. 1987. An IgG monoclonal antibody to group B meningococci cross-reacts with developmentally regulated polysialic acid units of glycoproteins in neural and extraneural tissues. *J Immunol,* 138**,** 4402-7.

FISCHER, H. & TOMASZ, A. 1985. Peptidoglycan cross-linking and teichoic acid attachment in *Streptococcus pneumoniae*. *J Bacteriol,* 163**,** 46-54.

FISCHER, W., BEHR, T., HARTMANN, R., PETER-KATALINIC, J. & EGGE, H. 1993. Teichoic acid and lipoteichoic acid of *Streptococcus pneumoniae* possess identical chain structures. A reinvestigation of teichoid acid (C polysaccharide). *Eur J Biochem,* 215**,** 851-7.

FLETCHER, L. D., BERNFIELD, L., BARNIAK, V., FARLEY, J. E., HOWELL, A., KNAUF, M., OOI, P., SMITH, R. P., WEISE, P., WETHERELL, M., XIE, X., ZAGURSKY, R., ZHANG, Y. & ZLOTNICK, G. W. 2004. Vaccine potential of the *Neisseria meningitidis* 2086 lipoprotein. *Infect Immun,* 72**,** 2088-100.

FORBES, K. J., BRUCE, K. D., BALL, A. & PENNINGTON, T. H. 1992. Variation in length and sequence of porin (ompP2) alleles of non-capsulate *Haemophilus influenzae*. *Mol Microbiol,* 6**,** 2107-12.

FORD, C. L., RANDAL-WHITIS, L. & ELLIS, S. R. 1999. Yeast proteins related to the p40/laminin receptor precursor are required for 20S ribosomal RNA processing and the maturation of 40S ribosomal subunits. *Cancer Res,* 59**,** 704-10.

FORSGREN, A., RIESBECK, K. & JANSON, H. 2008. Protein D of *Haemophilus influenzae*: a protective nontypeable *H. influenzae* antigen and a carrier for pneumococcal conjugate vaccines. *Clin Infect Dis,* 46**,** 726-31.

FOWLER, M., THOMAS, R. J., ATHERTON, J., ROBERTS, I. S. & HIGH, N. J. 2006. Galectin-3 binds to *Helicobacter pylori* O-antigen: it is upregulated and rapidly secreted by gastric epithelial cells in response to H-pylori adhesion. *Cellular Microbiology,* 8**,** 44-54.

FOXWELL, A. R., KYD, J. M. & CRIPPS, A. W. 1998. Nontypeable *Haemophilus influenzae*: pathogenesis and prevention. *Microbiol Mol Biol Rev,* 62**,** 294-308.

FROLET, C., BENIAZZA, M., ROUX, L., GALLET, B., NOIRCLERC-SAVOYE, M., VERNET, T. & DI GUILMI, A. M. 2010. New adhesin functions of surface-exposed pneumococcal proteins. *BMC Microbiol,* 10**,** 190.

FRYE, S. A., ASSALKHOU, R., COLLINS, R. F., FORD, R. C., PETERSSON, C., DERRICK, J. P. & TONJUM, T. 2006. Topology of the outer-membrane secretin PilQ from *Neisseria meningitidis*. *Microbiology,* 152**,** 3751-64.

FU, S., ZHANG, M., XU, J., OU, J., WANG, Y., LIU, H., LIU, J., CHEN, H. & BEI, W. 2013. Immunogenicity and protective efficacy of recombinant *Haemophilus parasuis* SH0165 putative outer membrane proteins. *Vaccine,* 31**,** 347-53.

FUJIMURA, Y., SUMIDA, M., SUGIHARA, K., TSUKAMOTO, S., YAMADA, K. & TACHIBANA, H. 2012. Green tea polyphenol EGCG sensing motif on the 67-kDa laminin receptor. *PLoS One,* 7**,** e37942.

FUKUMORI, T., TAKENAKA, Y., YOSHII, T., KIM, H. R., HOGAN, V., INOHARA, H., KAGAWA, S. & RAZ, A. 2003. CD29 and CD7 mediate galectin-3-induced type II T-cell apoptosis. *Cancer Res,* 63**,** 8302-11.

FUKUSHI, J., MAKAGIANSAR, I. T. & STALLCUP, W. B. 2004. NG2 proteoglycan promotes endothelial cell motility and angiogenesis via engagement of galectin-3 and alpha3beta1 integrin. *Mol Biol Cell,* 15**,** 3580-90.

GALDIERO, S., CAPASSO, D., VITIELLO, M., D'ISANTO, M., PEDONE, C. & GALDIERO, M. 2003. Role of surface-exposed loops of *Haemophilus influenzae* protein P2 in the mitogen-activated protein kinase cascade. *Infect Immun,* 71**,** 2798-809.

GALDIERO, S., VITIELLO, M., AMODEO, P., D'ISANTO, M., CANTISANI, M., PEDONE, C. & GALDIERO, M. 2006. Structural requirements for proinflammatory activity of porin P2 Loop 7 from *Haemophilus influenzae*. *Biochemistry,* 45**,** 4491-501.

GARCIA, E. & LOPEZ, R. 1997. Molecular biology of the capsular genes of *Streptococcus pneumoniae*. *FEMS Microbiol Lett,* 149**,** 1-10.

GARCIA, P., GARCIA, J. L., GARCIA, E. & LOPEZ, R. 1986. Nucleotide sequence and expression of the pneumococcal autolysin gene from its own promoter in *Escherichia coli*. *Gene,* 43**,** 265-72.

GARCIA, P., GONZALEZ, M. P., GARCIA, E., LOPEZ, R. & GARCIA, J. L. 1999. LytB, a novel pneumococcal murein hydrolase essential for cell separation. *Mol Microbiol,* 31**,** 1275-81.

GARPENHOLT, O., HUGOSSON, S., FREDLUND, H., GIESECKE, J. & OLCEN, P. 2000. Invasive disease due to *Haemophilus influenzae* type b during the first six years of general vaccination of Swedish children. *Acta Paediatr,* 89**,** 471-4.

GAUCZYNSKI, S., NIKLES, D., EL-GOGO, S., PAPY-GARCIA, D., REY, C., ALBAN, S., BARRITAULT, D., LASMEZAS, C. I. & WEISS, S. 2006. The 37-kDa/67-kDa laminin receptor acts as a receptor for infectious prions and is inhibited by polysulfated glycanes. *Journal of Infectious Diseases,* 194**,** 702-709.

GAUCZYNSKI, S., PEYRIN, J. M., HAIK, S., LEUCHT, C., HUNDT, C., RIEGER, R., KRASEMANN, S., DESLYS, J. P., DORMONT, D., LASMEZAS, C. I. & WEISS, S. 2001. The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. *Embo Journal,* 20**,** 5863-5875.

GEORGIADOU, M., CASTAGNINI, M., KARIMOVA, G., LADANT, D. & PELICIC, V. 2012. Large-scale study of the interactions between proteins involved in type IV pilus biology in *Neisseria meningitidis*: characterization of a subcomplex involved in pilus assembly. *Molecular Microbiology,* 84**,** 857-873.

GHAFFAR, F., BARTON, T., LOZANO, J., MUNIZ, L. S., HICKS, P., GAN, V., AHMAD, N. & MCCRACKEN, G. H., JR. 2004. Effect of the 7-valent pneumococcal conjugate vaccine on nasopharyngeal colonization by *Streptococcus pneumoniae* in the first 2 years of life. *Clin Infect Dis,* 39**,** 930-8.

GHOSH, A., KOLE, L., BANDYOPADHYAY, K., SARKAR, K. & DAS, P. K. 1996. Evidence of a laminin binding protein on the surface of *Leishmania donovani*. *Biochem Biophys Res Commun,* 226**,** 101-6.

GIESE, B., RODERBURG, C., SOMMERAUER, M., WORTMANN, S. B., METZ, S., HEINRICH, P. C. & MULLER-NEWEN, G. 2005. Dimerization of the cytokine receptors gp130 and LIFR analysed in single cells. *Journal of Cell Science,* 118**,** 5129-5140.

GILSDORF, J. R., MCCREA, K. W. & MARRS, C. F. 1997. Role of pili in *Haemophilus influenzae* adherence and colonization. *Infect Immun,* 65**,** 2997-3002.

GINSBERG, L. 2004. Difficult and recurrent meningitis. *J Neurol Neurosurg Psychiatry,* 75 Suppl 1**,** i16-21.

GIUFRE, M., MUSCILLO, M., SPIGAGLIA, P., CARDINES, R., MASTRANTONIO, P. & CERQUETTI, M. 2006. Conservation and diversity of HMW1 and HMW2 adhesin binding domains among invasive nontypeable *Haemophilus influenzae* isolates. *Infect Immun,* 74**,** 1161-70.

GIVANT-HORWITZ, V., DAVIDSON, B. & REICH, R. 2004. Laminin-induced signaling in tumor cells: the role of the M(r) 67,000 laminin receptor. *Cancer Res,* 64**,** 3572-9.

GOLDMAN, L. A., CUTRONE, E. C., KOTENKO, S. V., KRAUSE, C. D. & LANGER, J. A. 1996. Modifications of vectors pEF-BOS, pcDNA1 and pcDNA3 result in improved convenience and expression. *Biotechniques,* 21**,** 1013-5.

GOSINK, K. K., MANN, E. R., GUGLIELMO, C., TUOMANEN, E. I. & MASURE, H. R. 2000. Role of novel choline binding proteins in virulence of *Streptococcus pneumoniae*. *Infect Immun,* 68**,** 5690-5.

GRAF, J., IWAMOTO, Y., SASAKI, M., MARTIN, G. R., KLEINMAN, H. K., ROBEY, F. A. & YAMADA, Y. 1987. Identification of an amino acid sequence in laminin mediating cell attachment, chemotaxis, and receptor binding. *Cell,* 48**,** 989-96.

GRANDO, I. M., MORAES, C., FLANNERY, B., RAMALHO, W. M., HORTA, M. A., PINHO, D. L. & NASCIMENTO, G. L. 2015. Impact of 10-valent pneumococcal conjugate vaccine on pneumococcal meningitis in children up to two years of age in Brazil. *Cad Saude Publica,* 31**,** 276-84.

GRASS, S. & ST GEME, J. W., 3RD 2000. Maturation and secretion of the non-typable *Haemophilus influenzae* HMW1 adhesin: roles of the N-terminal and C-terminal domains. *Mol Microbiol,* 36**,** 55-67.

GRAY, L. D. & FEDORKO, D. P. 1992. Laboratory diagnosis of bacterial meningitis. *Clin Microbiol Rev,* 5**,** 130-45.

GRESCH, O. & ALTROGGE, L. 2012. Transfection of difficult-to-transfect primary mammalian cells. *Methods Mol Biol,* 801**,** 65-74.

GRIMWOOD, K., ANDERSON, P., ANDERSON, V., TAN, L. & NOLAN, T. 2000. Twelve year outcomes following bacterial meningitis: further evidence for persisting effects. *Archives of Disease in Childhood,* 83**,** 111-116.

GUERINA, N. G., LANGERMANN, S., SCHOOLNIK, G. K., KESSLER, T. W. & GOLDMANN, D. A. 1985. Purification and characterization of *Haemophilus influenzae* pili, and their structural and serological relatedness to *Escherichia coli* P and mannose-sensitive pili. *J Exp Med,* 161**,** 145-59.

GUO, N. H., KRUTZSCH, H. C., VOGEL, T. & ROBERTS, D. D. 1992. Interactions of a laminin-binding peptide from a 33-kDa protein related to the 67-kDa laminin receptor with laminin and melanoma cells are heparin-dependent. *J Biol Chem,* 267**,** 17743-7.

GUPTA, S. K., MASINICK, S., GARRETT, M. & HAZLETT, L. D. 1997. Pseudomonas aeruginosa lipopolysaccharide binds galectin-3 and other human corneal epithelial proteins. *Infect Immun,* 65**,** 2747-53.

HAGHI, F., PEERAYEH, S. N., SIADAT, S. D. & ZEIGHAMI, H. 2012. Recombinant outer membrane secretin PilQ(406-770) as a vaccine candidate for serogroup B *Neisseria meningitidis*. *Vaccine,* 30**,** 1710-1714.

HAKANSSON, A., ROCHE, H., MIRZA, S., MCDANIEL, L. S., BROOKS-WALTER, A. & BRILES, D. E. 2001. Characterization of binding of human lactoferrin to pneumococcal surface protein A. *Infection and Immunity,* 69**,** 3372-3381.

HALLSTROM, T. & RIESBECK, K. 2010. *Haemophilus influenzae* and the complement system. *Trends Microbiol,* 18**,** 258-65.

HAMMERSCHMIDT, S., TALAY, S. R., BRANDTZAEG, P. & CHHATWAL, G. S. 1997. SpsA, a novel pneumococcal surface protein with specific binding to secretory Immunoglobulin A and secretory component. *Molecular Microbiology,* 25**,** 1113-1124.

HANKINS, W. A., GWALTNEY, J. M., JR., HENDLEY, J. O., FARQUHAR, J. D. & SAMUELSON, J. S. 1982. Clinical and serological evaluation of a meningococcal polysaccharide vaccine groups A, C, Y, and W135. *Proc Soc Exp Biol Med,* 169**,** 54-7.

HANSEN, E. J., HART, D. A., MCGEHEE, J. L. & TOEWS, G. B. 1988. Immune enhancement of pulmonary clearance of nontypable *Haemophilus influenzae*. *Infect Immun,* 56**,** 182-90.

HARRISON, L. H. 2006. Prospects for vaccine prevention of meningococcal infection. *Clin Microbiol Rev,* 19**,** 142-64.

HARVEY, D., HOLT, D. E. & BEDFORD, H. 1999. Bacterial meningitis in the newborn: a prospective study of mortality and morbidity. *Semin Perinatol,* 23**,** 218-25.

HARVEY, R. M., HUGHES, C. E., PATON, A. W., TRAPPETTI, C., TWETEN, R. K. & PATON, J. C. 2014. The impact of pneumolysin on the macrophage response to *Streptococcus pneumoniae* is strain-dependent. *PLoS One,* 9**,** e103625.

HAUCK, C. R. & MEYER, T. F. 2003. 'Small' talk: Opa proteins as mediators of Neisseria-host-cell communication. *Curr Opin Microbiol,* 6**,** 43-9.

HAWKINS, B. T. & DAVIS, T. P. 2005. The blood-brain barrier/neurovascular unit in health and disease. *Pharmacol Rev,* 57**,** 173-85.

HELAINE, S., DYER, D. H., NASSIF, X., PELICIC, V. & FOREST, K. T. 2007. 3D structure/function analysis of PilX reveals how minor pilins can modulate the virulence properties of type IV pili. *Proc Natl Acad Sci U S A,* 104**,** 15888-93.

HENDRIXSON, D. R. & ST GEME, J. W., 3RD 1998. The *Haemophilus influenzae* Hap serine protease promotes adherence and microcolony formation, potentiated by a soluble host protein. *Mol Cell,* 2**,** 841-50.

HENRICHSEN, J. 1995. Six newly recognized types of *Streptococcus pneumoniae*. *J Clin Microbiol,* 33**,** 2759-62.

HERAS, B., EDELING, M. A., SCHIRRA, H. J., RAINA, S. & MARTIN, J. L. 2004. Crystal structures of the DsbG disulfide isomerase reveal an unstable disulfide. *Proc Natl Acad Sci U S A,* 101**,** 8876-81.

HERVA, E., LUOTONEN, J., TIMONEN, M., SIBAKOV, M., KARMA, P. & MAKELA, P. H. 1980. The effect of polyvalent pneumococcal polysaccharide vaccine on nasopharyngeal and nasal carriage of *Streptococcus pneumoniae*. *Scand J Infect Dis,* 12**,** 97-100.

HILDEBRAND, J. & AOUN, M. 2003. Chronic meningitis: still a diagnostic challenge. *J Neurol,* 250**,** 653-60.

HILL, D. J., GRIFFITHS, N. J., BORODINA, E. & VIRJI, M. 2010. Cellular and molecular biology of *Neisseria meningitidis* colonization and invasive disease. *Clin Sci (Lond),* 118**,** 547-64.

HILL, D. J., TOLEMAN, M. A., EVANS, D. J., VILLULLAS, S., VAN ALPHEN, L. & VIRJI, M. 2001. The variable P5 proteins of typeable and non-typeable *Haemophilus influenzae* target human CEACAM1. *Mol Microbiol,* 39**,** 850-62.

HILLERINGMANN, M., RINGLER, P., MULLER, S. A., DE ANGELIS, G., RAPPUOLI, R., FERLENGHI, I. & ENGEL, A. 2009. Molecular architecture of *Streptococcus pneumoniae* TIGR4 pili. *EMBO J,* 28**,** 3921-30.

HILTKE, T. J., SCHIFFMACHER, A. T., DAGONESE, A. J., SETHI, S. & MURPHY, T. F. 2003. Horizontal transfer of the gene encoding outer membrane protein P2 of nontypeable *Haemophilus influenzae*, in a patient with chronic obstructive pulmonary disease. *J Infect Dis,* 188**,** 114-7.

HILTKE, T. J., SETHI, S. & MURPHY, T. F. 2002. Sequence stability of the gene encoding outer membrane protein P2 of nontypeable *Haemophilus influenzae* in the human respiratory tract. *J Infect Dis,* 185**,** 627-31.

HIRABAYASHI, J. & KASAI, K. 1993. The family of metazoan metal-independent beta-galactoside-binding lectins: structure, function and molecular evolution. *Glycobiology,* 3**,** 297-304.

HIROSE, T. E., MALUF, E. M. & RODRIGUES, C. O. 2015. Pneumococcal meningitis: epidemiological profile pre- and post-introduction of the pneumococcal 10-valent conjugate vaccine. *J Pediatr (Rio J),* 91**,** 130-5.

HO, M. K. & SPRINGER, T. A. 1982. Mac-2, a novel 32,000 Mr mouse macrophage subpopulation-specific antigen defined by monoclonal antibodies. *J Immunol,* 128**,** 1221-8.

HODGSON, A., SMITH, T., GAGNEUX, S., AKUMAH, I., ADJUIK, M., PLUSCHKE, G., BINKA, F. & GENTON, B. 2001. Survival and sequelae of meningococcal meningitis in Ghana. *Int J Epidemiol,* 30**,** 1440-6.

HOLLENDER, C. A. & LIU, Z. 2010. Bimolecular fluorescence complementation (BiFC) assay for protein-protein interaction in onion cells using the helios gene gun. *J Vis Exp.*

HOLLINGSHEAD, S. K., BECKER, R. & BRILES, D. E. 2000. Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. *Infect Immun,* 68**,** 5889-900.

HOLST, J. 2007. Strategies for development of universal vaccines against meningococcal serogroup B disease: the most promising options and the challenges evaluating them. *Hum Vaccin,* 3**,** 290-4.

HONG, W., PENG, D., RIVERA, M. & GU, X. X. 2010. Protection against nontypeable *Haemophilus influenzae* challenges by mucosal vaccination with a detoxified lipooligosaccharide conjugate in two chinchilla models. *Microbes Infect,* 12**,** 11-8.

HOTOMI, M., IKEDA, Y., SUZUMOTO, M., YAMAUCHI, K., GREEN, B. A., ZLOTNICK, G., BILLAL, D. S., SHIMADA, J., FUJIHARA, K. & YAMANAKA, N. 2005. A recombinant P4 protein of *Haemophilus influenzae* induces specific immune responses biologically active against nasopharyngeal colonization in mice after intranasal immunization. *Vaccine,* 23**,** 1294-300.

HOUZELSTEIN, D., GONCALVES, I. R., FADDEN, A. J., SIDHU, S. S., COOPER, D. N., DRICKAMER, K., LEFFLER, H. & POIRIER, F. 2004. Phylogenetic analysis of the vertebrate galectin family. *Mol Biol Evol,* 21**,** 1177-87.

HOWARD, A. J., DUNKIN, K. T. & MILLAR, G. W. 1988. Nasopharyngeal carriage and antibiotic resistance of *Haemophilus influenzae* in healthy children. *Epidemiol Infect,* 100**,** 193-203.

HU, C. D., CHINENOV, Y. & KERPPOLA, T. K. 2002. Visualization of interactions among bZip and Rel family proteins in living cells using bimolecular fluorescence complementation. *Molecular Cell,* 9**,** 789-798.

HUANG, S. H. & JONG, A. 2009. Evolving role of laminin receptors in microbial pathogenesis and therapeutics of CNS infection. *Future Microbiol,* 4**,** 959-62.

HUANG, S. H. & JONG, A. Y. 2001. Cellular mechanisms of microbial proteins contributing to invasion of the blood-brain barrier. *Cell Microbiol,* 3**,** 277-87.

HUANG, S. H., STINS, M. F. & KIM, K. S. 2000. Bacterial penetration across the blood-brain barrier during the development of neonatal meningitis. *Microbes Infect,* 2**,** 1237-44.

HUGHES, R. C. 1999. Secretion of the galectin family of mammalian carbohydrate-binding proteins. *Biochim Biophys Acta,* 1473**,** 172-85.

HUNDT, C., PEYRIN, J. M., HAIK, S., GAUCZYNSKI, S., LEUCHT, C., RIEGER, R., RILEY, M. L., DESLYS, J. P., DORMONT, D., LASMEZAS, C. I. & WEISS, S. 2001. Identification of interaction domains of the prion protein with its 37-kDa/67-kDa laminin receptor. *EMBO J,* 20**,** 5876-86.

HUNG, M. C. & CHRISTODOULIDES, M. 2013. The biology of Neisseria adhesins. *Biology (Basel),* 2**,** 1054-109.

HYAMS, C., CAMBERLEIN, E., COHEN, J. M., BAX, K. & BROWN, J. S. 2010. The *Streptococcus pneumoniae* capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. *Infect Immun,* 78**,** 704-15.

IANNELLI, F., OGGIONI, M. R. & POZZI, G. 2002. Allelic variation in the highly polymorphic locus pspC of *Streptococcus pneumoniae*. *Gene,* 284**,** 63-71.

ISAACS, J. D., JACKSON, G. S. & ALTMANN, D. M. 2006. The role of the cellular prion protein in the immune system. *Clin Exp Immunol,* 146**,** 1-8.

JACKERS, P., MINOLETTI, F., BELOTTI, D., CLAUSSE, N., SOZZI, G., SOBEL, M. E. & CASTRONOVO, V. 1996. Isolation from a multigene family of the active human gene of the metastasis-associated multifunctional protein 37LRP/p40 at chromosome 3p21.3. *Oncogene,* 13**,** 495-503.

JAGER, V., BUSSOW, K., WAGNER, A., WEBER, S., HUST, M., FRENZEL, A. & SCHIRRMANN, T. 2013. High level transient production of recombinant antibodies and antibody fusion proteins in HEK293 cells. *BMC Biotechnol,* 13**,** 52.

JAIN, S., MOSCICKA, K. B., BOS, M. P., PACHULEC, E., STUART, M. C., KEEGSTRA, W., BOEKEMA, E. J. & VAN DER DOES, C. 2011. Structural characterization of outer membrane components of the type IV pili system in pathogenic Neisseria. *PLoS One,* 6**,** e16624.

JALALVAND, F., LITTORIN, N., SU, Y. C. & RIESBECK, K. 2014. Impact of immunization with Protein F on pulmonary clearance of nontypeable *Haemophilus influenzae*. *Vaccine,* 32**,** 2261-4.

JAMIESON, K. V., HUBBARD, S. R. & MERUELO, D. 2011. Structure-guided identification of a laminin binding site on the laminin receptor precursor. *J Mol Biol,* 405**,** 24-32.

JAMIESON, K. V., WU, J., HUBBARD, S. R. & MERUELO, D. 2008. Crystal structure of the human laminin receptor precursor. *J Biol Chem,* 283**,** 3002-5.

JANSON, H., RUAN, M. & FORSGREN, A. 1993. Limited diversity of the protein D gene (hpd) among encapsulated and nonencapsulated *Haemophilus influenzae* strains. *Infect Immun,* 61**,** 4546-52.

JANULCZYK, R., IANNELLI, F., SJOHOLM, A. G., POZZI, G. & BJORCK, L. 2000. Hic, a novel surface protein of *Streptococcus pneumoniae* that interferes with complement function. *Journal of Biological Chemistry,* 275**,** 37257-37263.

JEDRZEJAS, M. J. 2001. Pneumococcal virulence factors: structure and function. *Microbiol Mol Biol Rev,* 65**,** 187-207 ; first page, table of contents.

JESSOUROUN, E., DA SILVEIRA, I. F., LARANGEIRA, A. P., PEREIRA, S., FERNANDES, S. A., RABINOVITCH, L., FRASCH, C. E., CASTRO-FARIA-NETO, H. C. & BOZZA, P. T. 2004. Outer membrane vesicles (OMVs) and detoxified lipooligosaccharide (dLOS) obtained from Brazilian prevalent *N. meningitidis* serogroup B strains protect mice against homologous and heterologous meningococcal infection and septic shock. *Vaccine,* 22**,** 2617-25.

JODAR, L., FEAVERS, I. M., SALISBURY, D. & GRANOFF, D. M. 2002. Development of vaccines against meningococcal disease. *Lancet,* 359**,** 1499-508

.
JOHN, C. M., JARVIS, G. A., SWANSON, K. V., LEFFLER, H., COOPER, M. D., HUFLEJT, M. E. & GRIFFISS, J. M. 2002. Galectin-3 binds lactosaminylated lipooligosaccharides from *Neisseria gonorrhoeae* and is selectively expressed by mucosal epithelial cells that are infected. *Cellular Microbiology,* 4**,** 649-661.

JOHNSTON, R. B., JR. 1991. Pathogenesis of *pneumococcal pneumonia*. *Rev Infect Dis,* 13 Suppl 6**,** S509-17.

JONSON, A. B., NORMARK, S. & RHEN, M. 2005. Fimbriae, pili, flagella and bacterial virulence. *Contrib Microbiol,* 12**,** 67-89.

JONSSON, A. B., NYBERG, G. & NORMARK, S. 1991. Phase variation of gonococcal pili by frameshift mutation in pilC, a novel gene for pilus assembly. *EMBO J,* 10**,** 477-88.

JUGANARIU, G., MIFTODE, E., TEODOR, D., LECA, D. & DOROBAT, C. M. 2012. Clinical features and course of bacterial meningitis in children. *Rev Med Chir Soc Med Nat Iasi,* 116**,** 722-6.

KADIOGLU, A., TAYLOR, S., IANNELLI, F., POZZI, G., MITCHELL, T. J. & ANDREW, P. W. 2002. Upper and lower respiratory tract infection by *Streptococcus pneumoniae* is affected by pneumolysin deficiency and differences in capsule type. *Infect Immun,* 70**,** 2886-90.

KADIOGLU, A., WEISER, J. N., PATON, J. C. & ANDREW, P. W. 2008. The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. *Nat Rev Microbiol,* 6**,** 288-301.

KAHLER, C. M. & STEPHENS, D. S. 1998. Genetic basis for biosynthesis, structure, and function of meningococcal lipooligosaccharide (endotoxin). *Crit Rev Microbiol,* 24**,** 281-334.

KALLSTROM, H., LISZEWSKI, M. K., ATKINSON, J. P. & JONSSON, A. B. 1997. Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic Neisseria. *Molecular Microbiology,* 25**,** 639-647.

KANEDA, Y., KINOSHITA, K., SATO, M., SAEKI, Y., YAMADA, R., WATAYA-KANEDA, M. & TANAKA, K. 1998. The induction of apoptosis in HeLa cells by the loss of LBP-p40. *Cell Death Differ,* 5**,** 20-8.

KANEDA, Y., KINOSHITA, K., SATO, M., TANAKA, K. & KANEDA, Y. 1993. The analysis of 40 kDa nuclear protein, p40, in interphase cells and mitotic cells. *J Cell Sci,* 106 (Pt 3)**,** 741-8.

KAPLAN, S. L., MASON, E. O., JR., WALD, E. R., SCHUTZE, G. E., BRADLEY, J. S., TAN, T. Q., HOFFMAN, J. A., GIVNER, L. B., YOGEV, R. & BARSON, W. J. 2004. Decrease of invasive pneumococcal infections in children among 8 children's hospitals in the United States after the introduction of the 7-valent pneumococcal conjugate vaccine. *Pediatrics,* 113**,** 443-9.

KAR, G., GURSOY, A. & KESKIN, O. 2009. Human cancer protein-protein interaction network: a structural perspective. *PLoS Comput Biol,* 5**,** e1000601.

KAVANAUGH, D., KANE, M., JOSHI, L. & HICKEY, R. M. 2013. Detection of galectin-3 interaction with commensal bacteria. *Appl Environ Microbiol,* 79**,** 3507-10.

KAZMIN, D. A., CHINENOV, Y., LARSON, E. & STARKEY, J. R. 2003. Comparative modeling of the N-terminal domain of the 67 kDa laminin-binding protein: implications for putative ribosomal function. *Biochemical and Biophysical Research Communications,* 300**,** 161-166.

KAZMIN, D. A., HOYT, T. R., TAUBNER, L., TEINTZE, M. & STARKEY, J. R. 2000. Phage display mapping for peptide 11 sensitive sequences binding to laminin-1. *J Mol Biol,* 298**,** 431-45.

KERPPOLA, T. K. 2006. Visualization of molecular interactions by fluorescence complementation. *Nature Reviews Molecular Cell Biology,* 7**,** 449-456.

KERPPOLA, T. K. 2008. Bimolecular fluorescence complementation: visualization of molecular interactions in living cells. *Methods Cell Biol,* 85**,** 431-70.

KERPPOLA, T. K. 2009. Visualization of molecular interactions using bimolecular fluorescence complementation analysis: Characteristics of protein fragment complementation. *Chemical Society Reviews,* 38**,** 2876-2886.

KERPPOLA, T. K. 2013a. Multicolor bimolecular fluorescence complementation (BiFC) analysis of protein interactions with alternative partners. *Cold Spring Harb Protoc,* 2013**,** 798-803.

KERPPOLA, T. K. 2013b. Simultaneous visualization of multiple protein interactions using multicolor bimolecular fluorescence complementation (BiFC) analysis. *Cold Spring Harb Protoc,* 2013**,** 892-5.

KEYSERLING, H. L., POLLARD, A. J., DETORA, L. M. & GILMET, G. P. 2006. Experience with MCV-4, a meningococcal, diphtheria toxoid conjugate vaccine against serogroups A, C, Y and W-135. *Expert Rev Vaccines,* 5**,** 445-59.

KHAN, N. A., KIM, Y., SHIN, S. & KIM, K. S. 2007. FimH-mediated *Escherichia coli* K1 invasion of human brain microvascular endothelial cells. *Cell Microbiol,* 9**,** 169-78.

KHAN, N. A., SHIN, S., CHUNG, J. W., KIM, K. J., ELLIOTT, S., WANG, Y. & KIM, K. S. 2003. Outer membrane protein A and cytotoxic necrotizing factor-1 use diverse signaling mechanisms for *Escherichia coli* K1 invasion of human brain microvascular endothelial cells. *Microbial Pathogenesis,* 35**,** 35-42.

KHAN, N. A., WANG, Y., KIM, K. J., CHUNG, J. W., WASS, C. A. & KIM, K. S. 2002.

Cytotoxic necrotizing factor-1 contributes to *Escherichia coli* K1 invasion of the central nervous system. *J Biol Chem,* 277**,** 15607-12.

KHOW, O. & SUNTRARACHUN, S. 2012. Strategies for production of active eukaryotic proteins in bacterial expression system. *Asian Pac J Trop Biomed,* 2**,** 159-62.

KIELIAN, M., CHANEL-VOS, C. & LIAO, M. F. 2010. Alphavirus Entry and Membrane Fusion. *Viruses-Basel,* 2**,** 796-825.

KILIAN, M., REINHOLDT, J., LOMHOLT, H., POULSEN, K. & FRANDSEN, E. V. 1996. Biological significance of IgA1 proteases in bacterial colonization and pathogenesis: critical evaluation of experimental evidence. *APMIS,* 104**,** 321-38.

KILPATRICK, L. E., BRIDDON, S. J., HILL, S. J. & HOLLIDAY, N. D. 2010. Quantitative analysis of neuropeptide Y receptor association with beta-arrestin2 measured by bimolecular fluorescence complementation. *Br J Pharmacol,* 160**,** 892-906.

KIM, K. J., CHUNG, J. W. & KIM, K. S. 2005. 67-kDa laminin receptor promotes internalization of cytotoxic necrotizing factor 1-expressing *Escherichia coli* K1 into human brain microvascular endothelial cells. *Journal of Biological Chemistry,* 280**,** 1360-1368.

KIM, K. S. 2003. Pathogenesis of bacterial meningitis: from bacteraemia to neuronal injury. *Nat Rev Neurosci,* 4**,** 376-85.

KIM, K. S. 2006. Microbial translocation of the blood-brain barrier. *Int J Parasitol,* 36**,** 607-14.

KIM, K. S. 2008. Mechanisms of microbial traversal of the blood-brain barrier. *Nat Rev Microbiol,* 6**,** 625-34.

KING, P. 2012. *Haemophilus influenzae* and the lung (Haemophilus and the lung). *Clin Transl Med,* 1**,** 10.

KING, P. T. & SHARMA, R. 2015. The Lung Immune Response to Nontypeable *Haemophilus influenzae* (Lung Immunity to NTHi). *J Immunol Res,* 2015**,** 706376.

KINOSHITA, K., KANEDA, Y., SATO, M., SAEKI, Y., WATAYA-KANEDA, M., HOFFMANN, A. & KANEDA, Y. 1998. LBP-p40 binds DNA tightly through associations with histones H2A, H2B, and H4. *Biochemical and Biophysical Research Communications,* 253**,** 277-282.

KIRCHNER, M., HEUER, D. & MEYER, T. F. 2005. CD46-independent binding of neisserial type IV pili and the major pilus adhesin, PilC, to human epithelial cells. *Infection and Immunity,* 73**,** 3072-3082.

KIRCHNER, M. & MEYER, T. F. 2005. The PilC adhesin of the Neisseria type IV pilus-binding specificities and new insights into the nature of the host cell receptor. *Mol Microbiol,* 56**,** 945-57.

KLESHCHENKO, Y. Y., MOODY, T. N., FURTAK, V. A., OCHIENG, J., LIMA, M. F. & VILLALTA, F. 2004. Human galectin-3 promotes *Trypanosoma cruzi* adhesion to human coronary artery smooth muscle cells. *Infect Immun,* 72**,** 6717-21.

KLUGMAN, K. P., MADHI, S. A., HUEBNER, R. E., KOHBERGER, R., MBELLE, N., PIERCE, N. & VACCINE TRIALISTS, G. 2003. A trial of a 9-valent pneumococcal conjugate vaccine in children with and those without HIV infection. *N Engl J Med,* 349**,** 1341-8.

KODAMA, Y. & HU, C. D. 2010. An improved bimolecular fluorescence complementation assay with a high signal-to-noise ratio. *Biotechniques,* 49**,** 793-805.

KOHATSU, L., HSU, D. K., JEGALIAN, A. G., LIU, F. T. & BAUM, L. G. 2006. Galectin-3 induces death of Candida species expressing specific beta-1,2-linked mannans. *J Immunol,* 177**,** 4718-26.

KOTILAINEN, P., JALAVA, J., MEURMAN, O., LEHTONEN, O. P., RINTALA, E., SEPPALA, O. P., EEROLA, E. & NIKKARI, S. 1998. Diagnosis of meningococcal meningitis by broad-range bacterial PCR with cerebrospinal fluid. *J Clin Microbiol,* 36**,** 2205-9.

KRETZSCHMAR, H. A., PRUSINER, S. B., STOWRING, L. E. & DEARMOND, S. J. 1986. Scrapie Prion Proteins Are Synthesized in Neurons. *American Journal of Pathology,* 122**,** 1-5.

KRISTIANSEN, P. A., DIOMANDE, F., WEI, S. C., OUEDRAOGO, R., SANGARE, L., SANOU, I., KANDOLO, D., KABORE, P., CLARK, T. A., OUEDRAOGO, A. S., ABSATOU, K. B., OUEDRAOGO, C. D., HASSAN-KING, M., THOMAS, J. D., HATCHER, C., DJINGAREY, M., MESSONNIER, N., PREZIOSI, M. P., LAFORCE, M. & CAUGANT, D. A. 2011. Baseline meningococcal carriage in Burkina Faso before the introduction of a meningococcal serogroup A conjugate vaccine. *Clin Vaccine Immunol,* 18**,** 435-43.

KRZESLAK, A. & LIPPINSKA, A. 2004. Galectin-3 as a multifunctional protein. *Cellular & Molecular Biology Letters,* 9**,** 305-328.

KUBIET, M., RAMPHAL, R., WEBER, A. & SMITH, A. 2000. Pilus-mediated adherence of *Haemophilus influenzae* to human respiratory mucins. *Infect Immun,* 68**,** 3362-7.

KUKLINSKA, D. & KILIAN, M. 1984. Relative proportions of Haemophilus species in the throat of healthy children and adults. *Eur J Clin Microbiol,* 3**,** 249-52.

LAMBERTZ, C., GARVEY, M., KLINGER, J., HEESEL, D., KLOSE, H., FISCHER, R. & COMMANDEUR, U. 2014. Challenges and advances in the heterologous expression of cellulolytic enzymes: a review. *Biotechnol Biofuels,* 7**,** 135.

LANDOWSKI, T. H., DRATZ, E. A. & STARKEY, J. R. 1995a. Studies of the structure of the metastasis-associated 67 kDa laminin binding protein: fatty acid acylation and evidence supporting dimerization of the 32 kDa gene product to form the mature protein. *Biochemistry,* 34**,** 11276-87.

LANDOWSKI, T. H., UTHAYAKUMAR, S. & STARKEY, J. R. 1995b. Control pathways of the 67 kDa laminin binding protein: surface expression and activity of a new ligand binding domain. *Clin Exp Metastasis,* 13**,** 357-72.

LAPPANN, M., HAAGENSEN, J. A., CLAUS, H., VOGEL, U. & MOLIN, S. 2006. Meningococcal biofilm formation: structure, development and phenotypes in a standardized continuous flow system. *Mol Microbiol,* 62**,** 1292-309.

LEE, M. Y., PARK, E. G., CHOI, J. Y., CHEONG, H. S., CHUNG, D. R., PECK, K. R., SONG, J. H. & KO, K. S. 2010. 'Neisseria skkuensis' sp. nov., isolated from the blood of a diabetic patient with a foot ulcer. *J Med Microbiol,* 59**,** 856-9.

LEOWSKI, J. 1986. Mortality from acute respiratory infections in children under 5 years of age: global estimates. *World Health Stat Q,* 39**,** 138-44.

LEPOW, M. L., BEELER, J., RANDOLPH, M., SAMUELSON, J. S. & HANKINS, W. A. 1986. Reactogenicity and immunogenicity of a quadrivalent combined meningococcal polysaccharide vaccine in children. *J Infect Dis,* 154**,** 1033-6.

LESOT, H., KUHL, U. & MARK, K. 1983. Isolation of a laminin-binding protein from muscle cell membranes. *EMBO J,* 2**,** 861-5.

LEUCHT, C., VANA, K., RENNER-MULLER, I., DORMONT, D., LASMEZAS, C. I., WOLF, E. & WEISS, S. 2004. Knock-down of the 37-kDa/67-kDa laminin receptor in mouse brain by transgenic expression of specific antisense LRP RNA. *Transgenic Research,* 13**,** 81-85.

LEWIS, K. M., TURNER, R. J. & VINK, R. 2013. Blocking neurogenic inflammation for the treatment of acute disorders of the central nervous system. *Int J Inflam,* 2013**,** 578480.

LIEBMAN, J. M., BURBELO, P. D., YAMADA, Y., FRIDMAN, R. & KLEINMAN, H. K. 1993. Altered expression of basement-membrane components and collagenases in ascitic xenografts of OVCAR-3 ovarian cancer cells. *Int J Cancer,* 55**,** 102-9.

LIPPINCOTT-SCHWARTZ, J., ROBERTS, T. H. & HIRSCHBERG, K. 2000. Secretory protein trafficking and organelle dynamics in living cells. *Annual Review of Cell and Developmental Biology,* 16**,** 557-589.

LIU, F. T. & ORIDA, N. 1984. Synthesis of surface immunoglobulin E receptor in Xenopus oocytes by translation of mRNA from rat basophilic leukemia cells. *J Biol Chem,* 259**,** 10649-52.

LIU, T. Y., GOTSCHLICH, E. C., DUNNE, F. T. & JONSSEN, E. K. 1971. Studies on the meningococcal polysaccharides. II. Composition and chemical properties of the group B and group C polysaccharide. *J Biol Chem,* 246**,** 4703-12.

LO PASSO, C., ROMEO, A., PERNICE, I., DONATO, P., MIDIRI, A., MANCUSO, G., ARIGO, M., BIONDO, C., GALBO, R., PAPASERGI, S., FELICI, F., TETI, G. & BENINATI, C. 2007. Peptide mimics of the group B meningococcal capsule induce bactericidal and protective antibodies after immunization. *J Immunol,* 178**,** 4417-23.

LU, L., LAMM, M. E., LI, H. M., CORTHESY, B. & ZHANG, J. R. 2003. The human polymeric immunoglobulin receptor binds to *Streptococcus pneumoniae* via domains 3 and 4. *Journal of Biological Chemistry,* 278**,** 48178-48187.

LUDEWIGS, H., ZUBER, C., VANA, K., NIKLES, D., ZERR, I. & WEISS, S. 2007. Therapeutic approaches for prion disorders. *Expert Review of Anti-Infective Therapy,* 5**,** 613-630.

LUDWIG, G. V., KONDIG, J. P. & SMITH, J. F. 1996. A putative receptor for venezuelan equine encephalitis virus from mosquito cells. *Journal of Virology,* 70**,** 5592-5599.

LUIJKX, T. A., VAN GAANS-VAN DEN BRINK, J. A., VAN DIJKEN, H. H., VAN DEN DOBBELSTEEN, G. P. & VAN ELS, C. A. 2008. Hyperproliferation of B cells specific for a weakly immunogenic PorA in a meningococcal vaccine model. *Clin Vaccine Immunol,* 15**,** 1598-605.

LUKSIC, I., MULIC, R., FALCONER, R., ORBAN, M., SIDHU, S. & RUDAN, I. 2013. Estimating global and regional morbidity from acute bacterial meningitis in children: assessment of the evidence. *Croat Med J,* 54**,** 510-8.

LUO, R. S., MANN, B., LEWIS, W. S., ROWE, A., HEATH, R., STEWART, M. L., HAMBURGER, A. E., SIVAKOLUNDU, S., LACY, E. R., BJORKMAN, P. J., TUOMANEN, E. & KRIWACKI, R. W. 2005. Solution structure of choline binding protein A, the major adhesin of *Streptococcus pneumoniae*. *Embo Journal,* 24**,** 34-43.

MACE, S. E. 2008. Acute bacterial meningitis. *Emerg Med Clin North Am,* 26**,** 281-317, viii.

MAGNIFICO, A., TAGLIABUE, E., BUTO, S., ARDINI, E., CASTRONOVO, V., COLNAGHI, M. I. & MENARD, S. 1996. Peptide G, containing the binding site of the 67-kDa laminin receptor, increases and stabilizes laminin binding to cancer cells. *J Biol Chem,* 271**,** 31179-84.

MAIDEN, M. C., IBARZ-PAVON, A. B., URWIN, R., GRAY, S. J., ANDREWS, N. J., CLARKE, S. C., WALKER, A. M., EVANS, M. R., KROLL, J. S., NEAL, K. R., ALA'ALDEEN, D. A., CROOK, D. W., CANN, K., HARRISON, S., CUNNINGHAM, R., BAXTER, D., KACZMARSKI, E., MACLENNAN, J., CAMERON, J. C. & STUART, J. M. 2008. Impact of meningococcal serogroup C conjugate vaccines on carriage and herd immunity. *J Infect Dis,* 197**,** 737-43.

MAIER, B., KOOMEY, M. & SHEETZ, M. P. 2004. A force-dependent switch reverses type IV pilus retraction. *Proc Natl Acad Sci U S A,* 101**,** 10961-6.

MAIREY, E., GENOVESIO, A., DONNADIEU, E., BERNARD, C., JAUBERT, F., PINARD, E., SEYLAZ, J., OLIVO-MARIN, J. C., NASSIF, X. & DUMENIL, G. 2006. Cerebral microcirculation shear stress levels determine *Neisseria meningitidis* attachment sites along the blood-brain barrier. *J Exp Med,* 203**,** 1939-50.

MALINOFF, H. L. & WICHA, M. S. 1983. Isolation of a cell surface receptor protein for laminin from murine fibrosarcoma cells. *J Cell Biol,* 96**,** 1475-9.

MALORNY, B., MORELLI, G., KUSECEK, B., KOLBERG, J. & ACHTMAN, M. 1998. Sequence diversity, predicted two-dimensional protein structure, and epitope mapping of neisserial Opa proteins. *J Bacteriol,* 180**,** 1323-30.

MALYGIN, A. A., BONDARENKO, E. I., IVANISENKO, V. A., PROTOPOPOVA, E. V., KARPOVA, G. G. & LOKTEV, V. B. 2009. C-terminal fragment of human laminin-binding protein contains a receptor domain for venezuelan equine encephalitis and tick-borne encephalitis viruses. *Biochemistry (Mosc),* 74**,** 1328-36.

MANCHANDA, V., GUPTA, S. & BHALLA, P. 2006. Meningococcal disease: history, epidemiology, pathogenesis, clinical manifestations, diagnosis, antimicrobial susceptibility and prevention. *Indian J Med Microbiol,* 24**,** 7-19.

MANDRELL, R. E., KIM, J. J., JOHN, C. M., GIBSON, B. W., SUGAI, J. V., APICELLA, M. A., GRIFFISS, J. M. & YAMASAKI, R. 1991. Endogenous sialylation of the lipooligosaccharides of *Neisseria meningitidis*. *J Bacteriol,* 173**,** 2823-32.

MANDRELL, R. E. & ZOLLINGER, W. D. 1977. Lipopolysaccharide serotyping of *Neisseria meningitidis* by hemagglutination inhibition. *Infect Immun,* 16**,** 471-5.

MARC, M. M., KOROSEC, P., KOSNIK, M., KERN, I., FLEZAR, M., SUSKOVIC, S. & SORLI, J. 2004. Complement factors c3a, c4a, and c5a in chronic obstructive pulmonary disease and asthma. *Am J Respir Cell Mol Biol,* 31**,** 216-9.

MARKOWSKA, A. I., JEFFERIES, K. C. & PANJWANI, N. 2011. Galectin-3 protein modulates cell surface expression and activation of vascular endothelial growth factor receptor 2 in human endothelial cells. *J Biol Chem,* 286**,** 29913-21.

MARRS, C. F., KRASAN, G. P., MCCREA, K. W., CLEMANS, D. L. & GILSDORF, J. R. 2001. *Haemophilus influenzae* - human specific bacteria. *Front Biosci,* 6**,** E41-60.

MARTIGNONE, S., MENARD, S., BUFALINO, R., CASCINELLI, N., PELLEGRINI, R., TAGLIABUE, E., ANDREOLA, S., RILKE, F. & COLNAGHI, M. I. 1993. Prognostic significance of the 67-kilodalton laminin receptor expression in human breast carcinomas. *J Natl Cancer Inst,* 85**,** 398-402.

MARTIN, P. R., HOBBS, M., FREE, P. D., JESKE, Y. & MATTICK, J. S. 1993. Characterization of pilQ, a new gene required for the biogenesis of type 4 fimbriae in Pseudomonas aeruginosa. *Mol Microbiol,* 9**,** 857-68.

MARTNER, A., SKOVBJERG, S., PATON, J. C. & WOLD, A. E. 2009. *Streptococcus pneumoniae* autolysis prevents phagocytosis and production of phagocyte-activating cytokines. *Infect Immun,* 77**,** 3826-37.

MARUVADA, R., BLOM, A. M. & PRASADARAO, N. V. 2008. Effects of complement regulators bound to *Escherichia coli* K1 and Group B Streptococcus on the interaction with host cells. *Immunology,* 124**,** 265-76.

MATTICK, J. S. 2002. Type IV pili and twitching motility. *Annu Rev Microbiol,* 56**,** 289-314.

MCCLURE, C. A., FORD, M. W., WILSON, J. B. & ARAMINI, J. J. 2006. Pneumococcal conjugate vaccination in Canadian infants and children younger than five years of age: Recommendations and expected benefits. *Can J Infect Dis Med Microbiol,* 17**,** 19-26.

MCDANIEL, L. S., MCDANIEL, D. O., HOLLINGSHEAD, S. K. & BRILES, D. E. 1998. Comparison of the PspA sequence from *Streptococcus pneumoniae* EF5668 to the previously identified PspA sequence from strain Rx1 and ability of PspA from EF5668 to elicit protection against pneumococci of different capsular types. *Infection and Immunity,* 66**,** 4748-4754.

MCDANIEL, L. S., YOTHER, J., VIJAYAKUMAR, M., MCGARRY, L., GUILD, W. R. & BRILES, D. E. 1987. Use of Insertional Inactivation to Facilitate Studies of Biological Properties of Pneumococcal Surface Protein-a (Pspa). *Journal of Experimental Medicine,* 165**,** 381-394.

MCNEIL, L. K., ZAGURSKY, R. J., LIN, S. L., MURPHY, E., ZLOTNICK, G. W., HOISETH, S. K., JANSEN, K. U. & ANDERSON, A. S. 2013. Role of factor H binding protein in *Neisseria meningitidis* virulence and its potential as a vaccine candidate to broadly protect against meningococcal disease. *Microbiol Mol Biol Rev,* 77**,** 234-52.

MCVERNON, J., JOHNSON, P. D., POLLARD, A. J., SLACK, M. P. & MOXON, E. R. 2003. Immunologic memory in *Haemophilus influenzae* type b conjugate vaccine failure. *Arch Dis Child,* 88**,** 379-83.

MECHAM, R. P. 1991. Receptors for laminin on mammalian cells. *FASEB J,* 5**,** 2538-46.

MEISSNER, P., PICK, H., KULANGARA, A., CHATELLARD, P., FRIEDRICH, K. & WURM, F. M. 2001. Transient gene expression: Recombinant protein production with suspension-adapted HEK293-EBNA cells. *Biotechnology and Bioengineering,* 75**,** 197-203.

MELIN, M., TRZCINSKI, K., MERI, S., KAYHTY, H. & VAKEVAINEN, M. 2010. The capsular serotype of *Streptococcus pneumoniae* is more important than the genetic background for resistance to complement. *Infect Immun,* 78**,** 5262-70.

MENARD, S., CASTRONOVO, V., TAGLIABUE, E. & SOBEL, M. E. 1997. New insights into the metastasis-associated 67 kD laminin receptor. *Journal of Cellular Biochemistry,* 67**,** 155-165.

MENARD, S., TAGLIABUE, E. & COLNAGHI, M. I. 1998. The 67 kDa laminin receptor as a prognostic factor in human cancer. *Breast Cancer Res Treat,* 52**,** 137-45.

MENON, R. P. & HUGHES, R. C. 1999. Determinants in the N-terminal domains of galectin-3 for secretion by a novel pathway circumventing the endoplasmic reticulum-Golgi complex. *Eur J Biochem,* 264**,** 569-76.

MEREGHETTI, L., SITKIEWICZ, I., GREEN, N. M. & MUSSER, J. M. 2008. Extensive adaptive changes occur in the transcriptome of *Streptococcus agalactiae* (group B streptococcus) in response to incubation with human blood. *PLoS One,* 3**,** e3143.

MERZ, A. J., ENNS, C. A. & SO, M. 1999. Type IV pili of pathogenic Neisseriae elicit cortical plaque formation in epithelial cells. *Mol Microbiol,* 32**,** 1316-32.

MERZ, A. J. & SO, M. 2000. Interactions of pathogenic neisseriae with epithelial cell membranes. *Annu Rev Cell Dev Biol,* 16**,** 423-57.

MEYER, T. F. 1999. Pathogenic neisseriae: complexity of pathogen-host cell interplay. *Clin Infect Dis,* 28**,** 433-41.

MINTSERIS, J., PIERCE, B., WIEHE, K., ANDERSON, R., CHEN, R. & WENG, Z. P. 2007. Integrating statistical pair potentials into protein complex prediction. *Proteins-Structure Function and Bioinformatics,* 69**,** 511-520.

MITCHELL, A. M. & MITCHELL, T. J. 2010. *Streptococcus pneumoniae*: virulence factors and variation. *Clin Microbiol Infect,* 16**,** 411-8.

MIZUSHIMA, S. & NAGATA, S. 1990. Pef-Bos, a Powerful Mammalian Expression Vector. *Nucleic Acids Research,* 18**,** 5322-5322.

MONTUORI, N., MULLER, F., DE RIU, S., FENZI, G., SOBEL, M. E., ROSSI, G. & VITALE, M. 1999. Laminin receptors in differentiated thyroid tumors: restricted expression of the 67-kilodalton laminin receptor in follicular carcinoma cells. *J Clin Endocrinol Metab,* 84**,** 2086-92.

MOOK-KANAMORI, B. B., GELDHOFF, M., VAN DER POLL, T. & VAN DE BEEK, D. 2011. Pathogenesis and pathophysiology of pneumococcal meningitis. *Clin Microbiol Rev,* 24**,** 557-91.

MORAN, E. E., BURDEN, R., LABRIE, J. E., 3RD, WEN, Z., WANG, X. M., ZOLLINGER, W. D., ZHANG, L. & PINTO, V. B. 2012. Analysis of the bactericidal response to an experimental *Neisseria meningitidis* vesicle vaccine. *Clin Vaccine Immunol,* 19**,** 659-65.

MORAND, P. C., DRAB, M., RAJALINGAM, K., NASSIF, X. & MEYER, T. F. 2009. *Neisseria meningitidis* differentially controls host cell motility through PilC1 and PilC2 components of type IV Pili. *PLoS One,* 4**,** e6834.

MORLEY, S. L. & POLLARD, A. J. 2001. Vaccine prevention of meningococcal disease, coming soon? *Vaccine,* 20**,** 666-87.

MORONA, J. K., MORONA, R., MILLER, D. C. & PATON, J. C. 2002. *Streptococcus pneumoniae* capsule biosynthesis protein CpsB is a novel manganese-dependent phosphotyrosine-protein phosphatase. *J Bacteriol,* 184**,** 577-83.

MORONA, J. K., MORONA, R. & PATON, J. C. 2006. Attachment of capsular polysaccharide to the cell wall of *Streptococcus pneumoniae* type 2 is required for invasive disease. *Proc Natl Acad Sci U S A,* 103**,** 8505-10.

MORRIS, S. K., MOSS, W. J. & HALSEY, N. 2008. *Hoemophilus influenzae* type b conjugate vaccine use and effectiveness. *Lancet Infectious Diseases,* 8**,** 435-443.

MOSCHIONI, M., DONATI, C., MUZZI, A., MASIGNANI, V., CENSINI, S., HANAGE, W. P., BISHOP, C. J., REIS, J. N., NORMARK, S., HENRIQUES-NORMARK, B., COVACCI, A., RAPPUOLI, R. & BAROCCHI, M. A. 2008. *Streptococcus pneumoniae* contains 3 rlrA pilus variants that are clonally related. *J Infect Dis,* 197**,** 888-96.

MURPHY, T. F., FADEN, H., BAKALETZ, L. O., KYD, J. M., FORSGREN, A., CAMPOS, J., VIRJI, M. & PELTON, S. I. 2009. Nontypeable *Haemophilus influenzae* as a pathogen in children. *Pediatr Infect Dis J,* 28**,** 43-8.

NABORS, G. S., BRAUN, P. A., HERRMANN, D. J., HEISE, M. L., PYLE, D. J.,

GRAVENSTEIN, S., SCHILLING, M., FERGUSON, L. M., HOLLINGSHEAD, S. K., BRILES, D. E. & BECKER, R. S. 2000. Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules. *Vaccine,* 18**,** 1743-54.

NAGAI, T., IBATA, K., PARK, E. S., KUBOTA, M., MIKOSHIBA, K. & MIYAWAKI, A. 2002. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat Biotechnol,* 20**,** 87-90.

NAIMAN, H. L. & ALBRITTON, W. L. 1980. Counterimmunoelectrophoresis in the diagnosis of acute infection. *J Infect Dis,* 142**,** 524-31.

NAKAMURA, S., SHCHEPETOV, M., DALIA, A. B., CLARK, S. E., MURPHY, T. F., SETHI, S., GILSDORF, J. R., SMITH, A. L. & WEISER, J. N. 2011. Molecular basis of increased serum resistance among pulmonary isolates of non-typeable *Haemophilus influenzae*. *PLoS Pathog,* 7**,** e1001247.

NARKIO-MAKELA, M., HELLWAGE, J., TAHKOKALLIO, O. & MERI, S. 2001. Complement-regulator factor H and related proteins in otitis media with effusion. *Clin Immunol,* 100**,** 118-26.

NARUMI, K., INOUE, A., TANAKA, M., ISEMURA, M., SHIMO-OKA, T., ABE, T., NUKIWA, T. & SATOH, K. 1999. Inhibition of experimental metastasis of human fibrosarcoma cells by anti-recombinant 37-kDa laminin binding protein antibody. *Jpn J Cancer Res,* 90**,** 425-31.

NASSIF, X., BERETTI, J. L., LOWY, J., STENBERG, P., O'GAORA, P., PFEIFER, J., NORMARK, S. & SO, M. 1994. Roles of pilin and PilC in adhesion of *Neisseria meningitidis* to human epithelial and endothelial cells. *Proc Natl Acad Sci U S A,* 91**,** 3769-73.

NASSIF, X., MARCEAU, M., PUJOL, C., PRON, B., BERETTI, J. L. & TAHA, M. K. 1997. Type-4 pili and meningococcal adhesiveness. *Gene,* 192**,** 149-53.

NASSIF, X., PUJOL, C., MORAND, P. & EUGENE, E. 1999. Interactions of pathogenic Neisseria with host cells. Is it possible to assemble the puzzle? *Mol Microbiol,* 32**,** 1124-32.

NEDELEC, J., BOUCRAUT, J., GARNIER, J. M., BERNARD, D. & ROUGON, G. 1990. Evidence for autoimmune antibodies directed against embryonic neural cell adhesion molecules (N-CAM) in patients with group B meningitis. *J Neuroimmunol,* 29**,** 49-56.

NELSON, A. L., RIES, J., BAGNOLI, F., DAHLBERG, S., FALKER, S., ROUNIOJA, S., TSCHOP, J., MORFELDT, E., FERLENGHI, I., HILLERINGMANN, M., HOLDEN, D. W., RAPPUOLI, R., NORMARK, S., BAROCCHI, M. A. & HENRIQUES-NORMARK, B. 2007. RrgA is a pilus-associated adhesin in *Streptococcus pneumoniae*. *Mol Microbiol,* 66**,** 329-40.

NELSON, J., MCFERRAN, N. V., PIVATO, G., CHAMBERS, E., DOHERTY, C., STEELE, D. & TIMSON, D. J. 2008. The 67 kDa laminin receptor: structure, function and role in disease. *Bioscience Reports,* 28**,** 33-48.

NEWLACZYL, A. U. & YU, L. G. 2011. Galectin-3-A jack-of-all-trades in cancer. *Cancer Letters,* 313**,** 123-128.

NIEMINEN, J., KUNO, A., HIRABAYASHI, J. & SATO, S. 2007. Visualization of galectin-3 oligomerization on the surface of neutrophils and endothelial cells using fluorescence resonance energy transfer. *J Biol Chem,* 282**,** 1374-83.

NOVOTNY, L. A., CLEMENTS, J. D. & BAKALETZ, L. O. 2013. Kinetic analysis and evaluation of the mechanisms involved in the resolution of experimental nontypeable *Haemophilus influenzae*-induced otitis media after transcutaneous immunization. *Vaccine,* 31**,** 3417-26.

NUORTI, J. P., WHITNEY, C. G., CENTERS FOR DISEASE, C. & PREVENTION 2010. Prevention of pneumococcal disease among infants and children - use of 13-valent pneumococcal conjugate vaccine and 23-valent pneumococcal

polysaccharide vaccine - recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep,* 59**,** 1-18.

OCHIENG, J., FURTAK, V. & LUKYANOV, P. 2004. Extracellular functions of galectin-3. *Glycoconj J,* 19**,** 527-35.

OLYHOEK, A. J., SARKARI, J., BOPP, M., MORELLI, G. & ACHTMAN, M. 1991. Cloning and expression in *Escherichia coli* of opc, the gene for an unusual class 5 outer membrane protein from *Neisseria meningitidis* (meningococci/surface antigen). *Microb Pathog,* 11**,** 249-57.

ORIHUELA, C. J., MAHDAVI, J., THORNTON, J., MANN, B., WOOLDRIDGE, K. G., ABOUSEADA, N., OLDFIELD, N. J., SELF, T., ALA'ALDEEN, D. A. & TUOMANEN, E. I. 2009. Laminin receptor initiates bacterial contact with the blood brain barrier in experimental meningitis models. *J Clin Invest,* 119**,** 1638-46.

OULD-ABEIH, M. B., PETIT-TOPIN, I., ZIDANE, N., BARON, B. & BEDOUELLE, H. 2012. Multiple folding states and disorder of ribosomal protein SA, a membrane receptor for laminin, anticarcinogens, and pathogens. *Biochemistry,* 51**,** 4807-21.

OVIEDO-ORTA, E., AHMED, S., RAPPUOLI, R. & BLACK, S. 2015. Prevention and control of meningococcal outbreaks: The emerging role of serogroup B meningococcal vaccines. *Vaccine,* 33**,** 3628-35.

OZAKI, I., YAMAMOTO, K., MIZUTA, T., KAJIHARA, S., FUKUSHIMA, N., SETOGUCHI, Y., MORITO, F. & SAKAI, T. 1998. Differential expression of laminin receptors in human hepatocellular carcinoma. *Gut,* 43**,** 837-42.

PAN, X. L., YANG, Y. & ZHANG, J. R. 2014. Molecular basis of host specificity in human pathogenic bacteria. *Emerging Microbes & Infections,* 3.

PATTERSON, G. H., KNOBEL, S. M., SHARIF, W. D., KAIN, S. R. & PISTON, D. W. 1997. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophysical Journal,* 73**,** 2782-2790.

PEDELACQ, J. D., CABANTOUS, S., TRAN, T., TERWILLIGER, T. C. & WALDO, G. S. 2006. Engineering and characterization of a superfolder green fluorescent protein. *Nat Biotechnol,* 24**,** 79-88.

PEEK, J. A. & TAYLOR, R. K. 1992. Characterization of a periplasmic thiol:disulfide interchange protein required for the functional maturation of secreted virulence factors of Vibrio cholerae. *Proc Natl Acad Sci U S A,* 89**,** 6210-4.

PELLETIER, I. & SATO, S. 2002. Specific recognition and cleavage of galectin-3 by Leishmania major through species-specific polygalactose epitope. *Journal of Biological Chemistry,* 277**,** 17663-17670.

PELTOLA, H. 2000. Worldwide *Haemophilus influenzae* type b disease at the beginning of the 21st century: global analysis of the disease burden 25 years after the use of the polysaccharide vaccine and a decade after the advent of conjugates. *Clin Microbiol Rev,* 13**,** 302-17.

PELTOLA, H., KAYHTY, H., SIVONEN, A. & MAKELA, H. 1977. *Haemophilus influenzae* type b capsular polysaccharide vaccine in children: a double-blind field study of 100,000 vaccinees 3 months to 5 years of age in Finland. *Pediatrics,* 60**,** 730-7.

PEREZ-DORADO, I., GALAN-BARTUAL, S. & HERMOSO, J. A. 2012. Pneumococcal surface proteins: when the whole is greater than the sum of its parts. *Mol Oral Microbiol,* 27**,** 221-45.

PETTERSEN, E. F., GODDARD, T. D., HUANG, C. C., COUCH, G. S., GREENBLATT, D. M., MENG, E. C. & FERRIN, T. E. 2004. UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem,* 25**,** 1605-12.

PFISTER, H. W., BORASIO, G. D., DIRNAGL, U., BAUER, M. & EINHAUPL, K. M. 1992. Cerebrovascular complications of bacterial meningitis in adults. *Neurology,* 42**,** 1497-504.

PFLANZ, H., VANA, K., MITTEREGGER, G., RENNER-MULLER, I., PACE, C., KUCHENHOFF, H., KRETZSCHMAR, H. A., WOLF, E. & WEISS, S. 2009. Scrapie-Infected Transgenic Mice Expressing a Laminin Receptor Decoy Mutant Reveal a Prolonged Incubation Time Associated with Low Levels of PrPres. *Journal of Molecular Biology,* 388**,** 721-729.

PIERCE, B. G., HOURAI, Y. & WENG, Z. P. 2011. Accelerating Protein Docking in ZDOCK Using an Advanced 3D Convolution Library. *Plos One,* 6.

PIZARRO-CERDA, J. & COSSART, P. 2006. Bacterial adhesion and entry into host cells. *Cell,* 124**,** 715-727.

PLAUT, A. G. 1983. The IgA1 proteases of pathogenic bacteria. *Annu Rev Microbiol,* 37**,** 603-22.

POLLARD, A. J., PERRETT, K. P. & BEVERLEY, P. C. 2009. Maintaining protection against invasive bacteria with protein-polysaccharide conjugate vaccines. *Nat Rev Immunol,* 9**,** 213-20.

POOLMAN, J. T., BAKALETZ, L., CRIPPS, A., DENOEL, P. A., FORSGREN, A., KYD, J. & LOBET, Y. 2000. Developing a nontypeable *Haemophilus influenzae* (NTHi) vaccine. *Vaccine,* 19 Suppl 1**,** S108-15.

POPPERT, S., ESSIG, A., STOEHR, B., STEINGRUBER, A., WIRTHS, B., JURETSCHKO, S., REISCHL, U. & WELLINGHAUSEN, N. 2005. Rapid diagnosis of bacterial meningitis by real-time PCR and fluorescence in situ hybridization. *J Clin Microbiol,* 43**,** 3390-7.

POULSEN, K., REINHOLDT, J., JESPERSGAARD, C., BOYE, K., BROWN, T. A., HAUGE, M. & KILIAN, M. 1998. A comprehensive genetic study of streptococcal immunoglobulin A1 proteases: evidence for recombination within and between species. *Infect Immun,* 66**,** 181-90.

PRASADARAO, N. V., LYSENKO, E., WASS, C. A., KIM, K. S. & WEISER, J. N. 1999a. Opacity-associated protein A contributes to the binding of *Haemophilus influenzae* to chang epithelial cells. *Infect Immun,* 67**,** 4153-60.

PRASADARAO, N. V., SRIVASTAVA, P. K., RUDRABHATLA, R. S., KIM, K. S., HUANG, S. H. & SUKUMARAN, S. K. 2003. Cloning and expression of the *Escherichia coli* K1 outer membrane protein A receptor, a gp96 homologue. *Infect Immun,* 71**,** 1680-8.

PRASADARAO, N. V., WASS, C. A., STINS, M. F., SHIMADA, H. & KIM, K. S. 1999b. Outer membrane protein A-promoted actin condensation of brain microvascular endothelial cells is required for *Escherichia coli* invasion. *Infect Immun,* 67**,** 5775-83.

PRATHER, D. T., BAINS, M., HANCOCK, R. E., FILIATRAULT, M. J. & CAMPAGNARI, A. A. 2004. Differential expression of porins OmpP2A and OmpP2B of *Haemophilus ducreyi*. *Infect Immun,* 72**,** 6271-8.

PRINCE, S. M., ACHTMAN, M. & DERRICK, J. P. 2002. Crystal structure of the OpcA integral membrane adhesin from *Neisseria meningitidis*. *Proc Natl Acad Sci U S A,* 99**,** 3417-21.

PRON, B., TAHA, M. K., RAMBAUD, C., FOURNET, J. C., PATTEY, N., MONNET, J. P., MUSILEK, M., BERETTI, J. L. & NASSIF, X. 1997. Interaction of *Neisseria maningitidis* with the components of the blood-brain barrier correlates with an increased expression of PilC. *J Infect Dis,* 176**,** 1285-92.

PROTOPOPOVA, E. V., SOROKIN, A. V., KONOVALOVA, S. N., KACHKO, A. V., NETESOV, S. V. & LOKTEV, V. B. 1999. Human laminin binding protein as a cell receptor for the tick-borne encephalitis virus. *Zentralblatt Fur Bakteriologie*

-*International Journal of Medical Microbiology Virology Parasitology and Infectious Diseases,* 289**,** 632-638.

PRUSINER, S. B. 1998. Prions. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 13363-13383.

PUJOL, C., EUGENE, E., DE SAINT MARTIN, L. & NASSIF, X. 1997. Interaction of *Neisseria meningitidis* with a polarized monolayer of epithelial cells. *Infect Immun,* 65**,** 4836-42.

PUJOL, C., EUGENE, E., MARCEAU, M. & NASSIF, X. 1999. The meningococcal PilT protein is required for induction of intimate attachment to epithelial cells following pilus-mediated adhesion. *Proc Natl Acad Sci U S A,* 96**,** 4017-22.

QUATTRONI, P., LI, Y., LUCCHESI, D., LUCAS, S., HOOD, D. W., HERRMANN, M., GABIUS, H. J., TANG, C. M. & EXLEY, R. M. 2012. Galectin-3 binds *Neisseria meningitidis* and increases interaction with phagocytic cells. *Cell Microbiol,* 14**,** 1657-75.

RAETZ, C. R., GUAN, Z., INGRAM, B. O., SIX, D. A., SONG, F., WANG, X. & ZHAO, J. 2009. Discovery of new biosynthetic pathways: the lipid A story. *J Lipid Res,* 50 Suppl**,** S103-8.

RAHMAN, M., KALLSTROM, H., NORMARK, S. & JONSSON, A. B. 1997. PilC of pathogenic Neisseria is associated with the bacterial cell surface. *Mol Microbiol,* 25**,** 11-25.

RALPH, B. A., BRILES, D. E. & MCDANIEL, L. S. 1994. Cross-reactive protection eliciting epitopes of pneumococcal surface protein A. *Ann N Y Acad Sci,* 730**,** 361-3.

RAMSAY, M. E., ANDREWS, N., KACZMARSKI, E. B. & MILLER, E. 2001. Efficacy of meningococcal serogroup C conjugate vaccine in teenagers and toddlers in England. *Lancet,* 357**,** 195-6.

RAMSAY, M. E., ANDREWS, N. J., TROTTER, C. L., KACZMARSKI, E. B. & MILLER, E. 2003. Herd immunity from meningococcal serogroup C conjugate vaccination in England: database analysis. *BMJ,* 326**,** 365-6.

RAO, C. N., CASTRONOVO, V., SCHMITT, M. C., WEWER, U. M., CLAYSMITH, A. P., LIOTTA, L. A. & SOBEL, M. E. 1989. Evidence for a precursor of the high-affinity metastasis-associated murine laminin receptor. *Biochemistry,* 28**,** 7476-86.

RAO, N. C., BARSKY, S. H., TERRANOVA, V. P. & LIOTTA, L. A. 1983. Isolation of a tumor cell laminin receptor. *Biochem Biophys Res Commun,* 111**,** 804-8.

RAO, V. K., KRASAN, G. P., HENDRIXSON, D. R., DAWID, S. & ST GEME, J. W., 3RD 1999. Molecular determinants of the pathogenesis of disease due to non-typable *Haemophilus influenzae*. *FEMS Microbiol Rev,* 23**,** 99-129.

RAPPUOLI, R. 2000. Reverse vaccinology. *Curr Opin Microbiol,* 3**,** 445-50.

RAZ, A., AVIVI, A., PAZERINI, G. & CARMI, P. 1987. Cloning and expression of cDNA for two endogenous UV-2237 fibrosarcoma lectin genes. *Exp Cell Res,* 173**,** 109-16.

REA, V. E., ROSSI, F. W., DE PAULIS, A., RAGNO, P., SELLERI, C. & MONTUORI, N. 2012. 67 kDa laminin receptor: structure, function and role in cancer and infection. *Infez Med,* 20 Suppl 2**,** 8-12.

READ, R. C., WILSON, R., RUTMAN, A., LUND, V., TODD, H. C., BRAIN, A. P., JEFFERY, P. K. & COLE, P. J. 1991. Interaction of nontypable *Haemophilus influenzae* with human respiratory mucosa in vitro. *J Infect Dis,* 163**,** 549-58.

REDDY, M. S., BERNSTEIN, J. M., MURPHY, T. F. & FADEN, H. S. 1996. Binding between outer membrane proteins of nontypeable *Haemophilus influenzae* and human nasopharyngeal mucin. *Infection and Immunity,* 64**,** 1477-1479.

REINGOLD, A. L., BROOME, C. V., HIGHTOWER, A. W., AJELLO, G. W., BOLAN, G. A., ADAMSBAUM, C., JONES, E. E., PHILLIPS, C., TIENDREBEOGO, H. & YADA, A. 1985. Age-specific differences in duration of clinical protection

after vaccination with meningococcal polysaccharide A vaccine. *Lancet,* 2**,** 114-8.

REMAUT, H. & WAKSMAN, G. 2004. Structural biology of bacterial pathogenesis. *Curr Opin Struct Biol,* 14**,** 161-70.

RIBET, D. & COSSART, P. 2015. How bacterial pathogens colonize their hosts and invade deeper tissues. *Microbes and Infection,* 17**,** 173-183.

RICHMOND, P., GOLDBLATT, D., FUSCO, P. C., FUSCO, J. D., HERON, I., CLARK, S., BORROW, R. & MICHON, F. 1999. Safety and immunogenicity of a new *Neisseria meningitidis* serogroup C-tetanus toxoid conjugate vaccine in healthy adults. *Vaccine,* 18**,** 641-6.

RIEDMANN, E. M., LUBITZ, W., MCGRATH, J., KYD, J. M. & CRIPPS, A. W. 2011. Effectiveness of engineering the nontypeable *Haemophilus influenzae* antigen Omp26 as an S-layer fusion in bacterial ghosts as a mucosal vaccine delivery. *Hum Vaccin,* 7 Suppl**,** 99-107.

RIJKERS, G. T., VERMEER-DE BONDT, P. E., SPANJAARD, L., BREUKELS, M. A. & SANDERS, E. A. 2003. Return of *Haemophilus influenzae* type b infections. *Lancet,* 361**,** 1563-4.

RING, A., WEISER, J. N. & TUOMANEN, E. I. 1998. Pneumococcal trafficking across the blood-brain barrier. Molecular analysis of a novel bidirectional pathway. *J Clin Invest,* 102**,** 347-60.

RIZZO, M. A., SPRINGER, G. H., GRANADA, B. & PISTON, D. W. 2004. An improved cyan fluorescent protein variant useful for FRET. *Nature Biotechnology,* 22**,** 445-449.

ROBIDA, A. M. & KERPPOLA, T. K. 2009. Bimolecular fluorescence complementation analysis of inducible protein interactions: effects of factors affecting protein folding on fluorescent protein fragment association. *J Mol Biol,* 394**,** 391-409.

RODRIGUEZ, C. A., AVADHANULA, V., BUSCHER, A., SMITH, A. L., ST GEME, J. W., 3RD & ADDERSON, E. E. 2003. Prevalence and distribution of adhesins in invasive non-type b encapsulated *Haemophilus influenzae*. *Infect Immun,* 71**,** 1635-42.

ROFF, C. F. & WANG, J. L. 1983. Endogenous lectins from cultured cells. Isolation and characterization of carbohydrate-binding proteins from 3T3 fibroblasts. *J Biol Chem,* 258**,** 10657-63.

ROOS, K. L. 2000. Acute bacterial meningitis. *Semin Neurol,* 20**,** 293-306.

ROSANO, G. L. & CECCARELLI, E. A. 2014. Recombinant protein expression in *Escherichia coli*: advances and challenges. *Front Microbiol,* 5**,** 172.

ROSE, R. H., BRIDDON, S. J. & HOLLIDAY, N. D. 2010. Bimolecular fluorescence complementation: lighting up seven transmembrane domain receptor signalling networks. *Br J Pharmacol,* 159**,** 738-50.

ROSENOW, C., RYAN, P., WEISER, J. N., JOHNSON, S., FONTAN, P., ORTQVIST, A. & MASURE, H. R. 1997. Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*. *Molecular Microbiology,* 25**,** 819-829.

ROSZCZENKO, P., RADOMSKA, K. A., WYWIAL, E., COLLET, J. F. & JAGUSZTYN-KRYNICKA, E. K. 2012. A Novel Insight into the Oxidoreductase Activity of Helicobacter pylori HP0231 Protein. *Plos One,* 7.

RUDEL, T., SCHEURERPFLUG, I. & MEYER, T. F. 1995. Neisseria PilC protein identified as type-4 pilus tip-located adhesin. *Nature,* 373**,** 357-9.

RYLL, R. R., RUDEL, T., SCHEUERPFLUG, I., BARTEN, R. & MEYER, T. F. 1997. PilC of *Neisseria meningitidis* is involved in class II pilus formation and restores pilus assembly, natural transformation competence and adherence to epithelial cells in PilC-deficient gonococci. *Mol Microbiol,* 23**,** 879-92.

SA, E. C. C., GRIFFITHS, N. J. & VIRJI, M. 2010. *Neisseria meningitidis* Opc invasin binds to the sulphated tyrosines of activated vitronectin to attach to and invade human brain endothelial cells. *PLoS Pathog,* 6**,** e1000911.

SADARANGANI, M., POLLARD, A. J. & GRAY-OWEN, S. D. 2011. Opa proteins and CEACAMs: pathways of immune engagement for pathogenic Neisseria. *FEMS Microbiol Rev,* 35**,** 498-514.

SAEZ-LLORENS, X. & MCCRACKEN, G. H., JR. 2003. Bacterial meningitis in children. *Lancet,* 361**,** 2139-48.

SAHDEV, S., KHATTAR, S. K. & SAINI, K. S. 2008. Production of active eukaryotic proteins through bacterial expression systems: a review of the existing biotechnology strategies. *Molecular and Cellular Biochemistry,* 307**,** 249-264.

SAINT-LU, N., OORTWIJN, B. D., PEGON, J. N., ODOUARD, S., CHRISTOPHE, O. D., DE GROOT, P. G., DENIS, C. V. & LENTING, P. J. 2012. Identification of Galectin-1 and Galectin-3 as Novel Partners for Von Willebrand Factor. *Arteriosclerosis Thrombosis and Vascular Biology,* 32**,** 894-U87.

SALIH, M. A., AHMED, H. S., HOFVANDER, Y., DANIELSSON, D. & OLCEN, P. 1989. Rapid diagnosis of bacterial meningitis by an enzyme immunoassay of cerebrospinal fluid. *Epidemiol Infect,* 103**,** 301-10.

SAMPSON, J. S., O'CONNOR, S. P., STINSON, A. R., THARPE, J. A. & RUSSELL, H. 1994. Cloning and nucleotide sequence analysis of psaA, the *Streptococcus pneumoniae* gene encoding a 37-kilodalton protein homologous to previously reported *Streptococcus sp.* adhesins. *Infect Immun,* 62**,** 319-24.

SANCHEZ-BEATO, A. R., LOPEZ, R. & GARCIA, J. L. 1998. Molecular characterization of PcpA: a novel choline-binding protein of *Streptococcus pneumoniae*. *Fems Microbiology Letters,* 164**,** 207-214.

SANDERS, M. S., VAN WELL, G. T., OUBURG, S., MORRE, S. A. & VAN FURTH, A. M. 2011. Genetic variation of innate immune response genes in invasive pneumococcal and meningococcal disease applied to the pathogenesis of meningitis. *Genes Immun,* 12**,** 321-34.

SANJUAN, X., FERNANDEZ, P. L., MIQUEL, R., MUNOZ, J., CASTRONOVO, V., MENARD, S., PALACIN, A., CARDESA, A. & CAMPO, E. 1996. Overexpression of the 67-kD laminin receptor correlates with tumour progression in human colorectal carcinoma. *J Pathol,* 179**,** 376-80.

SANO, H., HSU, D. K., YU, L., APGAR, J. R., KUWABARA, I., YAMANAKA, T., HIRASHIMA, M. & LIU, F. T. 2000. Human galectin-3 is a novel chemoattractant for monocytes and macrophages. *Journal of Immunology,* 165**,** 2156-2164.

SATO, M., KINOSHITA, K., KANEDA, Y., SAEKI, Y., IWAMATSU, A., TANAKA, K. & KANEDA, Y. 1996. Analysis of nuclear localization of laminin binding protein precursor p40 (LBP/p40). *Biochemical and Biophysical Research Communications,* 229**,** 896-901.

SATO, M., SAEKI, Y., TANAKA, K. & KANEDA, Y. 1999. Ribosome-associated protein LBP/p40 binds to S21 protein of 40S ribosome: Analysis using a yeast two-hybrid system. *Biochemical and Biophysical Research Communications,* 256**,** 385-390.

SATO, S., ST-PIERRE, C., BHAUMIK, P. & NIEMINEN, J. 2009. Galectins in innate immunity: dual functions of host soluble beta-galactoside-binding lectins as damage-associated molecular patterns (DAMPs) and as receptors for pathogen-associated molecular patterns (PAMPs). *Immunol Rev,* 230**,** 172-87.

SATOH, K., NARUMI, K., ISEMURA, M., SAKAI, T., ABE, T., MATSUSHIMA, K., OKUDA, K. & MOTOMIYA, M. 1992. Increased expression of the 67kDa-laminin receptor gene in human small cell lung cancer. *Biochem Biophys Res Commun,* 182**,** 746-52.

SAYLE, R. A. & MILNERWHITE, E. J. 1995. Rasmol - Biomolecular Graphics for All. *Trends in Biochemical Sciences,* 20**,** 374-376.

SCHEIMAN, J., JAMIESON, K. V., ZIELLO, J., TSENG, J. C. & MERUELO, D. 2010a. Extraribosomal functions associated with the C terminus of the 37/67 kDa laminin receptor are required for maintaining cell viability. *Cell Death Dis,* 1**,** e42.

SCHEIMAN, J., TSENG, J. C., ZHENG, Y. & MERUELO, D. 2010b. Multiple Functions of the 37/67-kd Laminin Receptor Make It a Suitable Target for Novel Cancer Gene Therapy. *Molecular Therapy,* 18**,** 63-74.

SCHMIDT, H., HEIMANN, B., DJUKIC, M., MAZUREK, C., FELS, C., WALLESCH, C. W. & NAU, R. 2006. Neuropsychological sequelae of bacterial and viral meningitis. *Brain,* 129**,** 333-45.

SCHNEIDER, M. C., EXLEY, R. M., RAM, S., SIM, R. B. & TANG, C. M. 2007. Interactions between *Neisseria meningitidis* and the complement system. *Trends Microbiol,* 15**,** 233-40.

SCHOLTEN, R. J., KUIPERS, B., VALKENBURG, H. A., DANKERT, J., ZOLLINGER, W. D. & POOLMAN, J. T. 1994. Lipo-oligosaccharide immunotyping of *Neisseria meningitidis* by a whole-cell ELISA with monoclonal antibodies. *J Med Microbiol,* 41**,** 236-43.

SCHUT, E. S., DE GANS, J. & VAN DE BEEK, D. 2008. Community-acquired bacterial meningitis in adults. *Pract Neurol,* 8**,** 8-23.

SCHWARTZ, B., MOORE, P. S. & BROOME, C. V. 1989. Global epidemiology of meningococcal disease. *Clin Microbiol Rev,* 2 Suppl**,** S118-24.

SEEPERSAUD, R., HANNIFFY, S. B., MAYNE, P., SIZER, P., LE PAGE, R. & WELLS, J. A. 2005. Characterization of a novel leucine-rich repeat protein antigen from group B streptococci that elicits protective immunity. *Infection and Immunity,* 73**,** 1671-1683.

SELVA, L., CIRUELA, P., BLANCHETTE, K., DEL AMO, E., PALLARES, R., ORIHUELA, C. J. & MUNOZ-ALMAGRO, C. 2012. Prevalence and Clonal Distribution of pcpA, psrP and Pilus-1 among Pediatric Isolates of *Streptococcus pneumoniae*. *Plos One,* 7.

SERRUTO, D., BOTTOMLEY, M. J., RAM, S., GIULIANI, M. M. & RAPPUOLI, R. 2012. The new multicomponent vaccine against meningococcal serogroup B, 4CMenB: immunological, functional and structural characterization of the antigens. *Vaccine,* 30 Suppl 2**,** B87-97.

SEXTON, K., LENNON, D., OSTER, P., CRENGLE, S., MARTIN, D., MULHOLLAND, K., PERCIVAL, T., REID, S., STEWART, J. & O'HALLAHAN, J. 2004. The New Zealand Meningococcal Vaccine Strategy: a tailor-made vaccine to combat a devastating epidemic. *N Z Med J,* 117**,** U1015.

SHAPIRO, E. D., BERG, A. T., AUSTRIAN, R., SCHROEDER, D., PARCELLS, V., MARGOLIS, A., ADAIR, R. K. & CLEMENS, J. D. 1991. The protective efficacy of polyvalent pneumococcal polysaccharide vaccine. *N Engl J Med,* 325**,** 1453-60.

SHAW, G., MORSE, S., ARARAT, M. & GRAHAM, F. L. 2002. Preferential transformation of human neuronal cells by human adenoviruses and the origin of HEK 293 cells. *FASEB J,* 16**,** 869-71.

SHYU, Y. J. & HU, C. D. 2008. Fluorescence complementation: an emerging tool for biological research. *Trends in Biotechnology,* 26**,** 622-630.

SHYU, Y. J., LIU, H., DENG, X. & HU, C. D. 2006. Identification of new fluorescent protein fragments for bimolecular fluorescence complementation analysis under physiological conditions. *Biotechniques,* 40**,** 61-6.

SIIANOVA, E. 1992. [Expression of human LSB 32/67 KD gene in *E. coli* and analysis of its interactions with laminin]. *Biull Eksp Biol Med,* 113**,** 70-2.

SKERKER, J. M. & BERG, H. C. 2001. Direct observation of extension and retraction of type IV pili. *Proc Natl Acad Sci U S A,* 98**,** 6901-4.

SLANINA, H., SCHMUTZLER, M., CHRISTODOULIDES, M., KIM, K. S. & SCHUBERT-UNKMEIR, A. 2012. Effective plasmid DNA and small interfering RNA delivery to diseased human brain microvascular endothelial cells. *J Mol Microbiol Biotechnol,* 22**,** 245-57.

SMILLIE, W. G. 1938. The Biology of Pneumococcus. *American Journal of Public Health and the Nations Health,* 28**,** 663-664.

SMITH-VAUGHAN, H. C., SRIPRAKASH, K. S., MATHEWS, J. D. & KEMP, D. J. 1997. Nonencapsulated *Haemophilus influenzae* in Aboriginal infants with otitis media: prolonged carriage of P2 porin variants and evidence for horizontal P2 gene transfer. *Infect Immun,* 65**,** 1468-74.

SNAPE, M. D. & POLLARD, A. J. 2005. Meningococcal polysaccharide-protein conjugate vaccines. *Lancet Infect Dis,* 5**,** 21-30.

SOROKIN, A. V., MIKHAILOV, A. M., KACHKO, A. V., PROTOPOPOVA, E. V., KONOVALOVA, S. N., ANDRIANOVA, M. E., NETESOV, S. V., KORNEV, A. N. & LOKTEV, V. B. 2000. Human recombinant laminin-binding protein: isolation, purification, and crystallization. *Biochemistry (Mosc),* 65**,** 546-53.

SPARROW, C. P., LEFFLER, H. & BARONDES, S. H. 1987. Multiple soluble beta-galactoside-binding lectins from human lung. *J Biol Chem,* 262**,** 7383-90.

SPINOSA, M. R., PROGIDA, C., TALA, A., COGLI, L., ALIFANO, P. & BUCCI, C. 2007. The *Neisseria meningitidis* capsule is important for intracellular survival in human cells. *Infect Immun,* 75**,** 3594-603.

ST GEME, J. W., 3RD 2000. The pathogenesis of nontypable *Haemophilus influenzae* otitis media. *Vaccine,* 19 Suppl 1**,** S41-50.

ST GEME, J. W., 3RD 2002. Molecular and cellular determinants of non-typeable *Haemophilus influenzae* adherence and invasion. *Cell Microbiol,* 4**,** 191-200.

ST GEME, J. W., 3RD, CUTTER, D. & BARENKAMP, S. J. 1996. Characterization of the genetic locus encoding *Haemophilus influenzae* type b surface fibrils. *J Bacteriol,* 178**,** 6281-7.

ST GEME, J. W., 3RD, DE LA MORENA, M. L. & FALKOW, S. 1994. A *Haemophilus influenzae* IgA protease-like protein promotes intimate interaction with human epithelial cells. *Mol Microbiol,* 14**,** 217-33.

STEINBACH, W. J. & SHETTY, A. 2001. Use of the diagnostic bacteriology laboratory: a practical review for the clinician. *Postgraduate Medical Journal,* 77**,** 148-156.

STEPHENS, D. S. 1999. Uncloaking the meningococcus: dynamics of carriage and disease. *Lancet,* 353**,** 941-2.

STEPHENS, D. S. 2007. Conquering the meningococcus. *FEMS Microbiol Rev,* 31**,** 3-14.

STEPHENS, D. S. 2009. Biology and pathogenesis of the evolutionarily successful, obligate human bacterium *Neisseria meningitidis*. *Vaccine,* 27 Suppl 2**,** B71-7.

STROM, M. S. & LORY, S. 1993. Structure-function and biogenesis of the type IV pili. *Annu Rev Microbiol,* 47**,** 565-96.

SU, Y. C., RESMAN, F., HORHOLD, F. & RIESBECK, K. 2014. Comparative genomic analysis reveals distinct genotypic features of the emerging pathogen *Haemophilus influenzae* type f. *BMC Genomics,* 15**,** 38.

SWORDS, W. E., KETTERER, M. R., SHAO, J., CAMPBELL, C. A., WEISER, J. N. & APICELLA, M. A. 2001. Binding of the non-typeable *Haemophilus influenzae* lipooligosaccharide to the PAF receptor initiates host cell signalling. *Cell Microbiol,* 3**,** 525-36.

TACHIBANA, H., KOGA, K., FUJIMURA, Y. & YAMADA, K. 2004. A receptor for green tea polyphenol EGCG. *Nat Struct Mol Biol,* 11**,** 380-1.

TAHA, M. K., MORAND, P. C., PEREIRA, Y., EUGENE, E., GIORGINI, D., LARRIBE, M. & NASSIF, X. 1998. Pilus-mediated adhesion of *Neisseria*

meningitidis: the essential role of cell contact-dependent transcriptional upregulation of the PilC1 protein. *Mol Microbiol,* 28**,** 1153-63.

TAN, L. K., CARLONE, G. M. & BORROW, R. 2010. Advances in the development of vaccines against *Neisseria meningitidis*. *N Engl J Med,* 362**,** 1511-20.

TAN, Y. C., GILL, A. K. & KIM, K. S. 2015. Treatment strategies for central nervous system infections: an update. *Expert Opin Pharmacother,* 16**,** 187-203.

TARABOLETTI, G., BELOTTI, D., GIAVAZZI, R., SOBEL, M. E. & CASTRONOVO, V. 1993. Enhancement of metastatic potential of murine and human melanoma cells by laminin receptor peptide G: attachment of cancer cells to subendothelial matrix as a pathway for hematogenous metastasis. *J Natl Cancer Inst,* 85**,** 235-40.

TENENBAUM, T., SPELLERBERG, B., ADAM, R., VOGEL, M., KIM, K. S. & SCHROTEN, H. 2007. *Streptococcus agalactiae* invasion of human brain microvascular endothelial cells is promoted by the laminin-binding protein Lmb. *Microbes and Infection,* 9**,** 714-720.

TENG, C. H., CAI, M., SHIN, S., XIE, Y., KIM, K. J., KHAN, N. A., DI CELLO, F. & KIM, K. S. 2005. *Escherichia coli* K1 RS218 interacts with human brain microvascular endothelial cells via type 1 fimbria bacteria in the fimbriated state. *Infect Immun,* 73**,** 2923-31.

THEODORATOU, E., JOHNSON, S., JHASS, A., MADHI, S. A., CLARK, A., BOSCHI-PINTO, C., BHOPAL, S., RUDAN, I. & CAMPBELL, H. 2010. The effect of *Haemophilus influenzae* type b and pneumococcal conjugate vaccines on childhood pneumonia incidence, severe morbidity and mortality. *Int J Epidemiol,* 39 Suppl 1**,** i172-85.

THEPPARIT, C. & SMITH, D. R. 2004. Serotype-specific entry of dengue virus into liver cells: Identification of the 37-kilodalton/67-kilodalton high-affinity laminin receptor as a dengue virus serotype 1 receptor. *Journal of Virology,* 78**,** 12647-12656.

THIGPEN, M. C., WHITNEY, C. G., MESSONNIER, N. E., ZELL, E. R., LYNFIELD, R., HADLER, J. L., HARRISON, L. H., FARLEY, M. M., REINGOLD, A., BENNETT, N. M., CRAIG, A. S., SCHAFFNER, W., THOMAS, A., LEWIS, M. M., SCALLAN, E., SCHUCHAT, A. & EMERGING INFECTIONS PROGRAMS, N. 2011. Bacterial meningitis in the United States, 1998-2007. *N Engl J Med,* 364**,** 2016-25.

THORNTON, J. A., DURICK-EDER, K. & TUOMANEN, E. I. 2010. Pneumococcal pathogenesis: "innate invasion" yet organ-specific damage. *J Mol Med (Berl),* 88**,** 103-7.

TILTON, R. C., DIAS, F. & RYAN, R. W. 1984. Comparative evaluation of three commercial products and counterimmunoelectrophoresis for the detection of antigens in cerebrospinal fluid. *J Clin Microbiol,* 20**,** 231-4.

TIO, P. H., JONG, W. W. & CARDOSA, M. J. 2005. Two dimensional VOPBA reveals laminin receptor (LAMR1) interaction with dengue virus serotypes 1, 2 and 3. *Virology Journal,* 2.

TOHGO, A., TAKASAWA, S., MUNAKATA, H., YONEKURA, H., HAYASHI, N. & OKAMOTO, H. 1994. Structural determination and characterization of a 40 kDa protein isolated from rat 40 S ribosomal subunit. *FEBS Lett,* 340**,** 133-8.

TOMASZ, A. 1979. The mechanism of the irreversible antimicrobial effects of penicillins: how the beta-lactam antibiotics kill and lyse bacteria. *Annu Rev Microbiol,* 33**,** 113-37.

TOMMASSEN, J., VERMEIJ, P., STRUYVE, M., BENZ, R. & POOLMAN, J. T. 1990. Isolation of *Neisseria meningitidis* mutants deficient in class 1 (porA) and class 3 (porB) outer membrane proteins. *Infect Immun,* 58**,** 1355-9.

TONG, H. H., BLUE, L. E., JAMES, M. A. & DEMARIA, T. F. 2000. Evaluation of the virulence of a *Streptococcus pneumoniae* neuraminidase-deficient mutant in nasopharyngeal colonization and development of otitis media in the chinchilla model. *Infection and Immunity,* 68**,** 921-924.

TONG, H. H., JAMES, M., GRANTS, I., LIU, X., SHI, G. & DEMARIA, T. F. 2001. Comparison of structural changes of cell surface carbohydrates in the eustachian tube epithelium of chinchillas infected with a *Streptococcus pneumoniae* neuraminidase-deficient mutant or its isogenic parent strain. *Microbial Pathogenesis,* 31**,** 309-317.

TONJUM, T., FREITAG, N. E., NAMORK, E. & KOOMEY, M. 1995. Identification and characterization of pilG, a highly conserved pilus-assembly gene in pathogenic Neisseria. *Mol Microbiol,* 16**,** 451-64.

TONJUM, T. & KOOMEY, M. 1997. The pilus colonization factor of pathogenic neisserial species: organelle biogenesis and structure/function relationships--a review. *Gene,* 192**,** 155-63.

TROLLFORS, B., LAGERGARD, T., CLAESSON, B. A., THORNBERG, E., MARTINELL, J. & SCHNEERSON, R. 1992. Characterization of the serum antibody response to the capsular polysaccharide of *Haemophilus influenzae* type b in children with invasive infections. *J Infect Dis,* 166**,** 1335-9.

TROTTER, C. L., MCVERNON, J., RAMSAY, M. E., WHITNEY, C. G., MULHOLLAND, E. K., GOLDBLATT, D., HOMBACH, J., KIENY, M. P. & SUBGROUP, S. 2008. Optimising the use of conjugate vaccines to prevent disease caused by *Haemophilus influenzae* type b, *Neisseria meningitidis* and *Streptococcus pneumoniae*. *Vaccine,* 26**,** 4434-45.

TROTTER, C. L. & RAMSAY, M. E. 2007. Vaccination against meningococcal disease in Europe: review and recommendations for the use of conjugate vaccines. *FEMS Microbiol Rev,* 31**,** 101-7.

TUBULEKAS, I., BERGLUND, P., FLEETON, M. & LILJESTROM, P. 1997. Alphavirus expression vectors and their use as recombinant vaccines: A minireview. *Gene,* 190**,** 191-195.

TUNIO, S. A., OLDFIELD, N. J., BERRY, A., ALA'ALDEEN, D. A., WOOLDRIDGE, K. G. & TURNER, D. P. 2010. The moonlighting protein fructose-1, 6-bisphosphate aldolase of *Neisseria meningitidis*: surface localization and role in host cell adhesion. *Mol Microbiol,* 76**,** 605-15.

TUNKEL, A. R., HARTMAN, B. J., KAPLAN, S. L., KAUFMAN, B. A., ROOS, K. L., SCHELD, W. M. & WHITLEY, R. J. 2004. Practice guidelines for the management of bacterial meningitis. *Clin Infect Dis,* 39**,** 1267-84.

TWUMASI, P. A., JR., KUMAH, S., LEACH, A., O'DEMPSEY, T. J., CEESAY, S. J., TODD, J., BROOME, C. V., CARLONE, G. M., PAIS, L. B., HOLDER, P. K. & ET AL. 1995. A trial of a group A plus group C meningococcal polysaccharide-protein conjugate vaccine in African infants. *J Infect Dis,* 171**,** 632-8.

TZENG, Y. L., DATTA, A. K., STROLE, C. A., LOBRITZ, M. A., CARLSON, R. W. & STEPHENS, D. S. 2005. Translocation and surface expression of lipidated serogroup B capsular Polysaccharide in *Neisseria meningitidis*. *Infect Immun,* 73**,** 1491-505.

UEMURA, L., GRASSI, N. C. & CAZARIN, L. 2001. Pneumococcal endocarditis of subacute evolution. *Arq Bras Cardiol,* 76**,** 319-22.

ULANOVA, M. & TSANG, R. S. 2009. Invasive *Haemophilus influenzae* disease: changing epidemiology and host-parasite interactions in the 21st century. *Infect Genet Evol,* 9**,** 594-605.

UMEDA, D., YANO, S., YAMADA, K. & TACHIBANA, H. 2008. Green tea Polyphenol epigallocatechin-3-gallate signaling pathway through 67-kDa laminin receptor. *Journal of Biological Chemistry,* 283**,** 3050-3058.

UNKMEIR, A., LATSCH, K., DIETRICH, G., WINTERMEYER, E., SCHINKE, B., SCHWENDER, S., KIM, K. S., EIGENTHALER, M. & FROSCH, M. 2002. Fibronectin mediates Opc-dependent internalization of *Neisseria meningitidis* in human brain microvascular endothelial cells. *Mol Microbiol,* 46**,** 933-46.

VACCA, A., RIBATTI, D., RONCALI, L., LOSPALLUTI, M., SERIO, G., CARREL, S. & DAMMACCO, F. 1993. Melanocyte Tumor Progression Is Associated with Changes in Angiogenesis and Expression of the 67-Kilodalton Laminin Receptor. *Cancer,* 72**,** 455-461.

VAN ALPHEN, L., GEELEN-VAN DEN BROEK, L., BLAAS, L., VAN HAM, M. & DANKERT, J. 1991. Blocking of fimbria-mediated adherence of *Haemophilus influenzae* by sialyl gangliosides. *Infect Immun,* 59**,** 4473-7.

VAN ALPHEN, L., POOLE, J., GEELEN, L. & ZANEN, H. C. 1987. The erythrocyte and epithelial cell receptors for *Haemophilus influenzae* are expressed independently. *Infect Immun,* 55**,** 2355-8.

VAN DE BEEK, D. 2012. Progress and challenges in bacterial meningitis. *Lancet,* 380**,** 1623-4.

VAN DE BEEK, D. & DE GANS, J. 2004. Meningitis-associated hearing loss: protection by adjunctive antioxidant therapy. *Ann Neurol,* 55**,** 597-8; author reply 598.

VAN DE BEEK, D., DE GANS, J., TUNKEL, A. R. & WIJDICKS, E. F. 2006. Community-acquired bacterial meningitis in adults. *N Engl J Med,* 354**,** 44-53.

VAN DE BEEK, D., SCHMAND, B., DE GANS, J., WEISFELT, M., VAESSEN, H., DANKERT, J. & VERMEULEN, M. 2002. Cognitive impairment in adults with good recovery after bacterial meningitis. *J Infect Dis,* 186**,** 1047-52.

VAN DER ENDE, A., HOPMAN, C. T. P. & DANKERT, J. 2000. Multiple mechanisms of phase variation of PorA in *Neisseria meningitidis*. *Infection and Immunity,* 68**,** 6685-6690.

VAN DER LEY, P., HECKELS, J. E., VIRJI, M., HOOGERHOUT, P. & POOLMAN, J. T. 1991. Topology of outer membrane porins in pathogenic *Neisseria spp. Infect Immun,* 59**,** 2963-71.

VAN DER LEY, P. & STEEGHS, L. 2003. Lessons from an LPS-deficient *Neisseria meningitidis* mutant. *J Endotoxin Res,* 9**,** 124-8.

VAN SORGE, N. M. & DORAN, K. S. 2012. Defense at the border: the blood-brain barrier versus bacterial foreigners. *Future Microbiol,* 7**,** 383-94.

VAN ZELE, T., COPPIETERS, F., GEVAERT, P., HOLTAPPELS, G., VAN CAUWENBERGE, P. & BACHERT, C. 2009. Local complement activation in nasal polyposis. *Laryngoscope,* 119**,** 1753-8.

VANA, K. & WEISS, S. 2006. A trans-dominant negative 37kDa/67kDa laminin receptor mutant impairs PrP(Sc) propagation in scrapie-infected neuronal cells. *J Mol Biol,* 358**,** 57-66.

VANA, K., ZUBER, C., PFLANZ, H., KOLODZIEJCZAK, D., ZEMORA, G., BERGMANN, A. K. & WEISS, S. 2009. LRP/LR as an alternative promising target in therapy of prion diseases, Alzheimer's disease and cancer. *Infect Disord Drug Targets,* 9**,** 69-80.

VANDENBRULE, F. A., CASTRONOVO, V., MENARD, S., GIAVAZZI, R., MARZOLA, M., BELOTTI, D. & TARABOLETTI, G. 1996. Expression of the 67 kD laminin receptor in human ovarian carcinomas as defined by a monoclonal antibody, MLuC5. *European Journal of Cancer,* 32A**,** 1598-1602.

VENTICINQUE, L. & MERUELO, D. 2012. Comprehensive proteomic analysis of nonintegrin laminin receptor interacting proteins. *J Proteome Res,* 11**,** 4863-72.

VERHEUL, A. F., SNIPPE, H. & POOLMAN, J. T. 1993. Meningococcal lipopolysaccharides: virulence factor and potential vaccine component. *Microbiol Rev,* 57**,** 34-49.

VESIKARI, T., PRYMULA, R., MERRALL, E., KOHL, I., TONEATTO, D. & DULL, P. M. 2015. Meningococcal serogroup B vaccine (4CMenB): Booster dose in

previously vaccinated infants and primary vaccination in toddlers and two-year-old children. *Vaccine,* 33**,** 3850-8.

VIDARSSON, G., OVERBEEKE, N., STEMERDING, A. M., VAN DEN DOBBELSTEEN, G., VAN ULSEN, P., VAN DER LEY, P., KILIAN, M. & VAN DE WINKEL, J. G. 2005. Working mechanism of immunoglobulin A1 (IgA1) protease: cleavage of IgA1 antibody to *Neisseria meningitidis* PorA requires de novo synthesis of IgA1 Protease. *Infect Immun,* 73**,** 6721-6.

VIRJI, M. 2009. Pathogenic neisseriae: surface modulation, pathogenesis and infection control. *Nat Rev Microbiol,* 7**,** 274-86.

VIRJI, M., ALEXANDRESCU, C., FERGUSON, D. J., SAUNDERS, J. R. & MOXON, E. R. 1992. Variations in the expression of pili: the effect on adherence of *Neisseria meningitidis* to human epithelial and endothelial cells. *Mol Microbiol,* 6**,** 1271-9.

VIRJI, M., HECKELS, J. E., POTTS, W. J., HART, C. A. & SAUNDERS, J. R. 1989. Identification of epitopes recognized by monoclonal antibodies SM1 and SM2 which react with all pili of *Neisseria gonorrhoeae* but which differentiate between two structural classes of pili expressed by *Neisseria meningitidis* and the distribution of their encoding sequences in the genomes of Neisseria spp. *J Gen Microbiol,* 135**,** 3239-51.

VIRJI, M., MAKEPEACE, K., FERGUSON, D. J. & WATT, S. M. 1996. Carcinoembryonic antigens (CD66) on epithelial cells and neutrophils are receptors for Opa proteins of pathogenic neisseriae. *Mol Microbiol,* 22**,** 941-50.

VIRJI, M., MAKEPEACE, K., PEAK, I. R., FERGUSON, D. J., JENNINGS, M. P. & MOXON, E. R. 1995. Opc- and pilus-dependent interactions of meningococci with human endothelial cells: molecular mechanisms and modulation by surface polysaccharides. *Mol Microbiol,* 18**,** 741-54.

VIRJI, M., SAUNDERS, J. R., SIMS, G., MAKEPEACE, K., MASKELL, D. & FERGUSON, D. J. 1993. Pilus-facilitated adherence of *Neisseria meningitidis* to human epithelial and endothelial cells: modulation of adherence phenotype occurs concurrently with changes in primary amino acid sequence and the glycosylation status of pilin. *Mol Microbiol,* 10**,** 1013-28.

VIRKOLA, R., BRUMMER, M., RAUVALA, H., VAN ALPHEN, L. & KORHONEN, T. K. 2000. Interaction of fimbriae of *Haemophilus influenzae* type B with heparin-binding extracellular matrix proteins. *Infect Immun,* 68**,** 5696-701.

VITOVSKI, S., DUNKIN, K. T., HOWARD, A. J. & SAYERS, J. R. 2002. Nontypeable *Haemophilus influenzae* in carriage and disease: a difference in IgA1 protease activity levels. *JAMA,* 287**,** 1699-705.

VUONG, J., WANG, X., THEODORE, J. M., WHITMON, J., GOMEZ DE LEON, P., MAYER, L. W., CARLONE, G. M. & ROMERO-STEINER, S. 2013. Absence of high molecular weight proteins 1 and/or 2 is associated with decreased adherence among non-typeable *Haemophilus influenzae* clinical isolates. *J Med Microbiol,* 62**,** 1649-56.

WALTER, M., CHABAN, C., SCHUTZE, K., BATISTIC, O., WECKERMANN, K., NAKE, C., BLAZEVIC, D., GREFEN, C., SCHUMACHER, K., OECKING, C., HARTER, K. & KUDLA, J. 2004. Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant Journal,* 40**,** 428-438.

WANG, K. S., KUHN, R. J., STRAUSS, E. G., OU, S. & STRAUSS, J. H. 1992. High-Affinity Laminin Receptor Is a Receptor for Sindbis Virus in Mammalian-Cells. *Journal of Virology,* 66**,** 4992-5001.

WANG, M. H. & KIM, K. S. 2013. Cytotoxic Necrotizing Factor 1 Contributes to *Escherichia coli* Meningitis. *Toxins,* 5**,** 2270-2280.

WARNER, J. R. & MCINTOSH, K. B. 2009. How Common Are Extraribosomal Functions of Ribosomal Proteins? *Molecular Cell,* 34**,** 3-11.

WATANABE, N., BODIN, L., PANDEY, M., KRAUSE, M., COUGHLIN, S., BOUSSIOTIS, V. A., GINSBERG, M. H. & SHATTIL, S. J. 2008. Mechanisms and consequences of agonist-induced talin recruitment to platelet integrin alphaIIbbeta3. *J Cell Biol,* 181**,** 1211-22.

WATT, J. P., WOLFSON, L. J., O'BRIEN, K. L., HENKLE, E., DELORIA-KNOLL, M., MCCALL, N., LEE, E., LEVINE, O. S., HAJJEH, R., MULHOLLAND, K., CHERIAN, T., HIB & PNEUMOCOCCAL GLOBAL BURDEN OF DISEASE STUDY, T. 2009. Burden of disease caused by *Haemophilus influenzae* type b in children younger than 5 years: global estimates. *Lancet,* 374**,** 903-11.

WEBB, S. A. & KAHLER, C. M. 2008. Bench-to-bedside review: Bacterial virulence and subversion of host defences. *Crit Care,* 12**,** 234.

WEISER, J. N. 1998. Phase variation in colony opacity by *Streptococcus pneumoniae*. *Microb Drug Resist,* 4**,** 129-35.

WEISER, J. N., AUSTRIAN, R., SREENIVASAN, P. K. & MASURE, H. R. 1994. Phase Variation in Pneumococcal Opacity - Relationship between Colonial Morphology and Nasopharyngeal Colonization. *Infection and Immunity,* 62**,** 2582-2589.

WEISER, J. N., CHONG, S. T., GREENBERG, D. & FONG, W. 1995. Identification and characterization of a cell envelope protein of *Haemophilus influenzae* contributing to phase variation in colony opacity and nasopharyngeal colonization. *Mol Microbiol,* 17**,** 555-64.

WEISER, J. N., MARKIEWICZ, Z., TUOMANEN, E. I. & WANI, J. H. 1996. Relationship between phase variation in colony morphology, intrastrain variation in cell wall physiology, and nasopharyngeal colonization by *Streptococcus pneumoniae*. *Infection and Immunity,* 64**,** 2240-2245.

WESTERGARD, L., CHRISTENSEN, H. M. & HARRIS, D. A. 2007. The cellular prion protein (PrP(C)): its physiological function and role in disease. *Biochim Biophys Acta,* 1772**,** 629-44.

WEWER, U. M., LIOTTA, L. A., JAYE, M., RICCA, G. A., DROHAN, W. N., CLAYSMITH, A. P., RAO, C. N., WIRTH, P., COLIGAN, J. E., ALBRECHTSEN, R. & ET AL. 1986. Altered levels of laminin receptor mRNA in various human carcinoma cells that have different abilities to bind laminin. *Proc Natl Acad Sci U S A,* 83**,** 7137-41.

WHITCHURCH, C. B., HOBBS, M., LIVINGSTON, S. P., KRISHNAPILLAI, V. & MATTICK, J. S. 1991. Characterisation of a Pseudomonas aeruginosa twitching motility gene and evidence for a specialised protein export system widespread in eubacteria. *Gene,* 101**,** 33-44.

WILSON, R., READ, R. & COLE, P. 1992. Interaction of *Haemophilus influenzae* with mucus, cilia, and respiratory epithelium. *J Infect Dis,* 165 Suppl 1**,** S100-2.

WINN, W., JR, S. A., JANDA, W., KONEMAN, E., PROCOP, G., SCHRECKENBERGER, P. & WOODS, G. 2006. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. *Lippincott Williams & Wilkins, Philadelphia*.

WINTHER-LARSEN, H. C., HEGGE, F. T., WOLFGANG, M., HAYES, S. F., VAN PUTTEN, J. P. & KOOMEY, M. 2001. *Neisseria gonorrhoeae* PilV, a type IV pilus-associated protein essential to human epithelial cell adherence. *Proc Natl Acad Sci U S A,* 98**,** 15276-81.

WITHEROW, D. S. & CARSON, S. 2011. A laboratory-intensive course on the experimental study of protein-protein interactions. *Biochem Mol Biol Educ,* 39**,** 300-8.

WOLFGANG, M., LAUER, P., PARK, H. S., BROSSAY, L., HEBERT, J. & KOOMEY, M. 1998. PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated *Neisseria gonorrhoeae*. *Mol Microbiol,* 29**,** 321-30.

WOLFGANG, M., VAN PUTTEN, J. P., HAYES, S. F., DORWARD, D. & KOOMEY, M. 2000. Components and dynamics of fiber formation define a ubiquitous biogenesis pathway for bacterial pili. *EMBO J,* 19**,** 6408-18.

WONG, S. M., ST MICHAEL, F., COX, A., RAM, S. & AKERLEY, B. J. 2011. ArcA-regulated glycosyltransferase lic2B promotes complement evasion and pathogenesis of nontypeable *Haemophilus influenzae*. *Infect Immun,* 79**,** 1971-83.

WOPFNER, F., WEIDENHOFER, G., SCHNEIDER, R., VON BRUNN, A., GILCH, S., SCHWARZ, T. F., WERNER, T. & SCHATZL, H. M. 1999. Analysis of 27 mammalian and 9 avian PrPs reveals high conservation of flexible regions of the prion protein. *J Mol Biol,* 289**,** 1163-78.

WUEST, D. M., HARCUM, S. W. & LEE, K. H. 2012. Genomics in mammalian cell culture bioprocessing. *Biotechnol Adv,* 30**,** 629-38.

WYNN, R. F., LAING, R. B., LEEN, C. L., STATHAM, P. & EMMANUEL, F. X. 1994. A case of chronic meningococcal meningitis. *Clin Infect Dis,* 18**,** 829-30.

YAMAGUCHI, H. & MIYAZAKI, M. 2014. Refolding techniques for recovering biologically active recombinant proteins from inclusion bodies. *Biomolecules,* 4**,** 235-51.

YAMAMOTO, M., MCDANIEL, L. S., KAWABATA, K., BRILES, D. E., JACKSON, R. J., MCGHEE, J. R. & KIYONO, H. 1997. Oral immunization with PspA elicits protective humoral immunity against *Streptococcus pneumoniae* infection. *Infect Immun,* 65**,** 640-4.

YANG, R. Y., HSU, D. K. & LIU, F. T. 1996. Expression of galectin-3 modulates T-cell growth and apoptosis. *Proc Natl Acad Sci U S A,* 93**,** 6737-42.

YEO, H. J., COTTER, S. E., LAARMANN, S., JUEHNE, T., ST GEME, J. W., 3RD & WAKSMAN, G. 2004. Structural basis for host recognition by the *Haemophilus influenzae* Hia autotransporter. *EMBO J,* 23**,** 1245-56.

YEO, H. J., YOKOYAMA, T., WALKIEWICZ, K., KIM, Y., GRASS, S. & GEME, J. W., 3RD 2007. The structure of the *Haemophilus influenzae* HMW1 pro-piece reveals a structural domain essential for bacterial two-partner secretion. *J Biol Chem,* 282**,** 31076-84.

YI, K. & MURPHY, T. F. 1997. Importance of an immunodominant surface-exposed loop on outer membrane protein P2 of nontypeable *Haemophilus influenzae*. *Infect Immun,* 65**,** 150-5.

YIN, J. C., LI, G. X., REN, X. F. & HERRLER, G. 2007. Select what you need: A comparative evaluation of the advantages and limitations of frequently used expression systems for foreign genes. *Journal of Biotechnology,* 127**,** 335-347.

ZHANG, J., CARTER, J., SIU, S., O'NEILL, J. W., GATES, A. H., DELANEY, J. & MEHLIN, C. 2010. Fusion Partners as a Tool for the Expression of Difficult Proteins in Mammalian Cells. *Current Pharmaceutical Biotechnology,* 11**,** 241-245.

ZHANG, J. R., MOSTOV, K. E., LAMM, M. E., NANNO, M., SHIMIDA, S., OHWAKI, M. & TUOMANEN, E. 2000. The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells. *Cell,* 102**,** 827-837.

ZHANG, J. R. & TUOMANEN, E. 1999. Molecular and cellular mechanisms for microbial entry into the CNS. *J Neurovirol,* 5**,** 591-603.

ZHOU, J., LAW, D. K., SILL, M. L. & TSANG, R. S. 2007. Nucleotide sequence diversity of the bexA gene in serotypeable *Haemophilus influenzae* strains recovered from invasive disease patients in Canada. *J Clin Microbiol,* 45**,** 1996-9.

ZHOU, Y., YU, Q., QIN, X., BHAVSAR, D., YANG, L., CHEN, Q., ZHENG, W., CHEN, L. & LIU, J. 2015. Improving the Anticancer Efficacy of Laminin Receptor-Specific Therapeutic Ruthenium Nanoparticles (RuBB-Loaded EGCG-RuNPs) via ROS-Dependent Apoptosis in SMMC-7721 Cells. *ACS Appl Mater Interfaces*.

ZHU, L., PEARCE, D. & KIM, K. S. 2010. Prevention of *Escherichia coli* K1 penetration of the blood-brain barrier by counteracting the host cell receptor and signaling molecule involved in *E. coli* invasion of human brain microvascular endothelial cells. *Infect Immun,* 78**,** 3554-9.

ZHU, P., MORELLI, G. & ACHTMAN, M. 1999. The opcA and (psi)opcB regions in Neisseria: genes, pseudogenes, deletions, insertion elements and DNA islands. *Mol Microbiol,* 33**,** 635-50.

ZIDANE, N., OULD-ABEIH, M. B., PETIT-TOPIN, I. & BEDOUELLE, H. 2013. The folded and disordered domains of human ribosomal protein SA have both idiosyncratic and shared functions as membrane receptors. *Bioscience Reports,* 33**,** 113-124.

ZOLLINGER, W. D., POOLMAN, J. T. & MAIDEN, M. C. 2011. Meningococcal serogroup B vaccines: will they live up to expectations? *Expert Rev Vaccines,* 10**,** 559-61.

ZUBER, C., KNACKMAASS, S., ZEMORA, G., REUSCH, U., VLASOVA, E., DIEHL, D., MICK, V., HOFFMAN, K., NIKLES, D., FROEHLICH, T., ARNOLD, G. J., BRENIG, B., WOLF, E., LAHM, H., LITTLE, M. & WEISS, S. 2008a. Invasion of tumorigenic HT1080 cells is impeded by blocking or downregulating the 37-kDa/67-kDa laminin receptor. *Journal of Molecular Biology,* 378**,** 530-539.

ZUBER, C., KNACKMUSS, S., REY, C., REUSCH, U., ROTTGEN, P., FROHLICH, T., ARNOLD, G. J., PACE, C., MITTEREGGER, G., KRETZSCHMAR, H. A., LITTLE, M. & WEISS, S. 2008b. Single chain Fv antibodies directed against the 37 kDa/67 kDa laminin receptor as therapeutic tools in prion diseases. *Mol Immunol,* 45**,** 144-51.

ZUBER, C., KNACKMUSS, S., ZEMORA, G., REUSCH, U., VLASOVA, E., DIEHL, D., MICK, V., HOFFMANN, K., NIKLES, D., FROHLICH, T., ARNOLD, G. J., BRENIG, B., WOLF, E., LAHM, H., LITTLE, M. & WEISS, S. 2008c. Invasion of tumorigenic HT1080 cells is impeded by blocking or downregulating the 37-kDa/67-kDa laminin receptor. *J Mol Biol,* 378**,** 530-9.

ZUBER, C., MITTEREGGER, G., SCHUHMANN, N., REY, C., KNACKMUSS, S., RUPPRECHT, W., REUSCH, U., PACE, C., LITTLE, M., KRETZSCHMAR, H. A., HALLEK, M., BUNING, H. & WEISS, S. 2008d. Delivery of single-chain antibodies (scFvs) directed against the 37/67 kDa laminin receptor into mice via recombinant adeno-associated viral vectors for prion disease gene therapy. *Journal of General Virology,* 89**,** 2055-2061.

ZUGHAIER, S. M., TZENG, Y. L., ZIMMER, S. M., DATTA, A., CARLSON, R. W. & STEPHENS, D. S. 2004. *Neisseria meningitidis* lipooligosaccharide structure-dependent activation of the macrophage CD14/Toll-like receptor 4 pathway. *Infect Immun,* 72**,** 371-80.

Appendix

Formula of Commonly Used Buffers and Solutions

APPENDIX

Formula of Commonly Used Buffers and Solutions

Sodium dodecyl sulfate (SDS)-resolving buffer; (36.34 g Tris base, 8 ml 10% SDS add deionised waster (dH_2O) up to 200 ml, pH at 8.8).

SDS-staking buffer; (12.114 g Tris base, 8 ml 10% SDS) add dH_2O up to 200 ml, pH at 6.8.

SDS running buffer (10 ×); dissolve 30.3 g (0.25 M) Tris base, 187.7 g (2.5 M) Glycine, in 950 ml dH_2O, add 10 g SDS (1%), made up to 1000 ml with dH_2O, mix well.

SDS-sample buffer (5 x); 0.62 M Tris-Cl (pH 6.8), 5% SDS, 25% glycerol, 12.5% meracaptoethanol, 0.002% Bromophenol blue, or 7.8125 ml (2 M) Tris-Cl, 1.25 g SDS, 6.25 ml glycerol, 3.125 ml β-meracaptoethanol, tip of pipette Bromophenol blue, made up to 25 ml with dH_2O.

SDS-sample buffer (1 x); 2.72 g Tris, 25 ml of 20% SDS, 50% glycerol, 12.5% meracaptoethanol, 0.002% Bromophenol blue and add dH_2O up to 100 ml, pH at 6.8.

SDS-sample buffer (5 ×); 0.62 M Tris-Cl (pH 6.8), 5% SDS, 25% glycerol, 12.5% β-meracaptoethanol, 0.002% Bromophenol blue, or 7.8125 ml (2 M) Tris-Cl, 1.25 g SDS, 6.25 ml glycerol, 3.125 ml β-meracaptoethanol, tip of pipette Bromophenol blue, made up to 25 ml with dH_2O.

Resolving (separating) gel; 1.7 ml SDS-resolving buffer, 2.33 ml Acrylamide/Bis-Acrylamide (30%), 2.88 ml dH_2O, 30 µl 10% Ammonium persulfate (APS) and 30 µl Tetramethyl ethylenediamine (TEMED).

Staking gel; 1 ml SDS-stacking buffer, 0.8 ml Acrylamide/Bis-Acrylamide (30%), 2.18 ml dH_2O, 30 µl 10% APS and 30 µl TEMED.

Semi-dry blotting buffer; 5.82 g Tris base, 2.93 g Glycine, 3.75ml 10% SDS, 200 ml methanol and make up to 1000 ml with dH_2O.

Phosphate buffered saline solution (PBS); prepared by dissolving 1 tablet of Phosphate buffered saline (Dulbaco A, Oxoid) in 100 ml dH_2O and autoclave, this gives sodium chloride 0.16 mol, potassium chloride 0.003 mol, disodium hydrogen phosphate 0.008 mol and potassium dihydrogen phosphate 0.001 mol with a pH value of 7.3.

BSA; (Albumin from bovine serum, Sigma A3912) as lyophilized powder (M W *c.* 66 kDa) was prepared in sterile PBS according to the concentration needed.

Blocking buffer; 2.52 g skimmed milk powder, 50 ml PBS/0.05% Tween-20 (400 ml PBS with 200 µl Tween-20); or add 1 g BSA to 100 ml of PBS (1% BSA in PBS).

PBS-Tween washing buffer; 400 ml PBS/200 µl Tween-20.

LB (Luria-Bertani) broth; Tryptone 10 g, Yeast extract 5 g and Sodium chloride 10 g for 1000 ml dH_2O.

LB (Luria-Bertani) agar; Tryptone 10 g, Yeast extract 5 g, Sodium chloride 10g, Microbial tested agar 15 g for 1000 ml of dH_2O, pH 7.0 ± 0.2 at 25°C.

IPTG (Isopropyl-β-D thiogalactopyranoside); 1 M solution stock solution was prepared by dissolving 0.23 g of IPTG in 1 ml dH_2O, sterilize by filtration and store at -20°C.

DNA loading dye (6 ×); 10 mM Tris-HCl (pH 7.6), 0.03% Bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM Ethylenediamine tetraacetic acid (EDTA).

TAE buffer (50 × stock); 2 M Tris base (242 g) (life technologies), 2 M Acetic acid (57.1 ml glacial acetic acid [Fisher chemicals]) 50 mM EDTA (18.6 g) (pH 8.0) made up to 1000 ml with dH_2O.

Agarose gel; 0.8-1.0 % agarose was prepared by dissolving 0.8-1 g of agarose powder (sigma), 2 ml 50 × TAE buffer and made up to 100 ml with dH_2O; melt and add 5 µl ethidium bromide (10 mg ml^{-1}).

ELISA coating (carbonate) buffer; 0.1M Sodium Carbonate (Na2CO3) and 0.1M Sodium Bicarbonate (NaH_2CO3); pH 9.37.

Alkaline phosphatase buffer; 7.879g Tris-HCl, 2.92g NaCl, 0.1016g $MgCl_2.6H_2O$ in 500 ml of H2O, pH: 9.5.

RIPA buffer; 85% v/v 1M TE, 3.8% v/v 4M NaCl, 10% v/v 10% NP4O, 1% v/v 10% SDS, 5 mg ml-1 deoxycolate

Antibiotics; Antibiotics were purchased from Sigma-Aldrich UK, prepared according to the manufacturer's recommendations, sterilized by filtration and store at 4°C.