

Analysis of Complement Deposition on *Chlamydia trachomatis*

Nichlas Karred
Master's Thesis
Medicine with Industrial Specialization
Biomedicine
2018

Analysis of Complement Deposition on *Chlamydia trachomatis*

Master's Thesis
Medicine with Industrial Specialization
Biomedicine
2018

Nichlas Karred
Group 17gr9028
4th of September 2017 to 31th of May

Supervisor: Professor, DMsc, PhD Svend Birkelund
Co-supervisor: PhD fellow Mads Lausen Nielsen

Number of pages: 43
Number of supplementary: 1

Department of Health Science and Technology
Aalborg University

Table of content

Preface	1
Abstract	2
Dansk resumé (Danish Abstract)	3
1. Introduction	4
1.1 <i>Epidemiology</i>	4
1.2 <i>Structure of the chlamydial membrane</i>	4
1.3 <i>Developmental cycle of Chlamydiae</i>	5
1.4 <i>Immune responses to Chlamydial infections</i>	6
1.5 <i>The complement system</i>	7
1.6 <i>Activation of the complement cascade</i>	8
1.7 <i>Structure of complement C3</i>	9
1.8 <i>Fragments of complement C3</i>	9
1.9 <i>Chlamydiae and the complement system</i>	10
2. Material and methods	12
2.1 <i>Antibodies</i>	12
2.2 <i>Preparation of human serum</i>	12
2.3 <i>Chlamydiae culture and purification</i>	12
2.4 <i>Purification of peptides using C18 filter tips</i>	13
2.5 <i>Sample analysis using LC-MS/MS</i>	14
2.6 <i>Protein identification using MaxQuant</i>	14
2.7 <i>Immunoelectron microscopy</i>	14
2.8 <i>Immunofluorescence staining</i>	15
2.9 <i>Immunoblot analysis</i>	16
3. Results	17
3.1 <i>Analysis of complement C3 using by LC-MS/MS approach</i>	17
3.2 <i>Analysis of complement C3 using a bottom up LC-MS/MS approach</i>	17
3.3 <i>Investigating the deposition of complements C3 and C4 on C. trachomatis outer membrane</i>	21
3.4 <i>Confocal imaging of chlamydial Pmps</i>	24
3.5 <i>Immunoblot analysis using polyclonal anti-Pmp antibodies C. trachomatis</i>	26
4. Discussion	28
5. Summary	32
6. References	33
7. Supplementary	39

7.1 Schematic representation of the sequence coverage of C3 using Chymotrypsin.....39

7.2 Images of *C. trachomatis* after trypsination.....40



Preface

This thesis was done at the Department of Health, Science and Technology at Aalborg. The work was conducted from the 4th of September to the 31th of May at Laboratory for Medical Mass spectrometry under supervision by Professor, DMSc, PhD Svend Birkelund.

The author would like to thank Svend Birkelund for providing the facilities and instruments for this work. Thank you for being helpful, competent and for making this a great learning experience.

A special thank you to PhD Fellow Mads Lausen Nielsen for all your inputs and your supervision throughout this work.

Also a thank you to Assistant Professor, PhD Tue Bjerg Bennike for helping with searching MaxQuant and data analysis.

I would like thank Professor, DMSc Gunna Christiansen for providing the electron microscopy images, for critical reading and comments throughout the writing process.

Abstract

Chlamydia trachomatis are obligate intracellular gram-negative bacteria that are of major concern with regard to public health. They follow a biphasic development cycle and present as two morphologically distinct forms named: elementary bodies (EB) and reticulate bodies (RB). The integrity of the outer membrane is maintained by a set of crosslinked proteins through the chlamydial outer membrane complex (COMC).

The COMC contains proteins called polymorphic membrane proteins (Pmp). These Pmps are transmembranous autotransporters with an extracellular part that acts as an adhesins in the attachment of *Chlamydia* to host cells. The immune response against *Chlamydiae* involves cellular and humoral components of the immune system but infections tend to be chronic. Complement proteins are a central part of innate immunity but little is known about the activation and functions of these proteins in infections with *Chlamydiae*. Complement component C3 and its cleavage fragments are known to bind to the outer membrane but exact target of C3 is not known and the activity of complement varies between serotypes.

This study used liquid chromatography-tandem mass spectrometry to investigate the deposition of complement system proteins on the surface of *Chlamydia trachomatis* serovar L2 after incubation in normal human serum. Using this approach, the study found complement components C3b along with components of both the classic and alternative pathway as well as proteins known to inhibit the terminal complex. Immunoelectron microscopy confirmed the presence of C3b on intact *Chlamydia trachomatis* serovar L2 after serum incubation. The use of two different C3 specific antibodies on immunoelectron microscopy and immunoblot suggested the presence of fragments C3dg and iC3b. Immunoblot analysis showed that proteins from normal serum binds covalently to the outer membrane complex of *Chlamydia trachomatis* serovar L2 as high molecular protein bands. Further characterization using several Pmp-specific antibodies showed that PmpA is a target for these serum proteins.

Thus, this study suggests that complement C3 fragments are able bind to PmpA on the outer membrane of *Chlamydia trachomatis* serovar L2, but that this deposition of C3 does not necessarily lead to formation of MAC and complement mediated lysis.

Dansk resumé (Danish Abstract)

Chlamydia trachomatis er en obligat intracellulær gram negative bakterie af stor helbredsmæssig betydning. Dens livscyklus er bifasisk og findes som to morfologisk distinkte former: elementary bodies (EB) og reticulate bodies (RB). Bakteriens strukturelle integritet opretholdes af en ydermembran bestående af krydsbundne proteiner det betegnes COMC (Chlamydial Outer Membrane Complex).

COMC indeholder også de såkaldte polymorfe membranproteiner (Pmp). Disse proteiner er transmembrane autotransportere med en ekstracellulær komponent der virker som adhesin i under *Chlamydia*-bakteriens vedhæftning til værtscellen.

Immunsvaret mod infektioner med *Chlamydia*-bakterier involverer cellulære og humorale komponenter, men infektionerne ses ofte at være kroniske. Komplementproteiner er en central del af det innate immunforsvar, men meget lidt er kendt angående aktivering af komplement systemet og dettes funktion under *Chlamydia*-infektioner. Komplementkomponent C3 og dettes kløvningsprodukter har vist sig at binde til ydermembranen af *Chlamydia trachomatis* serovar L2, men hvilke strukturer den præcist binder er ikke kendt.

Dette studie anvendte Liquid chromatography-tandem massespektrometri til at undersøge aflejlingen af komplementproteiner på overfladen af *Chlamydia trachomatis* serovar L2 efter inkubation af bakterien i normalt humant serum. Studiet fandt ved brug af denne metode at komplementkomponent C3b samt komponenter fra den klassiske og alternative C3-konvertase og proteiner der inhiberer det terminale kompleks. Immunoelektronmikroskopi blev anvendt til at bekræfte tilstedeværelsen af C3b på intakte *Chlamydia trachomatis* serovar L2 efter seruminkubation. Der blev anvendt to forskellige C3 specifikke antistoffer til bade immunoelektronmikroskopi og immunoblot. Resultaterne herfra tydede på tilstedeværelsen af både C3dg og iC3b.

Immunoblotanalysen viste at proteiner fra normalt serum kan binde sig kovalent til ydermembranen på *Chlamydia trachomatis* serovar. Dette ses som højmolekylære bånd på immunoblots. Videre undersøgelse med flere Pmp-specifikke antistoffer viste at serumproteiner kan binde til PmpA. Dette studie antyder, at fragmenter af komplement C3 kan binde PmpA på ydermembranen af *Chlamydia trachomatis* serovar L2 men at dette ikke nødvendigvis fører til dannelse af et terminal kompleks med komplement-medieret lyse til følge.

1. Introduction

1.1 Epidemiology

Chlamydiae trachomatis (*C. trachomatis*) are obligate intracellular gram-negative bacteria that are of major concern with regard to public health. *C. trachomatis* serologically divided into 15 serovars (A, B, Ba, C-K and L1-3) that give rise to disease in humans (1). Serovar A-C cause trachoma that is highly prevalent in developing countries. Blinding trachoma is leading cause of visual impairments and blindness by infectious agents with an estimated prevalence of 1.2 million. (2)

The sexually transmitted disease (STD) known simply as *Chlamydia* is conferred by serovars D-K and remains a huge burden in health care settings around the world. As of 2016, genital Chlamydia infections are the most common STD worldwide with an estimated incidence of 131 million new cases every year (2). Although symptoms such as dysuria and urethral discharge are common, the condition is often asymptomatic and thereby insidious. If left untreated, serious damage to reproductive organs might ensue leading serious complications such as ectopic pregnancies and sterility in female patients (3).

A special case of chlamydial infections is lymphogranuloma venereum caused by Serovars L1-3. As with serovars D-K it is spread through sexual intercourse but unlike these serovars it infects lymphatic tissue in lymph nodes and not epithelial cells. Here it proliferates in resident phagocytes and in some cases disseminate and cause systemic disease. The condition is most commonly reported in homosexual males that present with acute disease, but the overall incidence is low (1).

1.2 Structure of the chlamydial membrane

Chlamydiae are gram negative bacteria in that they possess an outer membrane, a cell wall and an inner membrane. The cell wall is practically devoid of peptidoglycan and its existence in Chlamydiae was only recently confirmed.(4)

The integrity of the membrane is instead maintained by a set of cysteine rich proteins that are crosslinked through disulfide bonds, the so called chlamydial outer membrane complex (COMC). (5). The major outer membrane protein (MOMP) is the most abundant comprising more than 60% of COMC.(6) It contains variable domains that are immunogenic and differ between serovars. Other proteins that confer structural integrity to the bacteria are Omp2 and Omp3. A rough version of lipopolysaccharide (LPS) has also been found in the membrane in close relation to MOMP.(7)

The COMC also comprises a number of serovar specific proteins called polymorphic membrane proteins (Pmp). These proteins are autotransporters that span the outer membrane with an extracellular part that are believed to act as an adhesins in the attachment of Chlamydia to host cells. The genome of *C. trachomatis* codes for 9 Pmps (PmpA-I) that are all expressed. The amino acid sequence of Pmps varies between serovars but with some degree of homology. PmpD shows the highest degree of homology between *Chlamydia* species and serovars. Similarly, the sequence similarity between PmpA, PmpD and PmpI is 99% (8).

1.3 Developmental cycle of Chlamydiae

Chlamydiae follow a biphasic development cycle and are present as two morphologically and metabolically distinct forms named: elementary bodies (EB) and reticulate bodies (RB). EB are 300 nm in size with a round shape, a resilient membrane that allows them to survive extracellularly and the potential to infect host cells. Historically they have been thought of as a “spore-like” form of the bacterium that is metabolically inert. RB are the non-infectious, fully active, replicating organisms that are approximately 1 µm in diameter. This form is found exclusively intracellularly in epithelial cells.(9,10)

Adhesion to the host cell membranes is believed to be a two-step process that involves an initial and reversible electrostatic binding using heparan sulphate proteoglycans and a subsequent, irreversible binding to receptors on the host cell. (11,12) Entry into host cells is facilitated by reorganisation of actin filaments of the host cells in an GTPase dependent manner. This is achieved through type III secretion of a prepackaged protein, Translocated actin recruiting phosphoprotein (Tarp), that is injected into the host cell. Tarp is rapidly phosphorylated by host tyrosin kinases and induces actin polymerisation.(13,14). Inside the cell, the Chlamydiae reside in a specialized vesicle that is termed the chlamydial inclusion. The inclusion is able to avert fusion to lysosomes and instead associates with the Golgi apparatus. Inside the inclusion rapidly differentiates from the EB to RB and start replicating. After a period of replication, the RB revert to the EB and are released from the host cell by either lysis of the host cell or process called extrusion where the inclusion is detached from the host cell. (3)

1.4 Immune responses to Chlamydial infections

A successful immune response against Chlamydiae involves both cellular and humoral components of the immune system.(15) Studies in mice form the basis of our understanding of these infections. The exact manner in which chlamydial infections are resolved is not fully understood and a detailed review of all possible scenarios is beyond the scope of this study. Instead, a brief outline of major players in chlamydia immunity based on current literature is provided below.

The resolution of primary chlamydial infection relies heavily on CD4+ T-cells as well as anti-chlamydial antibodies.(16) CD4+ T-cells recognize chlamydial antigens on MHC II of antigen presenting cells (APC) which leads to maturation of the T-cells to Type 1 Helper T-cells (Th1).(17) Early infections with Chlamydiae are controlled by an innate immune response that starts with the recruitment of neutrophils and natural killer cells (NK cells) as a response to cytokine secretion from resident dendritic cells (DC) and epithelial cells.(18,19) NK cells are cytotoxic lymphocytes that target cells infected with intracellular pathogens, but they also release of IFN- γ which hints a possible role in stimulating Th1 immune response of CD4+ cells.(20)

The role of IFN γ is two-fold: it has been shown to indirectly inhibit the growth of *C. trachomatis* *in vitro* cell culture and it stimulates phagocytosis in macrophages (21).

Chlamydiae enters DC in the same manner as they infect epithelial cells, but they do not readily propagate inside DC. The ability of DC to present Chlamydial antigens on MHC receptors for T-cells has been described and is regarded as important for protective immunity against future infections.(22) For this reason, DC have received a lot of attention in the search for an effective vaccine against *Chlamydia* infections.

The role of macrophages against invading pathogens is well recognized but their role with respect to chlamydial infections remain poorly documented. Macrophages have been shown to migrate to areas of chlamydial infection and are also activated by INF γ . Activated macrophages display increased autophagocytosis which in turn leads to increased antigen-presentation.(23) Additionally, infection with Chlamydia induces production of pro-inflammatory cytokines IL-1 α , IL-1 β , IL-6, IL-8, IL-10 and IL-12p70 *in vitro*. (19,24) While Chlamydia are able to enter into macrophages, they are not able to survive as they are unable to avoid fusion with lysosomes.(25,26)

B-cells have been known to play a part in chlamydial infection for some time and antibodies against chlamydia have been known to be elevated for 40 years.(27) While it is generally accepted that antibodies have a protective role in secondary infections, it is still disputed whether antibodies have a role in primary infections with Chlamydia. Mice deficient in both CD4+ T-cells and B-cells are not able to control secondary chlamydia infections, while mice deficient in only CD+4 T-cells were able eliminate Chlamydia.

1.5 The complement system

The complement system consists of over 30 proteins and is present in serum as well as locally in the tissues of the human body. Since the discover of the first complement factor it has been recognized as an important part of the innate immune system. (28) More recently it has been suggested that some of these proteins also has a place in the adaptive immune response. The primary site of production of complement proteins is the liver, but several cells have shown to produce complement as well including monocytes, T-cells and B-cells.(29)

The main functions of the complement system are recruitment of immune cells to areas of infection, opsonization of pathogens and lysis of pathogens by creation of the so-called membrane associated attack complex (MAC) or terminal complex. This is achieved through proteolytic cleavage into several bioactive fragments that act as either anaphylatoxins or opsonins.

1.6 Activation of the complement cascade

Complement is activated through three pathways: the classic-, lectin- and the alternative pathway. (Fig.1.1) The classic pathway is activated by complement factor C1 (consisting of C1q, C1r and C1s). Binding of C1 leads to formation of the classic C3 convertase (C4b2a).(30) The C3 convertase is able to cleave C3 to the anaphylatoxin C3a and the opsonin C3b. This C3b can bind to the C3 convertase to form the C5 convertase (C4b2aC3b) that cleaves C5 to C5a and C5b. C5b initiates formation of the terminal complex through binding of C6-9. This creates transmembrane pores in the pathogen membrane and subsequent lysis of the cell.(31)

The lectin pathway also leads to the formation of the classic C3 convertase and differs only in the initiation which is achieved through mannose-binding lectin (MBL) or ficolin that bind to pathogen associated carbohydrates and not antibodies. (32)

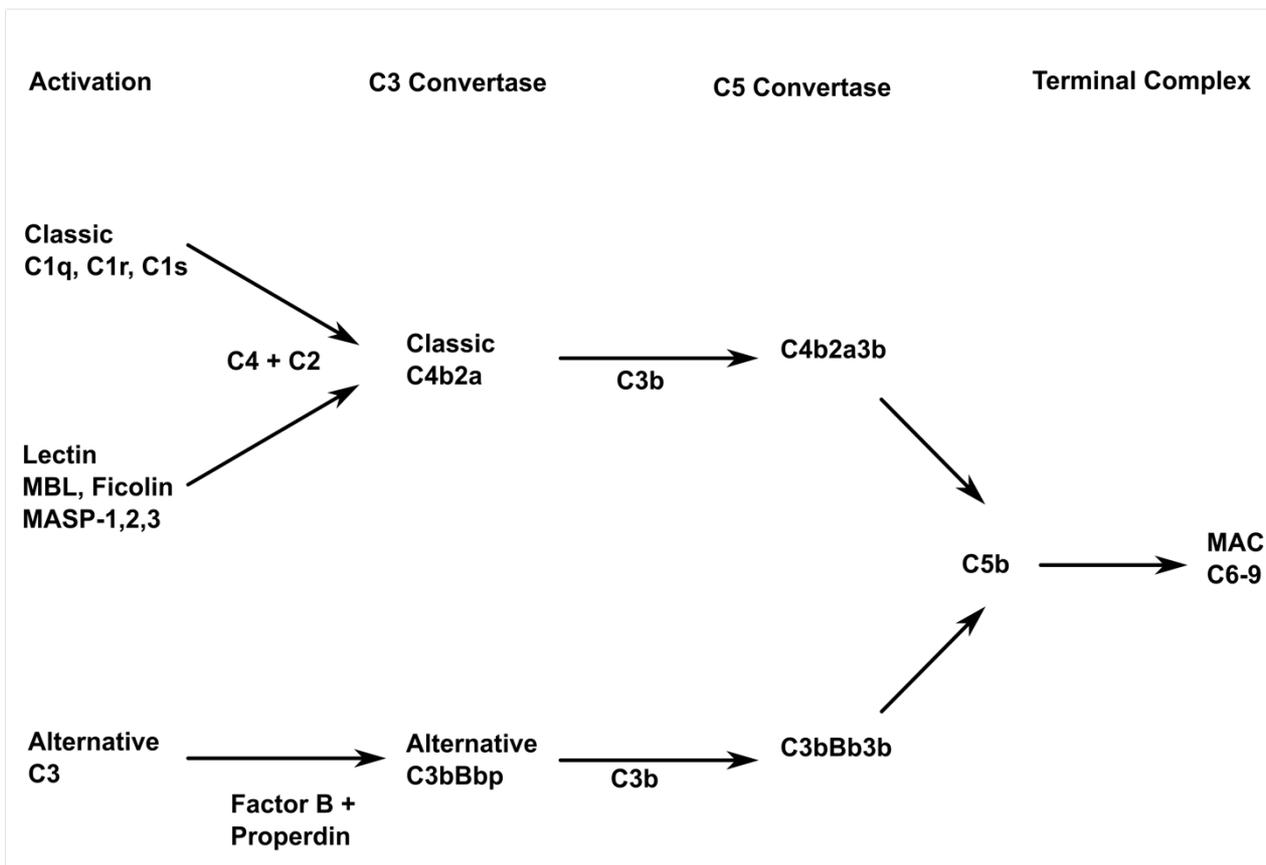


Figure 1.1 Activation of the complement cascade.

The alternative activation pathway differs from the classical and the lectin pathway by relying solely on the autoactivation of complement C3. Once deposited, C3 is able to initiate its own C3

convertase (C3bBbp). Since only C3b is required for generation of a functioning C3 convertase it is possible for alternative C3 convertases to act in concert with the classic C3 convertase and increase the efficiency of the complement system. For this reason, the alternative pathway is known as the “amplification loop”.(33)

Virtually every step of the complement cascade is regulated. The soluble proteins C1 inhibitor (C1-IN) and C4 binding protein (C4-BP) inhibit formation of the classic C3 convertase by binding C1q and C4 respectively. Complement factor I (FI) is able to cleave C3 in the presence of complement factor H or membrane bound CD55 and thereby stop formation of the alternative C3 convertase. In case that a C5 convertase has been initiated, the formation of MAC can be blocked by the two serum proteins Clusterin and Vitronectin. (34)

1.7 Structure of complement C3

Complement C3 is the most abundant of all complement system proteins. It is synthesized as a single chain of 1663 peptides consisting of a 22-peptide signal peptide, a β -chain, a tetra-arginine linker and an α -chain in this order. The mature peptide consists only of an α - and β -chain linked together by a disulphide bond. The α -chain contains several internal disulphide bonds one of which is located between amino-terminal and the carboxy-terminal regions which allows the C3dg region to be cleaved off. (35)

1.8 Fragments of complement C3

Native C3 is sequentially cleaved into several smaller fragments with different biological functions (Fig.1.2). Cleavage by C3 convertase results in the 9 kDa C3a and the 185 kDa C3b. C3 contains an internal thioester bond in the α -chain. Upon cleavage into C3b, this thioester is exposed and C3b is able to bind to structures on the pathogen membrane.(36) Cleavage of C3b to iC3b by FI results in removal of the 3 kDa C3f fragment from the α -chain. This inhibits the formation of MAC but complement receptors (CR) 1, 3 and 4 on phagocytes and DC still recognize iC3b and it still leads to enhanced phagocytosis.(37) Further degradation of iC3b to C3c and C3dg by FI can also occur once bound to CR1 or in the presence of FH. The C3c fragment is released from the cell surface with C3dg covalently attached through the thioester bond to the surface of the pathogen.(38)

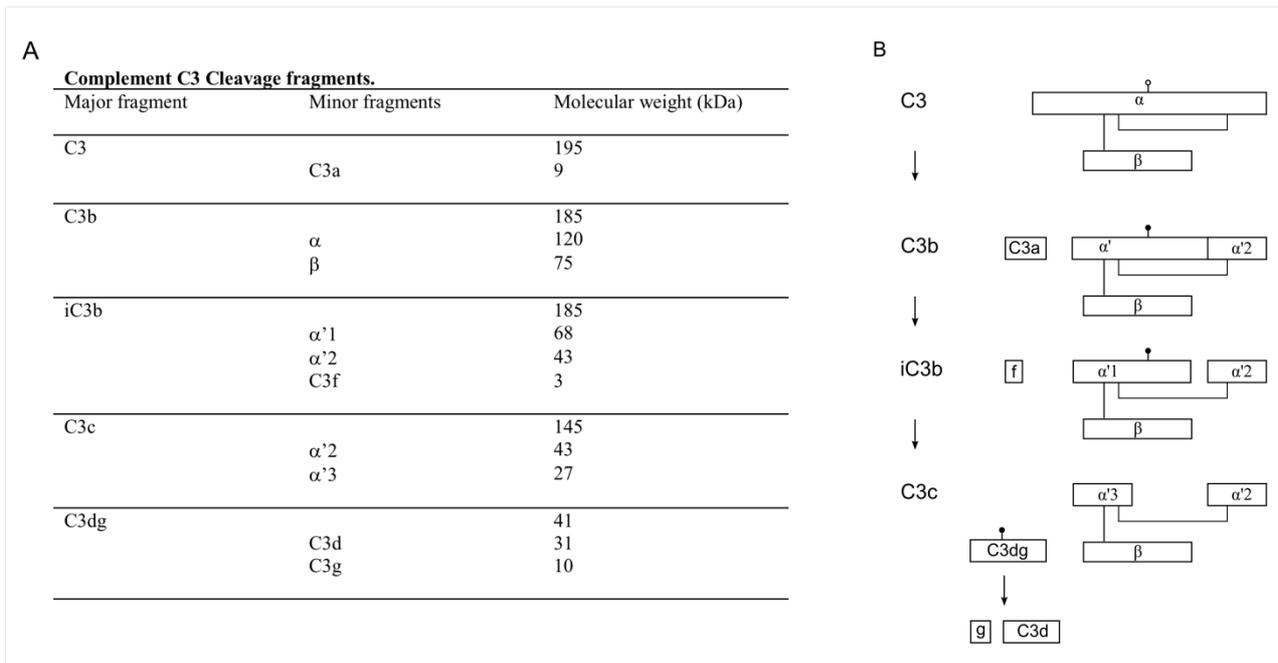


Figure 1.2 A) Known biological cleavage fragments of complement C3 and molecular weights. B) Schematic representation of the successive proteolytic cleavage of C3.

1.9 Chlamydiae and the complement system

Evidence suggest that the complement system participate in immunological clearance of chlamydial infections. In vivo studies, using a *C. psittaci* lung infection model, have shown that complement C3 is important in controlling the clinical outcome of this particular infections, but the differences were lower in *C. trachomatis* indicating that there are species-related complement sensitivities (39,40). These studies offer no functional explanation for the observed differences in complement sensitivities but call for in depth analyses of this discrepancy (40). Complement activation by *Chlamydia* has been demonstrated in vitro and complement has been shown to enhance the destruction of *Chlamydiae* (41,42) and studies with immunocompetent human serum has been shown to possess anti-chlamydial activity (43).

These early studies of inactivation of *Chlamydiae* in serum proposed that complement was activated by the classic pathway (43). This was later challenged by Hall and colleagues who suggested that the effect of complement on *C. trachomatis* was primarily conferred by the alternative pathway.(44) The exact contribution of each activation pathway is therefore unknown. Furthermore, only C3b and iC3b have been shown to bind to *C. trachomatis*.(26,44) These factors are known to be cleaved by soluble FI with FH as a cofactor.(37)

Chlamydia Protease-like activity factor (CPAF) *Chlamydia* RB has been shown to cleave C3 and factor B in vitro which might confer some protection from complement and the anti-chlamydial effect of complement appears to be mediated primarily by early complement proteins and not by C5-9 (45). This indicates that a terminal complex is not formed. Proteins such as clusterin and vitronectin have been shown to bind late complement proteins C7,8,9 and C9 respectively and thereby inhibit MAC.

The aim of the study was to detect complement system proteins on the membrane of *C. trachomatis* with the purpose of a bottom up Liquid Chromatography coupled with tandem mass spectrometry LC-MS/MS approach to identify peptides from complement fragments or regulatory proteins.

2. Material and methods

2.1 Antibodies

Primary polyclonal rabbit anti-human- C1q, C3c, C3d and C4c were all purchased from Agilent Technologies (Agilent Technologies, Glostrup, Denmark). Primary polyclonal rabbit anti-Pmp (A, C, D, E, F, G, H, I) (unpublished), polyclonal rabbit anti-COMC (pAb17) (46), monoclonal mouse anti-MOMP (47), monoclonal mouse anti-HSP60 were all generated by prof. Svend Birkelund. Monoclonal rat anti-C3d was kindly provided by Yaseelan Palarasah from University of Southern Denmark (48).

Anti-rabbit IgG Alkaline phosphatase (AP) were acquired from and Sigma-Aldrich (Sigma-Aldrich, St. Louis, MI). For microscopy, secondary FITC-conjugated anti-rabbit and anti-mouse were acquired from Jackson ImmunoResearch (Jackson ImmunoResearch, PA, USA). For immunoelectron microscopy goat anti-rabbit conjugated with 10 nm colloidal gold (British BioCell, Cardiff, UK) was used.

2.2 Preparation of human serum

Human serum was obtained from one seronegative donor at Aalborg University. Briefly, blood was drawn from the donor in untreated S-Monovette® 9 ml Z vial (Sarstedt, Germany). The blood was allowed to coagulate at room temperature for 30 min and subsequently centrifuged at 2000 x g for 10 min at 4°C. Serum fraction was aspirated and stored in -80°C aliquots designated Normal human serum (NHS), thawed as needed and kept on ice. Heat inactivation was done by heating serum to 56°C for 30 min, centrifuged at 20.000 x g for 15 min at 4°C and designated heat inactivated serum (HIHS). The use of human serum was approved by The North Denmark Region Committee on Health Research Ethics under case no. N-20150073.

2.3 Chlamydiae culture and purification

C. trachomatis L2/434/Bu were purchased from American Type Culture Collection (ATCC, VA, USA), expanded in a monolayer of McCoy cells (ATCC) for three days and disrupted using a cell scraper. *Chlamydiae* were released from the host cells through extrusion using a syringe with a needle. *Chlamydiae* were separated in EB from RB fractions by Visipaque® (GE Healthcare, density gradient centrifugation using an ultra-centrifuge.

Briefly, the suspension was centrifuged for 5 minutes at 800 x g and 4°C to remove host cell debris. The supernatant was placed on a cushion of 30% Visipaque® in HEPES buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 130 mM NaCl) and centrifuged at 45.000 x G in a sorval T-865 rotor for 30 min and 4°C. This yielded semi purified pellet of *Chlamydiae* that was resuspended in Phosphate buffered saline (PBS) supplemented with 1mM CaCl and 1mM MgCl. Five µL DNase and RNase were added and tubes were incubated for 30 min at 37°C. HEPES was added and the suspension was placed on a step gradient of 30% Visipaque, 40% Visipaque and lastly 44% Visipaque in HEPES-buffer. The gradient was centrifuged at 100.000 x G for 60 min and 4°C which yielded two distinct bands of *Chlamydiae*, the EB and RB fractions. EB and RB were stored at -80°C. Purity of the EB and RB were assessed by negative staining using Transmission Electron Microscopy (TEM) according to Caldwell(6).

Preparation of peptides for LC-MS/MS

Purified *C. trachomatis* EB were incubated in either NHS or HHS for 30 min at 37°C and washed three times in PBS by centrifugation at 20.000 x g and 4°C. *C. trachomatis* was resuspended in 200µL triethylammonium bicarbonate (TEAB) buffer. 4µg Pierce trypsin protease MS grade (Thermo Scientific, Waltham, MA) or Pierce chymotrypsin protease MS grade (Thermo Scientific, Waltham, MA) was added to each sample and incubated for 30 min and 120 min at 37°C. The solutions were centrifuged at 20.000 x g at 4°C. The pelleted *C. trachomatis* were stored at -20°C and the supernatant was transferred to a separate tube and incubated for another 120 min at 37°C to ensure proper protein digestion. Reduction of disulfide linkages and alkylation was achieved by incubation for 30 minutes at 37°C with 50 mM Tris(2-carboxyethyl)phospine (TCEP) and 50 mM Chloroacetamide (CAA), respectively.

2.4 Purification of peptides using C18 filter tips

The solution was then passed through Pierce C18 filter tips for purification and desalting according to manufacturer's protocol. Briefly, C18 tips were wetted using 70% (v/v) acetonitrile (ACN) and washed with 0.1% (v/v) trifluoroacetic acid (TFA). TFA was added to all samples to a final concentration of 0.5% (v/v) to maintain peptides in their protonated form prior to purification. The C18 tips were loaded with sample from the top using a second pipette. This step was repeated three times for maximal yield. The samples were desalted/washed with 0.1% TFA (v/v) and 2% ACN (v/v). The peptides were eluted using 50µL 0.1% (v/v) and 70% ACN (v/v) into LoBind microcentrifuge tubes (Eppendorf) and dried by evaporation using a vacuum centrifuge overnight.

Peptide samples were reconstituted in loading buffer for liquid chromatography (LC) consisting of 2% ACN, 0.1% TFA, 0.1% formic acid (FA).

2.5 Sample analysis using LC-MS/MS

Peptide sample analysis was performed using the Ultimate 3000 UPLC system (Thermo Scientific, Waltham, MA) coupled with the timsTOF Mass spectrometer (Bruker Daltronics).

Reverse phase separation was done on a 15 cm C18 column with gradient ramp starting at 98% solvent A (0.1% FA and 0.1% TFA) and 2% solvent B (0.1 % FA in ACN). Solvent B was increased over to 30% over 30 min at a flow rate of 300 nl/min and an oven temperature of 60°C. The eluted peptides were ionised and analysed in triplicates by time of flight tandem MS using the timsTOF Mass spectrometer (Bruker Daltronics).

2.6 Protein identification using MaxQuant

The RAW files from timsTOF were searched with Maxquant v1.6.1.0 against the SwissProt human proteome and Uniprot Chlamydia trachomatis L2 proteome. Standard settings in MaxQuant were applied with the following modifications: carbamidomethyl of cysteines (static), amino-terminal acetylation (variable), oxidation of methionine (variable) and deamidation (NQR).

Identified peptides and proteins were filtered with a false discovery rate (FDR) <1% using the MaxQuant forward/reverse database search.

2.7 Immunoelectron microscopy

Immunoelectron microscopy (IEM) was performed according to Lausen et al. 2018 (26) using a JEOL 1010 transmission electron microscope (Jeol, Tokyo, Japan). Briefly, purified *C. trachomatis* EB in solution was mixed with either NHS or HHS (1/10 the volume of EB). Five µL of the EB solution was transferred to glow discharged, carbon coated 400 mesh nickel grids and subsequently washed three times using PBS drops (pH 6.5). Blocking was performed using one drop of 1% ovalbumin (Sigma-Aldrich) in PBS. The samples were incubated at 37°C for 30 minutes in either rabbit anti-C3c, anti-C3d or rabbit anti-C4c diluted 1:200 in ovalbumin. The samples were washed three times on PBS drops and incubated in 10nm colloidal gold-conjugated goat anti-rabbit antibody in ovalbumin (1:25) for 30 minutes at 37°C. The samples were washed as previously described and incubated on three drops of 0.5% cold fish gelatin (Sigma-Aldrich) in PBS, 5 min each, washed in three drops of PBS and one drop of water. Lastly, the samples were stained with

one drop of 0.5% phosphotungstic acid. Images were acquired with an Olympus KeenView digital camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Transmission immunoelectron microscopy (TEM) was performed according to Lausen et al. 2018 using a JEOL 1010 transmission electron microscope operated at 60kV (Jeol, Tokyo, Japan). Five μL of the EB solution was transferred to glow discharged, carbon coated 400 mesh nickel grids and stained with one drop of 1% phosphotungstic acid pH 7.0 (pH) for 1 min and dried. Imaging was also performed using the KeenView digital camera. Sice determination was done using a carbron replica grid with 2160 lines per mm (SPI, West Chester, PA , USA)

2.8 Immunofluorescence staining

McCoy cells were cultured in standard cell medium consisting of RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamin and 0.01 mg/mL hexamycin. Cells were seeded on glass cover slips at 50,000 cells per well in a 24 wells plate and incubated at 37°C with 5% CO₂ overnight or until an appropriate confluence level was reached.

Cells were infected with EB diluted 1:4000 in RPMI for 1 hr. EB-containing media was removed, washed three times with PBS and medium was replaced with standard medium supplemented with 1 $\mu\text{g}/\text{mL}$ cycloheximide. The infected McCoy cells were fixed at three timepoints: 24 hours post infection (hpi), 36 hpi and 43 hpi.

Fixation was done by removing the media, washing once with PBS and fixed for 20 min in 3.7% (v/v) formaldehyde at 4°C.

All samples were permeabilised using 0.2% (v/v) Triton X-100 in PBS for 7 min at room temperature and blocked for 15 min at 37°C in a blocking solution consisting of 0.1% (w/v) BSA in PBS. All antibodies were diluted 1:200 (anti-MOMP 1:20) in blocking solution and samples were incubated for 30 min at 37°C followed by three washes in PBS. Secondary antibody was added and incubated for 30 min at 37°C. Unbound antibody was removed by three washes with PBS. Counter staining was performed with 2 $\mu\text{g}/\text{ml}$ To-Pro-3-iodide (Thermo Scientific) in PBS and incubated for 12 minutes at RT. The cover coverslips were washed twice in PBS and finally mounted on glass slides with an antifading agent. Microscopic analysis was done using the Leica TCS SP5 confocal microscope and images were acquired in Leica Application Suite - Advanced Fluorescence (Leica Microsystems) (LAS AF). All image processing was done using LAS X.

2.9 Immunoblot analysis

Briefly, 100 µl purified EB were incubated in an equal volume of either NHS or HIHS for 30 min at 37°C. Unbound serum proteins were subsequently washed away through three 15 min centrifugation steps at 20,000 x g 4°C. The supernatant was discarded after each centrifugation and the pelleted EB were resuspended in ice cold PBS. All EB samples were lysed in LDS sample buffer (Expedeon, SD, USA) with 5% (w/v) β-mercaptoethanol, heated for 5 minutes at 95°C and stored at -20 °C.

Gel electrophoresis was performed using polyacrylamide gels made using Hoefer mould. Stacking gel solution consisted of: 5% acrylamide, 20% (v/v) SDS and 0.5M Tris HCL at pH 6.8. Resolving gel solution consisted of either 8% and 12% acrylamide, 20% (v/v) SDS, 1.5M Tris HCL at pH 8.8. Polymerisation was catalysed using TEMED and ammonium persulfate was used as free radical initiator. Cast polyacrylamide gels were kept at 4°C for storage.

The Hoefer electrophoresis chamber was used for electrophoresis using a standard Tris Glycine SDS running buffer (Tris 25mM, Glycine 192mM, SDS 0.1% (w/v)) at 10°C. Protein separation was achieved using constant voltage at 150V for the first 30 minutes or until the wave front had passed through the stacking gel and into the resolving gel. The voltage was then increased to 180V for the remainder of the runtime.

Transfer of separated proteins to a nitrocellulose membrane was done using a (Hoefer) blotting chamber in transfer buffer consisting of Tris 25mM, glycine 192mM and 20% methanol (v/v). Cooling was applied at 4°C and voltage set at 100V for 2 hrs.

Membranes were blocked for 30 minutes in TBS with 3% gelatin (Bio Rad) at 37°C. After blocking, membranes were rinsed in deionized water and incubated in primary antibody (1:1.000) solution overnight at 4°C. Unbound antibody was removed by washing three times in TBS containing 0.05% Tween 20 (v/v). Membranes were incubated in secondary antibody solutions (1:30,000) for 60 minutes at 37°C followed by three washes as described above. Finally, the membranes were incubated in BCIP/NBT (Kem-En-Tec Diagnostics, Taastrup, Denmark) solution for approximately 10 minutes and the blots were imaged using a scanner.

3. Results

3.1 Analysis of complement C3 using by LC-MS/MS approach

The purpose of this study was to identify serum proteins related to the complement system on the surface of *C. trachomatis*. To investigate this, we used LC-MS/MS on *C. trachomatis* incubated in human serum. Because this approach has not been used to study complement deposition before, we decided to test whether our method was sensitive enough to detect any complement components at all. This was done using purified complement component C3, as this is the most abundant complement component and because fragments complement C3 binds to *C. trachomatis* (6,26). To ensure that all known biological cleavage fragments from C3 were detectable using our method we first analysed trypsin digested purified C3. This yielded a total of 104 peptides from C3 of which 99 were unique peptides. (See Fig.3.1) Sequence coverage was 63.2% and covered all biologically relevant cleavage fragments.

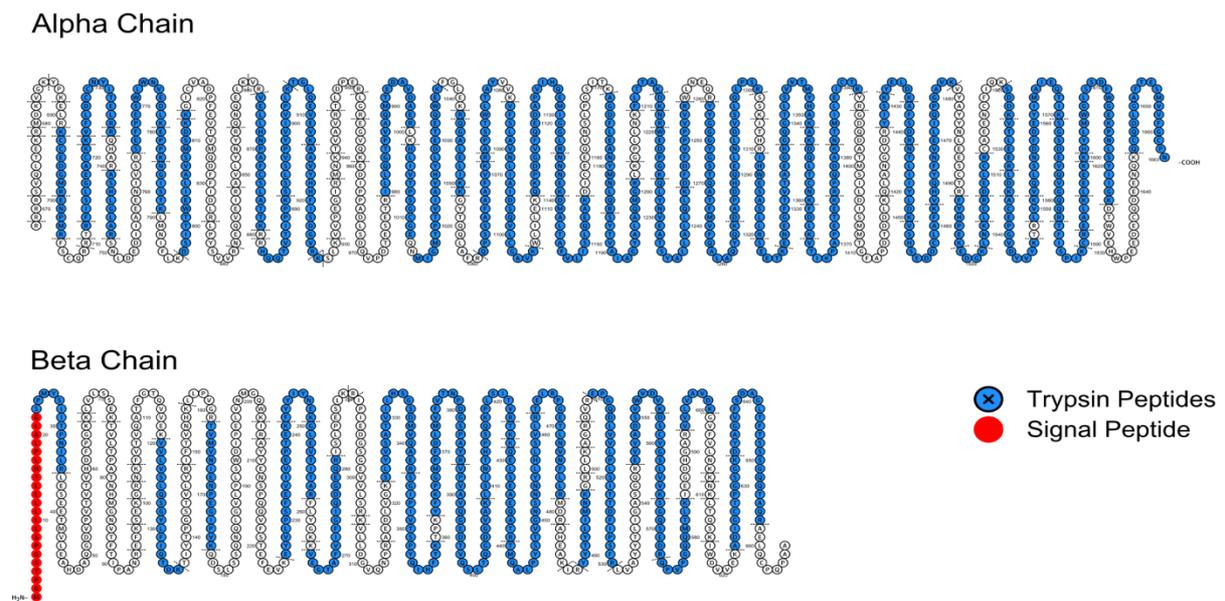


Figure 3.1 Schematic showing the coverage of tryptic peptides of C3 (colored in blue)

3.2 Analysis of complement C3 using a bottom up LC-MS/MS approach

We then proceeded to examine the deposition of complement on the outer membrane of *C. trachomatis* serovar L2. *C. trachomatis* EB were incubated in NHS, washed thoroughly, and incubated in a trypsin solution for up to two hrs. The resulting peptides were purified using C18

filter tips and analysed in triplicate using LC-MS/MS. As we were only interested in the surface deposition of serum proteins, we performed transmission electron microscopy to assess the integrity of the EB after incubation in the trypsin solution. (Fig. 3.2) shows EB after digestion using trypsin and the deterioration of the EB membrane over a period of two hours. No noticeable changes are on the membrane after 15 and 30 min (30 min not shown). The deterioration of the outer membrane and after 120 min was noticeable with the membranes of the EB rupturing (Fig. 3.2 Bottom right) After 60 min EB were still intact but with beginning deterioration and “roughening” similar to appear the 120 min sample (Fig. 3.2. Bottom left).

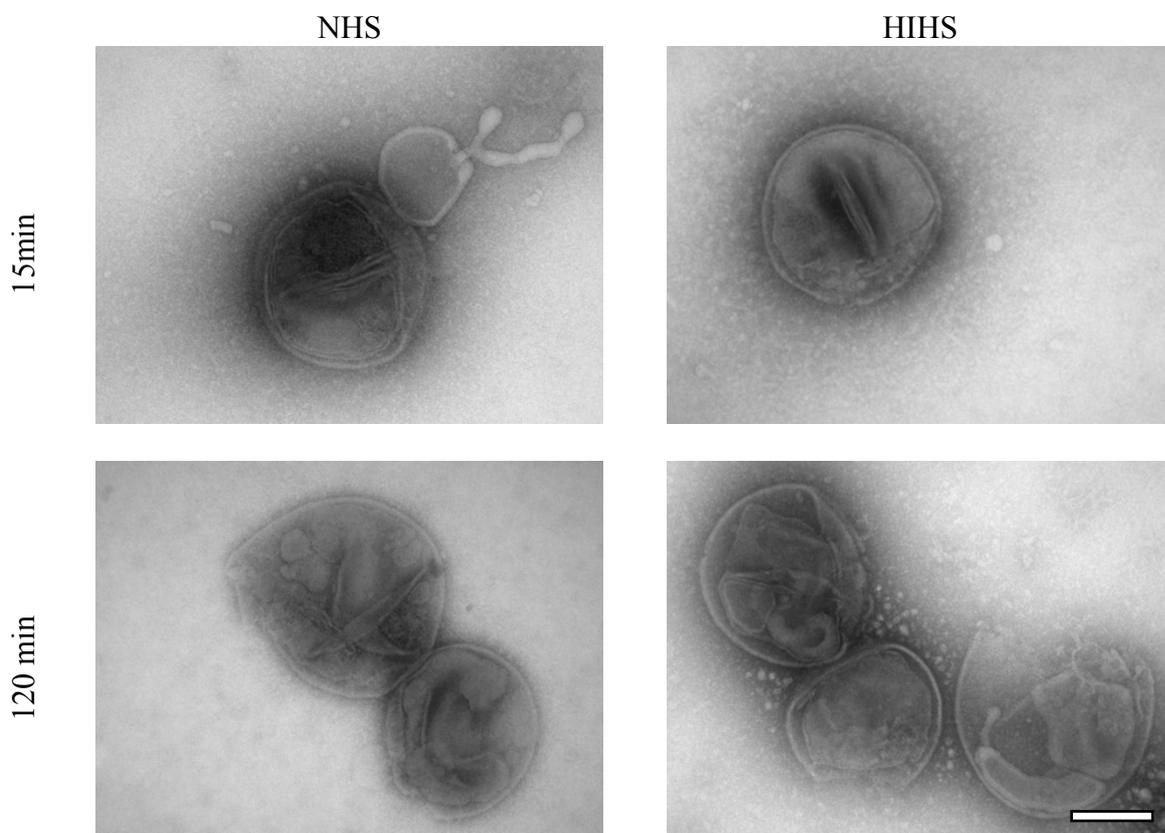


Figure 3.2 Transmission electron microscopy of *C. trachomatis* EB incubated for 30 min in NHS (left) or HIHS (right) . After the first incubation the EB were incubated in trypsin for 15, 30, 60 or 120 min to ensure that the EB were still intact. Scalebar 200 nm

We were able to identify 14 unique peptides of complement C3 from all known biological cleavage fragments. Information about these peptides are summarized in (Table 3.1).

We also performed this experiment with EB incubated in HIHS and we found no peptides related to any complement proteins. We showed that we are able to detect peptides originating from the C3a fragment by LC-MS/MS (Fig. 3.1) and we find no peptides from this region in any of our samples.

This is in line with what we expected, as this fragment is cleaved off from C3 before binding to the surface is possible.

Table 3.1 Complement C3 peptides identified from *C. trachomatis*

Sequence	Length	Missed cleavage	MS/MS count	Score	Fragment	Protease
GQGTLVVVMTYHAK	14	0	81	285.83	C3c α' 2	Trypsin
ILLQGTPVAQMTEDAVDAER	20	0	80	340.73	C3g	Trypsin
SEETKENEFTVTAEGK	17	1	61	402.38	C3c α' 2	Trypsin
EGVQKEDIPPADLSDQVPDTESETR	25	1	21	262.55	C3g	Trypsin
DFDFVPPVVR	10	0	15	174.27	C3d	Trypsin
AGDFLEANYMNLQR	14	0	13	234.67	C3d	Trypsin
IHWESASLLR	10	0	13	163.16	C3f	Trypsin
GYTQQLAFR	9	0	3	148.98	C3d	Trypsin
ENEGFTVTAEGK	12	0	3	181.44	C3c α' 2	Trypsin
SSLSVPYVIVPLK	13	0	2	81.865	C3c α' 1	Trypsin
TGLQEVEVK	9	0	2	72.23	C3c α' 1	Trypsin
QGALELIK	9	0	1	72.038	C3d	Trypsin
TIYTPGSTVLYR	12	0	1	78.098	C3 β	Trypsin
NRWEDPGKQLY	11	2	20	159.79	C3d	Chymotrypsin
NLDVSLQLPSR	11	0	11	130.66	C3d	Chymotrypsin
SEETKENEFG	10	1	6	174.02	C3c α' 2	Chymotrypsin

From a biological stand point it would be interesting to know which fragments of the complement C3 is present on the surface of *C. trachomatis* as each fragment serves a different function. Interestingly, using trypsin we were able to detect the peptide sequence IHWESASLLR originating from complement component C3f. This particular fragment is only present in active C3b. A representative mass spectrum of this sequence is presented below as Fig. 3.3.

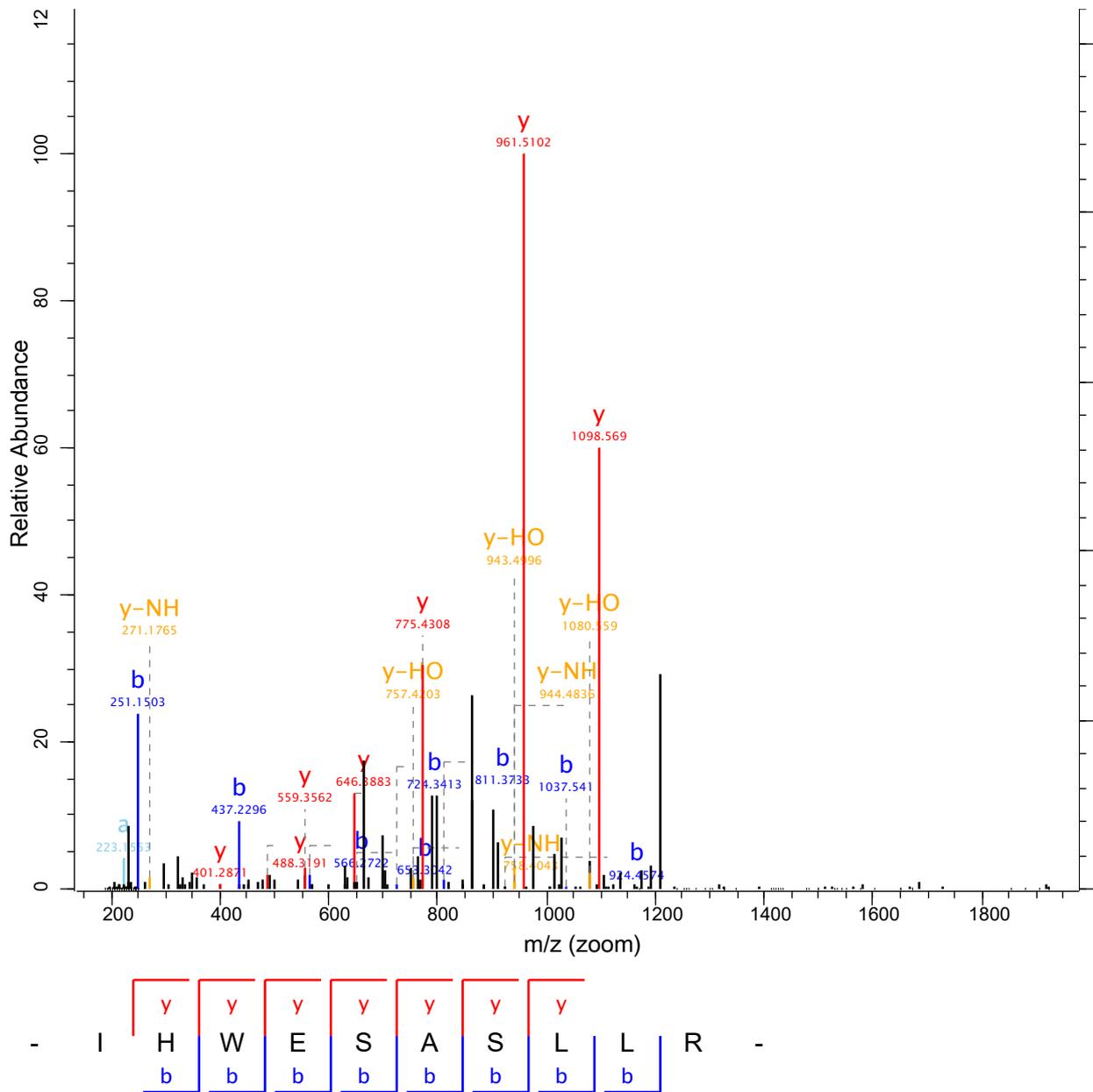


Figure 3.2 Mass spectrum of the sequence IHWESASLLR from fragment C3f.

Several of the biological cleavage sites of complement C3 are tryptic cleavage sites (N-terminal of lysine or arginine). For this reason, trypsin digestion does not provide information about which fragments are present as a result of natural cleavage of C3. We then repeated the experiment using chymotrypsin in the hope of generating peptides containing these natural cleavage sites. Here we found 120 unique peptides from our purified C3 control and were also able to detect all C3 fragments with a sequence coverage of 53.9% Using chymotrypsin, we were able to identify

another 3 unique peptides from serum incubated *C. trachomatis* (Table 1). We were unable to detect any peptides from the natural cleavage sites of C3. We were also not able to detect any peptide from C3f on the surface of *C. trachomatis* after chymotrypsin digestion.

In addition to the identification of complement C3 we also identified 2 peptides of both C4 (α -chain and γ -chain) and Factor B (both fragment Bb) of the classical and alternative complement activation pathways, respectively. Furthermore, MAC inhibitory proteins vitronectin and clusterin and apolipoprotein A1, E were found. (A full list of recovered peptides from the Maxquant search is attached as Supplementary)

To sum up: We were able to identify peptides from all biological fragments of complement C3 except C3a. This is in line with the idea that binding of C3b requires removal of the soluble C3a. The identification of C4 was interesting as it was not possible to find any reference to C4 binding in *C. trachomatis*. For this reason, we chose to include C4 in our further investigation.

3.3 Investigating the deposition of complements C3 and C4 on C. trachomatis outer membrane

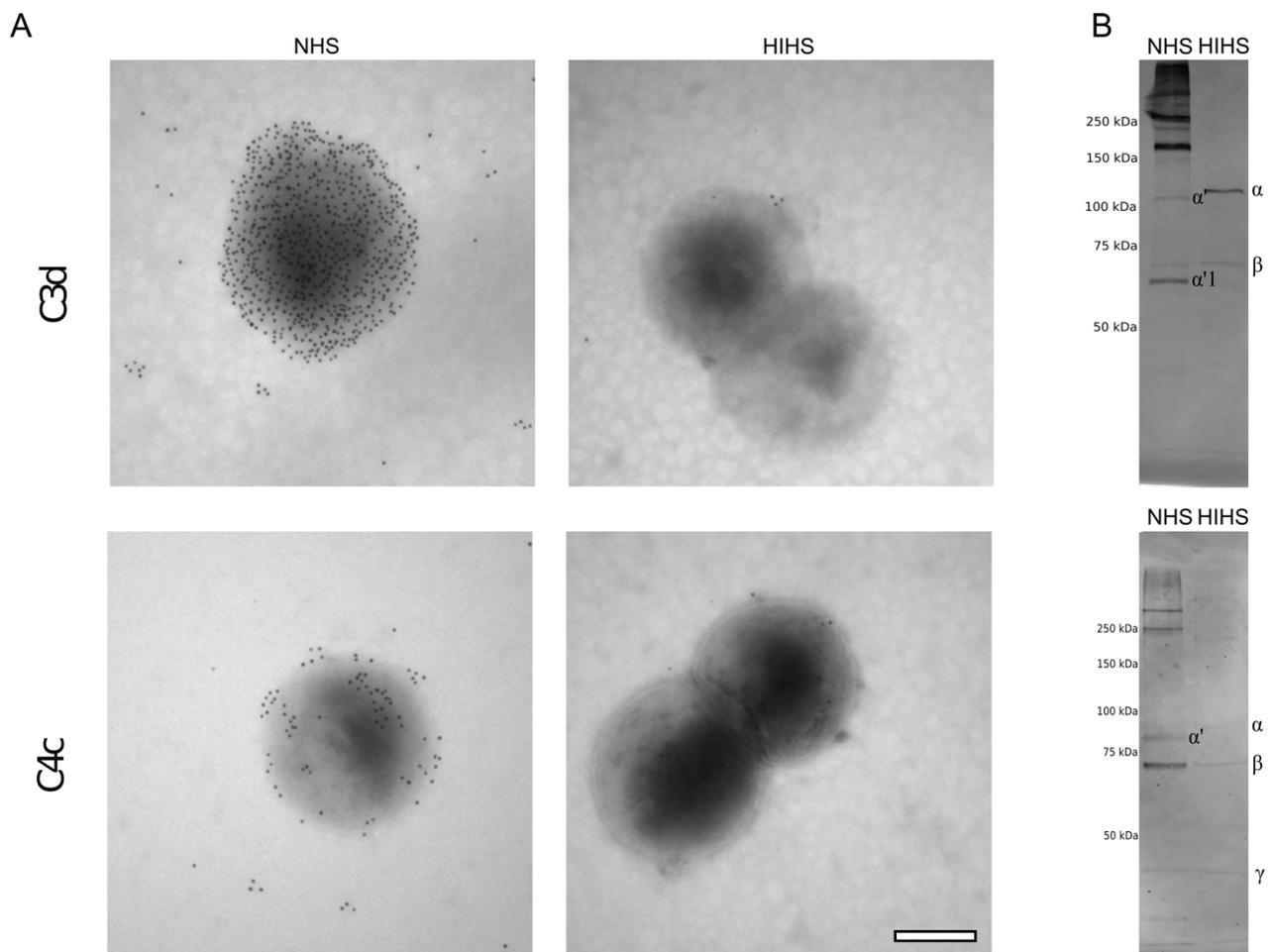
Our data suggested that this method was indeed able to detect complement proteins bound to the outer membrane of *C. trachomatis* EB using LC-MS/MS. We wanted to confirm that these proteins were in fact present on the surface of EB after incubation in NHS and that this was not the case after heat inactivation. Furthermore, it is known that both complement components C3 and C4, upon activation bind covalently to their targets. We used immunoelectron microscopy and gold-labelled anti-C3d and anti-C4c to visualize the deposition of these complement factors on the surface of EB and immunoblot to investigate whether the presence of complement resulted in protein bands of higher molecular weight (Fig. 3.3A).

Fig. 3.3B shows immunoblots of *C. trachomatis* incubated in NHS and HIHS that both C3 (top) and C4 (bottom). In the right lane of the C3 blot we see two distinct protein bands at 120 kDa and 75 kDa which corresponds well with α - and β -chain of uncleaved C3 after incubation in HIHS. This changes markedly when incubated in NHS (left lane). The 75 kDa β -chain undergoes no cleavage and remains unchanged. Instead, the intensity of the intact α -chain decreased, and several new high

molecular weight bands were generated as well as a band between 75 kDa and 50 kDa which most likely is the 68 kDa α' 1 fragment from iC3b.

These high molecular bands are most prominent at roughly 160 kDa and 250 kDa. The exact identity of these new does not correlate with any known C3 fragments and must originate from the binding of C3 to chlamydial outer membrane proteins.

Complement C4 consists of 3 subunits: a 98 kDa α -chain, a 73 kDa β -chain and a 33 kDa γ -chain.(49) Activation results in cleavage of the α -chain and release of the 8,6 kDa C4a.(50) All of these fragments appear in both the NHS and HIHS lanes albeit at higher intensity for NHS. A shift of the 98 kDa band on the right to slightly lower molecular weight corresponding to the cleavage of C4a could indicate binding of C4b to the chlamydial surface. We also see two high molecular bands with the one being 250 kDa and the other even larger.



3.4 Confocal imaging of chlamydial Pmps

The results presented above suggest that complement proteins and in particular complement C3 bind covalently to high molecular structures in the outer membrane of *C. trachomatis* EB. To test whether serum proteins, including complement factors, bind to structures on the surface of EB we performed an immunoblot analysis on NHS treated chlamydial EB using antibodies generated to each of the polymorphic membrane proteins (Pmp) (fig. 3.5). This revealed that binding of serum proteins to the chlamydial surface does happen and yields two protein bands at some 200kDa and two bands at 250kDa.

Pmp are among the largest proteins at the chlamydial surface at around 95 – 190 kDa. Binding of complement proteins to Pmps therefore might account for the high molecular bands presented above. Polyclonal rabbit anti-Pmp antibodies generated by Allan Shaw and Prof. Svend Birkelund (unpublished) were used to investigate whether this was in fact the case.

In order to verify the binding of these anti-Pmp antibodies to the surface of *C. trachomatis* EB we performed immunofluorescent staining and confocal microscopy of chlamydial inclusions in McCoy cells.

Pmps are located in the outer membrane and should only stain as green ring structures surrounding the bacteria. To ensure that our anti-Pmp antibodies were specific to outer membrane structures we used monoclonal anti-MOMP specific to MOMP in order to visualize the outer membrane. To test whether anti-Pmp antibodies bind to structures inside *C. trachomatis*, we used the intracellular HSP60 as a control.

We were able to identify *C. trachomatis* Pmps: A, C, D, H with high signal intensity. Pmps G and I were detectable yet at much lower intensity and finally PmpF was not detectable using the current set-up. In all samples where inclusions were identified, the *Chlamydiae* appeared as fine ring structures similar to that of MOMP (Fig. 3.5 Second row), indicating that the antibodies recognized structures on the outer membrane. Fig. 3.5 (bottom row) shows the images comparing PmpD with the mouse serum prior to immunization.

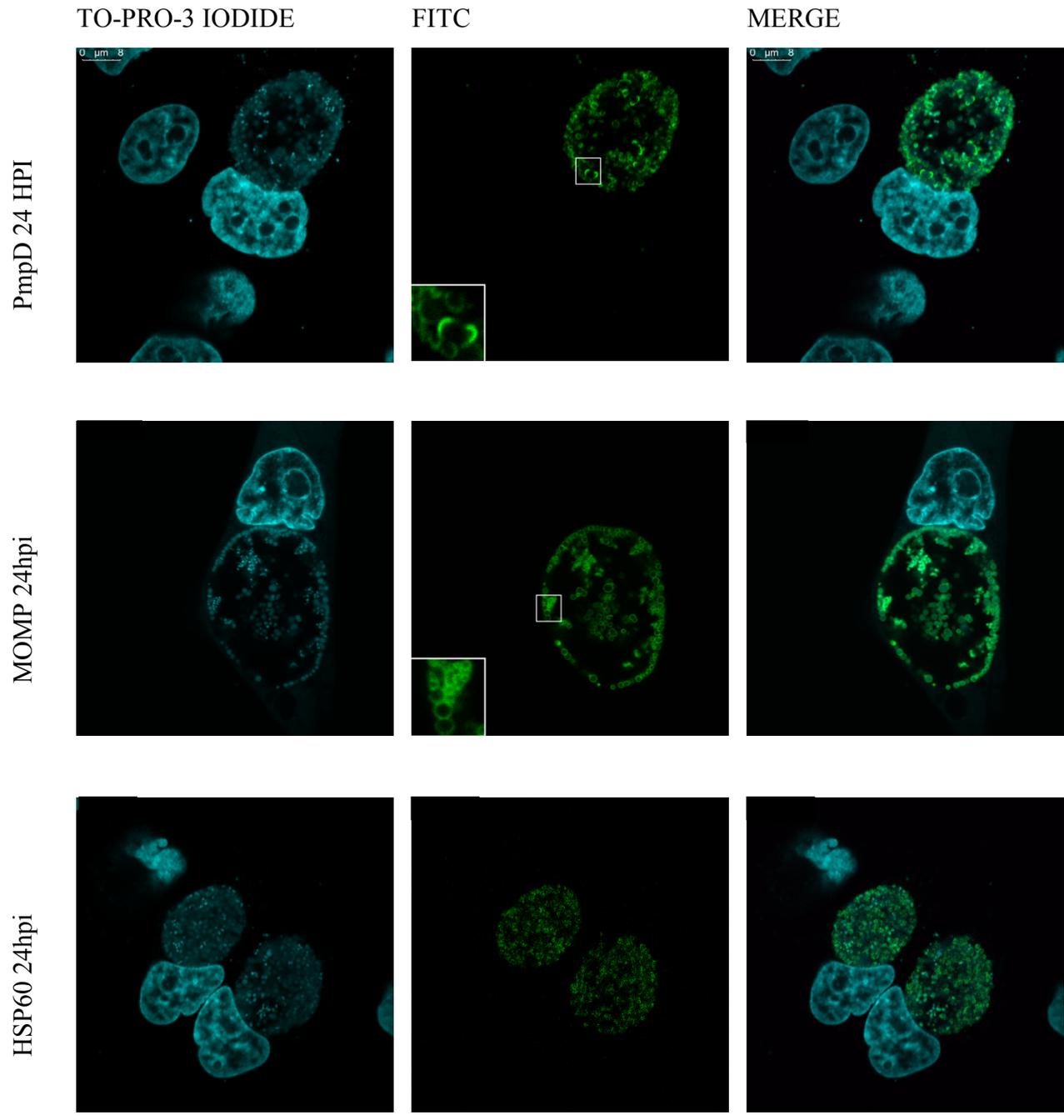




Figure 3.5 Immunofluorescence images of McCoy cells infected with *C. trachomatis* serovar L2 using confocal microscopy at 24 hpi. Top row represents anti-PmpD from left: TO-PRO-3-iodide nuclear stain, anti-PmpD-FITC, merge. Second row anti-MOMP, Third row anti-HSP60, bottom row represents pre-immunisation rabbit serum control. Scale bar in lower right corner indicates 20 μm .

3.5 Immunoblot analysis using polyclonal anti-Pmp antibodies *C. trachomatis*

Seeing as our anti-COMC immunoblot showed similar high molecular bands as C3 and C4 we went on to test our Pmp antibodies in immunoblotting as shown in Fig. 3.6. Despite the differences in signal intensity between antibodies, we decided to include all antibodies in our immunoblot analysis in order to test the antibodies on the denatured proteins. It became apparent that our antibodies behaved in more or less the same intensities on immunoblot as in our confocal imaging except PmpC and PmpI that showed lower signal compared to confocal imaging.

Briefly, *C. trachomatis* EB incubated in NHS or HIHS were lysed in sample buffer, separated on polyacrylamide gels, and transferred to nitrocellulose membranes. The membranes cut into strips that were then incubated overnight in primary antibody solution and developed using AP-conjugated secondary antibodies. This revealed that serum proteins bind to PmpA yielding several faint, yet sharply defined protein bands at 250 kDa and higher. Comparing PmpA bands to bands to anti-COMC (PAb-19) revealed that the two bands that appear at 200 kDa on anti-COMC are not present on any blots stained with anti-Pmp antibodies.

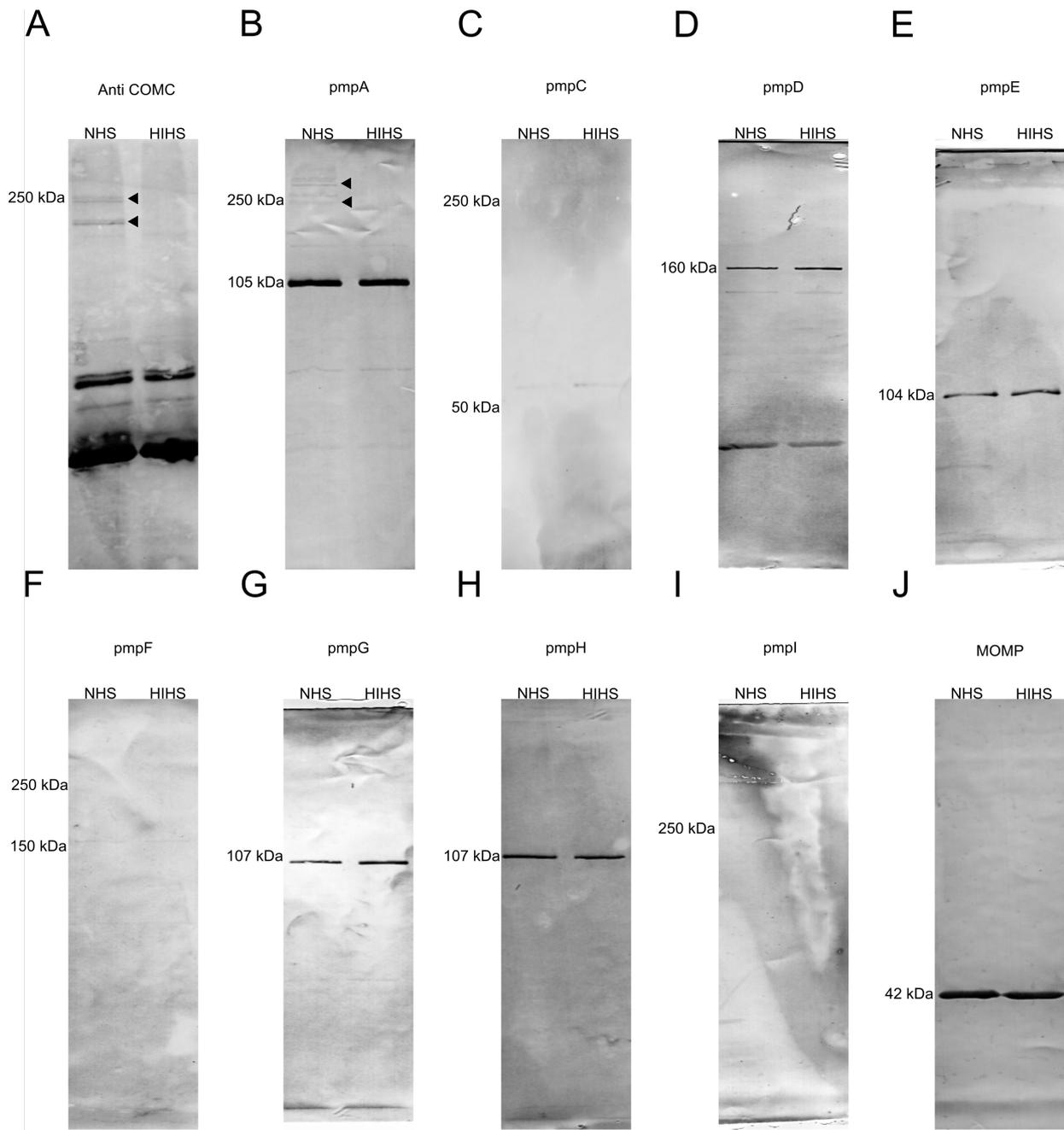


Figure 3.6 Immunoblots of *C. tracomatis* EB incubated in NHS (left) and HIHS (right): A) PAb17 anti-COMC B) anti-PmpA, C) anti-PmpC, D) anti-PmpD, E) anti-PmpE, F) anti-PmpF, G) anti-PmpG, H) anti-PmpH, I) anti-PmpI, J) anti-MOMP. Arrows point to high-molecular bands that are believed to originate from serum proteins.

4. Discussion

We have shown that by LC-MS/MS it is possible to detect complement proteins on the surface of intact *C. trachomatis* serovar L2 after incubation in human serum. Using this method, we were able to identify peptides from all known biological fragments of complement component C3. The presence of C3b on the chlamydial surface was established by the identification of fragment C3f. We confirmed the presence of C3 by immunoelectron microscopy and immunoblot analysis and our data suggest that complement proteins are able to bind to PmpA of *C. trachomatis*.

Activation of the complement cascade has been demonstrated by several groups, but some controversy exists over which of the known pathways most efficiently leads to activation of *Chlamydiae*. Early observations indicated that activation of complement was antibody dependent, suggesting involvement of the classic pathway (42) while Hall and colleagues demonstrated that the alternative pathway was more effective at initiating the complement cascade (44).

We were able to find two peptides from the fragment Bb of complement factor B, one of the proteins responsible for the initiating the alternative C3 convertase. We also find C3f which is only present in C3b. This indicates that the alternative convertase is present in accordance with the findings of Hall and colleagues. The lack of properdin prevents us from determining if this is indeed a stable C3 convertase. It should be noted that properdin is a highly glycosylated protein and this might be the reason why we are unable to find it using mass spectrometry as it will bind strongly to a C18 column. Properdin has been shown to bind to *C. pneumoniae* and it is likely that it also binds to *C. trachomatis*. (51)

While the classic pathway has been proposed as the primary activator based on serum survivability of *C. trachomatis* (43), the deposition of C4 has never been reported on *C. trachomatis* using mass spectrometry. The present study was able to recover peptides from the alpha and gamma chains of complement C4. The alpha chain of C4 contains the reactive thioester domain that conveys the binding potential, similar to complement C3. This indicates that either C4b or iC4b is present on the membrane. The iC4b fragment is not able to initiate the classical C3 convertase but just as C3b and iC3b it is able to bind to CR1 and CR3 on monocytes and macrophages and act as an opsonin (52). Regardless of the means of activation, looking at the immunoelectron images presented in this report, it is apparent that cleavage and deposition of C3 occurs and it is plausible that both the classic and alternative C3 convertase contribute.

It is possible that C3b is cleaved to iC3b which would stop formation MAC. The use of immunoelectron microscopy to visualize the *Chlamydiae* after serum incubation also showed that the bacteria were intact after 30 min with no sign of MAC formation despite deposition of complement C3 and C4. This is in line with the previous reports that MAC formation is not necessary for chlamydial inactivation (45).

Additionally, we were not able to find any late complement components using our setup. Instead, we were able to detect the complement inhibitory proteins vitronectin and clusterin, both of which are known to inhibit formation of MAC. Clusterin has shown to bind surface bound C7, C8 and C9 and is thereby able to inhibit MAC formation (53). Similarly, vitronectin has been proposed to bind nascent C5-7 complexes before binding to pathogen membranes and inhibit C9, and thus stop polymerisation of these molecules. Vitronectin has previously been shown to facilitate adhesion in *C. trachomatis* (54). It was not possible to find any reference to clusterin deposition on *Chlamydiae* but the gram negative bacterium *Pseudomonas Aeruginosa* has been shown to recruit clusterin.(55) These results point towards opsonization as the primary means of clearing *C. trachomatis* and are supported by the differences presented on immunoelectron microscopy and immunoblot analysis. On immunoelectron microscopy we see a clear difference between the loosely bound anti-C3c and the tightly bound anti-C3d which indicates that the C3c chain might be cleaved off and that a substantial amount of either C3dg or C3d is deposited. This was investigated by immunoblot analysis using monoclonal anti-C3d specific to cleaved C3d. As we were not able to detect any protein bands it suggests that the deposited C3 fragment is predominantly C3dg. The finding of the α' 1 fragment on immunoblots using polyclonal anti-C3d also supports the presence of iC3b.

Complement C3b, iC3b and C3dg are all known opsonins that are recognized by complement receptors on human phagocytes (29). These opsonins are able to bind covalently to their targets via the active thioester domain (31). This study demonstrated that these opsonins are able to bind covalently to *C. trachomatis* using immunoblots with polyclonal antibodies against C3c and C3d on *C. trachomatis* incubated in NHS and HIHS. Based on immunoblots using polyclonal antibodies against the COMC and several Pmps we demonstrated that serum protein bind binding to structures at 200 kDa and 250 kDa for COMC when comparing NHS with HIHS. Protein bands on PmpA blots showed a similar migration towards 250 kDa and several low intensity, yet distinct bands. Interestingly, we saw no protein binding to PmpD or PmpI despite the strong sequence homology between these proteins and PmpA.(56)

Hall and colleagues demonstrated that five high molecular weight protein bands appeared after serum incubation. Based on immunoblot analysis they suggested that complement C3 principally binds to MOMP or multimers of MOMP (44). At the time, this was a plausible explanation as only LPS and MOMP had been reported on *C. trachomatis* and the existence of Pmps in this pathogen was first shown seven years later (5). Still, they were unable to account for one protein band at 166 kDa. The molecular weight of this protein complexed with molecular weights of Pmps could account for the unknown protein band as proposed in Lausen et al (26).

We did not find any such binding on MOMP using immunoblotting and this inconsistency is most likely due to the differences in antibody specificity as this has previously been shown using monoclonal antibodies against MOMP. (47)

The use of this bottom up approach does allow for quantification at protein level but as we are interested in specific we are not able to quantify on these data. However, we are able to assess the relative abundance of C3 and C4 on the basis of immunoelectron microscopy.

High abundant proteins are common contaminants in mass spectrometric analyses. The concentration of C3 in serum is high, and as such there is a risk that these results are affected by left over complement from inadequate washing. While we do identify a single peptide from serum albumin, the most abundant serum protein, in one HIHS sample, we would like to point to the fact that the concentration of serum albumin is up to ten times higher than the most abundant complement component, C3. Therefore, it is highly unlikely that the results presented here are a product of poor washing.

This highlights an important consideration when reviewing the data presented in this study. Our setup is designed to investigate complement proteins that bind to the surface of *C. trachomatis* EB and to remove serum proteins. Additionally, the peptides that were identified were only searched against human proteins and as such we are unable to comment on potential effects exerted by chlamydial proteins. It should be noted that proteins that only associate with the EB through weak binding might be washed away. This means that anaphylatoxins from the complement cascade cannot be analysed using this method. It should be addressed, that certain peptides are not analysed well with LC-MS/MS due to poor ionisation or post translational modification. An example of this could be properdin as this protein is highly glycosylated which might explain why we are unable to detect it.

Despite the limitations presented above, this LC-MS/MS approach is an appropriate method for investigating complement system proteins on *C. trachomatis* and the uncertainties that exist regarding the data might be solved by isolating the PmpA high molecular bands using in-gel digest. Based on LC-MS/MS, immunoelectron microscopy and immunoblotting we suggest that these proteins are fragments of complement component C3. We cannot exclude the possibility of C4 binding as this protein also produces protein bands in the same region at 250 kDa on immunoblot analysis but based on immunoelectron microscopy it is apparent that C3 fragments are the predominant complement factor and that they are bound to PmpA.

5. Summary

This study showed that LC-MS/MS was able to detect deposition of complement system proteins on the surface of *Chlamydia trachomatis* serovar L2 after incubation in normal human serum. Using this approach, the study found complement components C3b along with components of both the classic and alternative pathway as well as proteins known to inhibit the terminal complex.

Immunoelectron microscopy confirmed the presence of C3b on intact *Chlamydia trachomatis* serovar L2 after serum incubation. The use of two different C3 specific antibodies on immunoelectron microscopy and immunoblot suggested the presence of fragments C3dg and iC3b. Immunoblot analysis showed that serum proteins binds covalently to the outer membrane complex of *Chlamydia trachomatis* serovar L2 as high molecular protein bands. Investigation of these protein bands using several Pmp-specific antibodies showed that PmpA is a target for these serum proteins.

Thus, this study suggests that complement C3 fragments is able to bind PmpA on the outer membrane of *Chlamydia trachomatis* serovar L2, but deposition of C3 does not necessarily lead to formation of MAC and complement mediated lysis.

6. References

1. Ceovic R, Gulin SJ. Lymphogranuloma venereum: Diagnostic and treatment challenges. Vol. 8, Infection and Drug Resistance. 2015. p. 39–47.
2. WHO. Global Health Sector Strategy on Sexually Transmitted Infections 2016-2021. World Heal Organ [Internet]. 2016;1(June):63. Available from: http://apps.who.int/iris/bitstream/10665/183117/1/9789241508841_eng.pdf
3. Reyburn H. WHO Guidelines for the Treatment of Chlamydia trachomatis. World Heal Organ [Internet]. 2016;340(may28 1):c2637–c2637. Available from: <http://apps.who.int/iris/bitstream/handle/10665/246165/9789241549714-eng.pdf;jsessionid=7EDA0B673861E121DBF1465EEA5F9953?sequence=1><http://www.ncbi.nlm.nih.gov/pubmed/25103303><http://www.ncbi.nlm.nih.gov/pubmed/20511305><http://www.bmj.com/cgi/doi/10>
4. Liechti GW, Kuru E, Hall E, Kalinda A, Brun Y V., Vannieuwenhze M, et al. A new metabolic cell-wall labelling method reveals peptidoglycan in Chlamydia trachomatis. Nature [Internet]. 2014;506(7489):507–10. Available from: <http://dx.doi.org/10.1038/nature12892>
5. Mygind PH, Christiansen G, Roepstorff P, Birkelund S. Membrane proteins PmpG and PmpH are major constituents of Chlamydia trachomatis L2 outer membrane complex. FEMS Microbiol Lett. 2000;186(2):163–9.
6. Caldwell HD, Kromhout J, Schachter J. Purification and Partial Characterization of the Major Outer Membrane Protein of Chlamydia trachomatis. 1981;31(3):1161–76.
7. Birkelund S, Morgan-Fisher M, Timmerman E, Gevaert K, Shaw AC, Christiansen G. Analysis of proteins in Chlamydia trachomatis L2 outer membrane complex, COMC. FEMS Immunol Med Microbiol. 2009;55(2):187–95.
8. Vasilevsky S, Stojanov M, Greub G, Baud D, Vasilevsky S, Stojanov M, et al. Chlamydial polymorphic membrane proteins : regulation , function and potential vaccine candidates. 2016;5594(February):11–22.
9. Elwell C, Mirrashidi K, Engel J, Francisco S. Chlamydia cell biology and pathogenesis. Nat Rev Microbiol. 2016;14(6):385–400.
10. AbdelRahman YM, Belland RJ. The chlamydial developmental cycle. FEMS Microbiol Rev. 2005;29(5):949–59.
11. Zhang JP, Stephens RS. Mechanism of *C. trachomatis* attachment to eukaryotic host cells.

- Cell. 1992;69(5):861–9.
12. Dautry-Varsat A, Subtil A, Hackstadt T. Recent insights into the mechanisms of Chlamydia entry. *Cell Microbiol.* 2005;7(12):1714–22.
 13. Jewett TJ, Fischer ER, Mead DJ, Hackstadt T. Chlamydial TARP is a bacterial nucleator of actin. *Proc Natl Acad Sci U S A* [Internet]. 2006;103(42)(42):15599–604. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1622868&tool=pmcentrez&rendertype=abstract>
 14. Dai W, Li Z. Conserved type III secretion system exerts important roles in Chlamydia trachomatis. *Int J Clin Exp Pathol.* 2014;7(9):5404–14.
 15. Morrison SG, Su H, Caldwell HD, Morrison RP. Immunity to murine Chlamydia trachomatis genital tract reinfection involves B cells and CD4+ T cells but not CD8+ T cells. *Infect Immun.* 2000;68(12):6979–87.
 16. Rank RG, Soderberg LSF, Barron AL. Chronic chlamydial genital infection in congenitally athymic nude mice. *Infect Immun.* 1985;48(3):847–9.
 17. Redgrove KA, McLaughlin EA. The role of the immune response in chlamydia trachomatis infection of the male genital tract: A double-edged sword. *Front Immunol.* 2014;5(OCT):1–22.
 18. Vasilevsky S, Greub G, Nardelli-Haeffliger D, Baud D. Genital Chlamydia trachomatis: Understanding the roles of innate and adaptive immunity in vaccine research. *Clin Microbiol Rev.* 2014;27(2):346–70.
 19. Hvid M, Baczynska A, Deleuran B, Fedder J, Knudsen HJ, Christiansen G, et al. Interleukin-1 is the initiator of Fallopian tube destruction during Chlamydia trachomatis infection. *Cell Microbiol.* 2007;9(12):2795–803.
 20. Hook CE, Matyszak MK, Hill Gaston JS. Infection of epithelial and dendritic cells by Chlamydia trachomatis results in IL-18 and IL-12 production, leading to interferon- γ production by human natural killer cells. *FEMS Immunol Med Microbiol.* 2005;45(2):113–20.
 21. Brunham RC, Rey-Ladino J. Immunology of Chlamydia infection: Implications for a Chlamydia trachomatis vaccine. *Nat Rev Immunol.* 2005;5(2):149–61.
 22. Matyszak MK, Young JL, Gaston JS, Hill Gaston JS. Uptake and processing of *Chlamydia trachomatis* by human dendritic cells. *Eur J Immunol* [Internet]. 2002;32(3):742–51. Available from: <http://dx.doi.org/10.1002/1521-> _____

- 4141(200203)32:3<%5Cnhttp://3.0.co%5Cnhttp://2-9
23. Al-zeer MA, Al-younes HM, Lauster D, Lubad MA, Meyer TF. Autophagy restricts *Chlamydia trachomatis* growth in human macrophages via IFNG-inducible guanylate binding proteins. *Autophagy*. 2013;9(1):50–62.
 24. Bas S, Neff L, Vuillet M, Spenato U, Seya T, Matsumoto M, et al. The Proinflammatory Cytokine Response to *Chlamydia trachomatis* Elementary Bodies in Human Macrophages Is Partly Mediated by a Lipoprotein, the Macrophage Infectivity Potentiator, through TLR2/TLR1/TLR6 and CD14. *J Immunol* [Internet]. 2008;180(2):1158–68. Available from: <http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.180.2.1158>
 25. Sun HS, Eng EWY, Jeganathan S, Sin AT-W, Patel PC, Gracey E, et al. *Chlamydia trachomatis* vacuole maturation in infected macrophages. *J Leukoc Biol* [Internet]. 2012;92(4):815–27. Available from: <http://www.jleukbio.org/cgi/doi/10.1189/jlb.0711336>
 26. Lausen M, Christiansen G, Karred N, Winther R, Poulsen TBG, Palarasah Y, et al. Complement C3 opsonization of *Chlamydia trachomatis* facilitates uptake in human monocytes. *Microbes Infect* [Internet]. 2018; Available from: <http://www.ncbi.nlm.nih.gov/pubmed/29729435%0Ahttp://linkinghub.elsevier.com/retrieve/pii/S1286457918300960>
 27. Barenfanger J, MacDonald AB. The Role of Immunoglobulin in the Neutralization of Trachoma Infectivity. *J Immunol* [Internet]. 1974;113(5):160–1617. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/4214333>
 28. Sarma JV, Ward P a. The Compliment System. NIH Public Access author Manusc. 2012;343(343(1)):227–35.
 29. Dunkelberger JR, Song WC. Complement and its role in innate and adaptive immune responses. *Cell Res* [Internet]. 2010;20(1):34–50. Available from: <http://dx.doi.org/10.1038/cr.2009.139>
 30. Lachmann PJ, Hughes-Jones NC. Initiation of complement activation. *Springer Semin Immunopathol*. 1984;7(2–3):143–62.
 31. Holers VM. Complement and Its Receptors: New Insights into Human Disease. *Annu Rev Immunol* [Internet]. 2014;32(1):433–59. Available from: <http://www.annualreviews.org/doi/10.1146/annurev-immunol-032713-120154>
 32. Gál P, Barna L, Kocsis A, Závodszy P. Serine proteases of the classical and lectin pathways: Similarities and differences. *Immunobiology*. 2007;212(4–5):267–77.

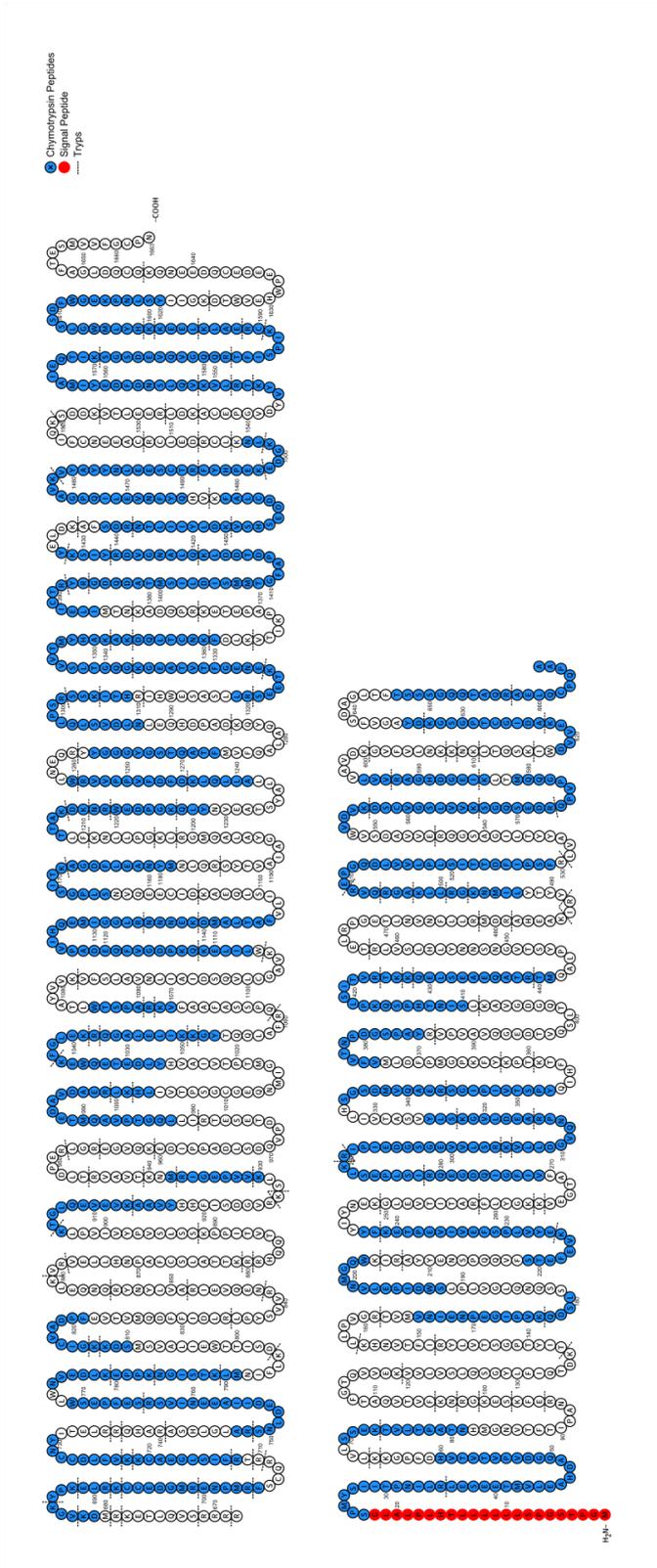
33. Thurman JM, Holers VM. The Central Role of the Alternative Complement Pathway in Human Disease. *J Immunol* [Internet]. 2006;176(3):1305–10. Available from: <http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.176.3.1305>
34. Zipfel PF, Skerka C. Complement regulators and inhibitory proteins. *Nat Rev Immunol* [Internet]. 2009;9(10):729–40. Available from: <http://dx.doi.org/10.1038/nri2620>
35. Sahu A, Lambris JD. Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. *Immunol Rev.* 2001;180(4):35–48.
36. Pangburn MK, Schreiber RD, Müller-Eberhard HJ. Formation of the initial C3 convertase of the alternative complement pathway. Acquisition of C3b-like activities by spontaneous hydrolysis of the putative thioester in native C3. *J Exp Med* [Internet]. 1981;154(3):856–67. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2186450&tool=pmcentrez&rendertype=abstract>
37. Wagner E, Frank MM. Therapeutic potential of complement modulation. *Nat Rev Drug Discov.* 2010;9(1):43–56.
38. Ross GD, Newman SL, Lambris JD, Devery-Pocius JE, Cain JA, Lachmann PJ. Generation of three different fragments of bound C3 with purified factor I or serum. II. Location of binding sites in the C3 fragments for factors B and H, complement receptors, and bovine conglutinin. *J Exp Med.* 1983;158(2):334–52.
39. Bode J, Dutow P, Sommer K, Janik K, Glage S, Tümmler B, et al. A New Role of the Complement System: C3 Provides Protection in a Mouse Model of Lung Infection with Intracellular *Chlamydia psittaci*. *PLoS One.* 2012;7(11).
40. Dutow P, Wask L, Bothe M, Fehlhaber B, Laudeley R, Rheinheimer C, et al. An optimized, fast-to-perform mouse lung infection model with the human pathogen *Chlamydia trachomatis* for in vivo screening of antibiotics, vaccine candidates and modified host-pathogen interactions. *Pathog Dis.* 2016;74(2):1–15.
41. Fedorko DP, Clark RB, Nachamkin I, Dalton HP. Complement dependent in vitro neutralization of *Chlamydia trachomatis* by a subspecies-specific monoclonal antibody. *Med Microbiol Immunol.* 1987;176(4):225–8.
42. Osborn MF, Johnson AP, Taylor-Robinson D. Susceptibility of different serovars of *Chlamydia trachomatis* to inactivation by normal human serum. *Genitourin Med.* 1985;61(4):244–6.

43. Johnson AP, Osborn MF, Rowntree S, Thomas BJ, Taylor-Robinson D. A study of inactivation of *Chlamydia trachomatis* by normal human serum. *Br J Vener Dis* [Internet]. 1983;59(6):369–72. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=6416608
44. Hall RT, Strugnell T, Wu X, Devine D V., Stiver HG. Characterization of kinetics and target proteins for binding of human complement component C3 to the surface-exposed outer membrane of *Chlamydia trachomatis* serovar L2. *Infect Immun*. 1993;61(5):1829–34.
45. Lin JSL, Yan LL, Ho Y, Rice PA. Early complement components enhance neutralization of *Chlamydia trachomatis* infectivity by human sera. *Infect Immun*. 1992;60(6):2547–50.
46. Birkelund S, Johnsen H, Christiansen G. *Chlamydia trachomatis* serovar L2 induces protein tyrosine phosphorylation during uptake by HeLa cells. *Infect Immun*. 1994;62(11):4900–8.
47. Birkelund S, Lundemose AG, Christiansen G. Chemical Cross-Linking of *Chlamydia trachomatis*. 1988;56(3):654–9.
48. Rasmussen KJ, Skjoedt MO, Vitved L, Skjoedt K, Palarasah Y. A novel antihuman C3d monoclonal antibody with specificity to the C3d complement split product. *J Immunol Methods* [Internet]. 2017;444:51–5. Available from: <http://dx.doi.org/10.1016/j.jim.2017.02.002>
49. Andoh A, Fujiyama Y, Kimura T, Uchihara H, Sakumoto H, Okabe H, et al. Molecular characterization of complement components (C3, C4, and factor B) in human saliva. *J Clin Immunol*. 1997;17(5):404–7.
50. Gorski JP, Hugli TE, Müller-Eberhard HJ. C4a: the third anaphylatoxin of the human complement system. *Proc Natl Acad Sci U S A*. 1979;76(10):5299–302.
51. Cortes C, Ferreira VP, Pangburn MK. Native properdin binds to *Chlamydia pneumoniae* and promotes complement activation. *Infect Immun*. 2011;79(2):724–31.
52. Murata K, Baldwin WM, Lambris JD. Mechanisms of complement activation, C4d deposition, and their contribution to the pathogenesis of antibody mediated rejection. *Transplant Rev*. 2009;23(3):139–50.
53. Tschopp J, Chonn A, Hertig S, French LE. Clusterin, the human apolipoprotein and complement inhibitor, binds to complement C7, C8 beta, and the b domain of C9. *J Immunol* [Internet]. 1993;151(4):2159–65. Available from: <http://www.jimmunol.org/content/151/4/2159.abstract> _____

54. Singh B, Su YC, Riesbeck K. Vitronectin in bacterial pathogenesis: A host protein used in complement escape and cellular invasion. *Mol Microbiol.* 2010;78(3):545–60.
55. Hallström T, Uhde M, Singh B, Skerka C, Riesbeck K, Zipfel PF. *Pseudomonas aeruginosa* uses Dihydrolipoamide dehydrogenase (Lpd) to bind to the human terminal pathway regulators vitronectin and clusterin to inhibit terminal pathway complement attack. *PLoS One.* 2015;10(9):1–21.
56. Carrasco JA, Tan C, Rank RG, Hsia R ching, Bavoil PM. Altered developmental expression of polymorphic membrane proteins in penicillin-stressed *Chlamydia trachomatis*. *Cell Microbiol.* 2011;13(7):1014–25.

7. Supplementary

7.1 Schematic representation of the equence coverage of C3 using Chymotrypsin

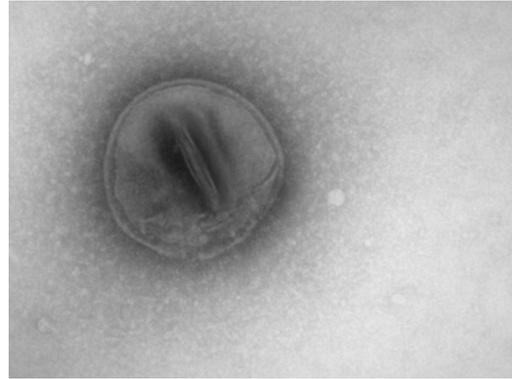
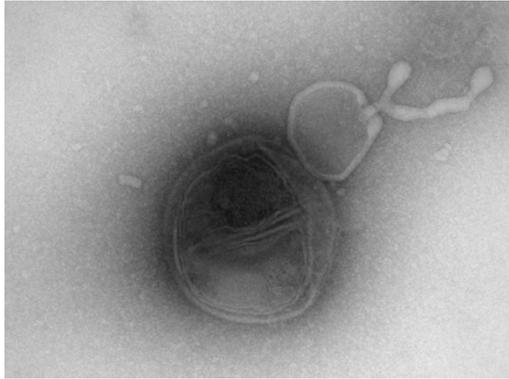


7.2 Images of *C. trachomatis* after trypsination

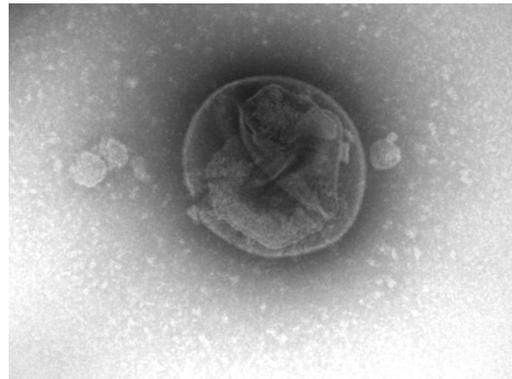
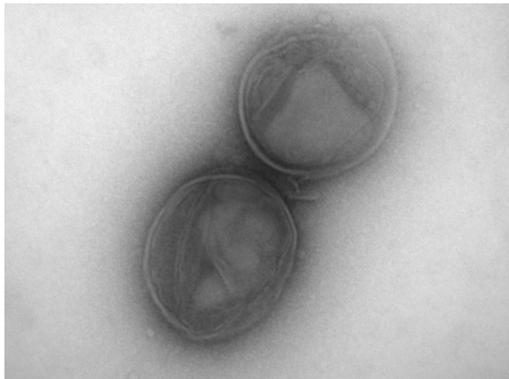
NHS

HIHS

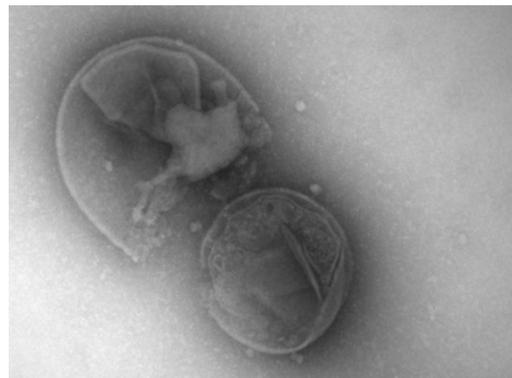
Trypsin 15min



Trypsin 30min



Trypsin 60min



Trypsin 120 min