CHLAMYDIAL INFERTILITY IN WOMEN: DIAGNOSIS, EPIDEMIOLOGY AND IMMUNE RESPONSE

Shruti Menon

Bachelor of Science (Biotechnology) (Honours)

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Institute of Health and Biomedical Innovation

School of Biomedical Sciences

Faculty of Health

Queensland University of Technology

2016

Keywords

C. trachomatis, female Infertility, sequelae, immunopathogenesis, sero-epidemiology, serological diagnosis, cytokines, Samoa

Abstract

Chlamydia (C.) trachomatis is one of the most common sexually transmitted pathogens in the world. In women, chlamydial infections can result in the progression from acute infection to disease sequelae such as cervicitis, pelvic inflammatory disease and tubal factor infertility (TFI). The scarring and fibrosis of fallopian tubes caused by *C. trachomatis* infections account for 10%-30% of all cases of female infertility.

Currently, *C. trachomatis*-related tubal infertility is diagnosed using laparoscopy and hysterosalpinography (HSG). The invasive nature of these diagnostic tests renders them unsuitable for routine use; and the high cost associated with these procedures restricts their availability to women in low-resource settings and developing nations. Serological diagnostic assays have been reported by several studies as a good alternative to these invasive strategies in diagnosing chlamydial infertility in women. However, commercial serological diagnostic assays are not used due to lack of sensitivity or specificity, and reported cross-reactivity of antibodies with other chlamydial species. Therefore, through this study, a novel multi-antigen peptide based ELISA was developed that can detect *C. trachomatis*-related TFI in women. Using existing commercial serological assays, the prevalence and risk factors associated with *C. trachomatis*-related infertility were determined in a female population from a developing nation, Samoa. This study also investigated and identified the immune markers associated with *C. trachomatis*-related TFI from the peripheral blood mononuclear cells (PBMC) of women.

The novel multi-antigen peptide based ELISA developed through this study, the QUT *Chlamydia* infertility test exhibited high specificity in identifying women with tubal pathology (100%) and *C. trachomatis*-related infertility (94%). However, this limited the assay sensitivity to 16% and 27% respectively. The test was developed and evaluated on a development cohort (n=262), which included an infertile cohort (women with TFI and other forms of infertility; n=97), acute *C. trachomatis* infections cohort (n=112) and fertile cohort (n=53). The specificity and sensitivity was higher than existing commercial serological assays such as MEDAC

MOMP, MEDAC Infertile, ANILabsystems and Microimmunofluorescence assay (MIF) .

While numerous efforts have been undertaken to diagnose and control *C. trachomatis*-related infertility, the underlying mechanism leading to the disease progression is yet to be understood. Transcriptomic and cytokine assays of stimulated PBMCs from women with chlamydial infertility (n=31) identified that some women launched a proinflammatory (CXCL-10, CXCL11, HLA-A and IL-1β) response to *C. trachomatis* infection.

In addition, the study further evaluates the prevalence and risk factors associated with chlamydial infertility in a high-prevalence, low-resource setting such as Samoa. The prevalence of *C. trachomatis* estimated by PCR in sexually active women between the ages of 18-29 (n=239) was 35.6%. The prevalence of *C. trachomatis*-related infertility was determined using commercial serological assays, which determined that 50% of self-reported infertile women (n=85), were positive for chlamydial infertility. Amongst commercial serological assays, MEDAC MOMP was the only test that could effectively identify women with self-reported infertility from those without ($p<0.05$). This shows that the prevalence of *C. trachomatis* infections and its sequelae is very high in Samoa. Thus, routine testing and treatment strategies would ideally reduce the disease burden from infertility within this population.

Research publications associated with this thesis

Menon, S., Timms, P., Allan, J. A., Alexander, K., Rombauts, L., Horner, P., Keltz, M., Hocking, J., and Huston, W. M. (2015) Human and Pathogen Factors Associated with *Chlamydia trachomatis*-Related Infertility in Women, *Clinical Microbiology Reviews 28*, 969-985.

Menon, S., Alexander, K., Timms, P., Allan, J. A., and Huston, W. M. (2016) CXCL10, CXCL11, HLA-A and IL-1beta are induced in peripheral blood mononuclear cells from women with Chlamydia trachomatis related infertility, *Pathogens and disease 74*, ftv099.

Additional research publications

Cunningham, K., Stansfield, S. H., Patel, P., Menon, S., Kienzle, V., Allan, J. A., and Huston, W. M. (2013) The IL-6 response to Chlamydia from primary reproductive epithelial cells is highly variable and may be involved in differential susceptibility to the immunopathological consequences of chlamydial infection, *BMC immunology 14*, 50.

Research Presentations

Oral Presentation

- Menon, S., Stansfield, S.H., Timms, P. and Huston, W. M. (2014) Serological and cellular responses in women with chlamydial infertility, In *2014 Australian Chlamydia Conference*, USC Innovation Centre, University of the Sunshine Coast.
- Menon, S., Stansfield, S.H., Timms, P. and Huston, W. M. (2014) Development of multi-antigen peptide ELISA for chlamydial infertility in women, In *Institute of Health and Biomedical Innovation Annual Postgraduate Student Conference: IHBI Inspires* Radisson Resort, Gold Coast, QLD Australia.

Poster Presentation

- Menon, S., Stansfield, S.H., Allan, J.A., Rombauts, L., Weston, G., Alexander, K., Timms, P. and Huston, W. M. (2014) Development of a multi-antigen peptide ELISA for Chlamydial infertility In *The Thirteenth International Symposium on Human Chlamydial Infections* Asilomar Conference Grounds, Pacific Grove, California USA.

- Menon, S., Stansfield, S.H., Allan, J.A., Timms, P. and Huston, W. M. (2013) Identification analysis of novel peptide antigens for a *Chlamydia trachomatis* infertility using a whole proteome-peptide antibody interaction strategy In *Australian Society for Microbiology Annual Scientific Meeting 2013*, Adelaide Convention Centre, Adelaide, South Australia.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

APC	Antigen-presenting cell
ATP	Adenosine triphosphate
AUC	Area under the curve
BLAST	Basic local alignment search tool
CAT	Chlamydia antibody testing
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CP	*C. pneumoniae*
CS	*C. psittaci*
CT	*C. trachomatis*
CPAF	Chlamydial protease/proteasome-like activity factor
cHSP60	Chlamydial heat shock protein60
CXCL	(C-X-C motif) ligand
DAPI	4', 6-Diamidino-2-Phenylindole
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EB	Elementary body
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
FCS	Fetal calf serum
GAPDH	Glyceraldehyde-3-phosphate de hydrogenase
gDNA	Genomic DNA

HeLa	Henrietta Lacks immortal cell line
HLA	Human Leukocyte Antigen
HEp-2	Human epithelial type 2 cell line
h PI	Hours post-infection
HRP	Horseradish peroxidase
HtrA	High temperature requirement protein A
IL	Interleukin
IFN	Interferon
Ig	Immunoglobulin
IVF	In vitro fertilization
kDA	Kilo Dalton
LPS	Lipopolysaccharide
LGT	Lower genital tract
MBP	Mannose binding protein
MHC	Major Histocompatibility complex
MIF	Microimmunofluorescence
MOI	Multiplicity of infection
MOMP	Major outer membrane protein
mRNA	Messenger Ribonucleic acid
NAATs	Nucleic acid amplification tests
ng	Nanograms
NK	Natural killer
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OD	Optical density
OMP	Outer membrane protein

PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCB	Phosphate citrate buffer
PCR	Polymerase chain reaction
PID	Pelvic inflammatory disease
qRT-PCR	Quantitative real time polymerase chain reaction
RB	Reticulate body
ROC	Receiver operating characteristic
IFU	Inclusion forming units
TFI	Tubal factor infertility
TLR	Toll like receptor
TNF	Tumour necrosis factor
UGT	Upper genital tract
UV	Ultraviolet
WHO	World health Organization

Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

QUT Verified Signature

Signature:

Date: ____30.03.2016_____

Acknowledgements

I would like to thank my principal supervisor Dr. Willa Huston for giving me an opportunity to work on an exciting and challenging project. I greatly appreciate the guidance and support she has provided me through my PhD journey. I would like to sincerely thank her for constantly believing in me and encouraging me to pursue my goals.

I would also like to extend my gratitude to Professor Peter Timms for his guidance through the project. His support during the writing process and his advice on my project has been hugely valuable. I would also like to thank Dr. Kimberly Alexander for her continued support and guidance during the course of my project. I am grateful to Dr. Scott Stansfield for his guidance on the statistical aspects of this project. Special mention also to Professor Louise Hafner and Dr. Kerry Armstrong for their valuable inputs and comments on my thesis.

I would like to thank my friends at Infectious Disease Program and Institute of Health and Biomedical Innovation for creating a fun and supportive environment to work in. I would especially like to thank my friends in the Protease group who I've shared my best times with; Vanissa Ong, James Marsh, Amba Lawrence, Anu Chacko, Bryan Wee, Mark Thomas and Pooja Patel.

None of this would have been possible without the unconditional support and love of my parents. I would like to extend my deepest gratitude to Acha, Amma and Malu who motivated and encouraged me to follow my dreams. I would also like to thank my extended family for all their support and encouragement through the PhD journey.

Last, but not the least, I would like to extend my heartfelt thanks to my beloved husband, Ajay, for being my rock and supporting me during difficult times.

Chapter 1: Introduction

1.1 BACKGROUND

C. trachomatis is one of the most common sexually transmitted bacteria in the world. The asymptomatic nature of the disease causes it to go undiagnosed and untreated, thus resulting in long -term sequelae such as pelvic inflammatory disease, ectopic pregnancy and infertility. The current techniques used to diagnose these sequelae in women are laparoscopy and hysterosalpinography. These techniques are invasive, expensive and prone to misdiagnoses [1]. Therefore, there is a need for a diagnostic assay that is non-invasive, cost-effective and has high diagnostic performance in detecting women with *C. trachomatis*-related infertility. Serological assays are a useful technique in diagnosing ectopic pregnancies, pelvic inflammatory diseases and recurrent miscarriages [2]. However, due to its lack of specificity owing to its high rate of cross-reactivity between other chlamydial species, it is not used in IVF clinics as the first line of infertility investigation. Additionally, they are not adept to differentiate women with acute infections from women with *C. trachomatis*-related infertility. Therefore, this study aimed to develop a novel multi-antigen peptide ELISA that has high specificity in identifying women with *C. trachomatis*-related infertility.

1.2 CONTEXT

Through this study, a novel peptide based ELISA, The QUT *Chlamydia* infertility test was developed that showed high specificity in diagnosing women with *C. trachomatis* –related infertility. The assay was robust and reproducible, and also outperformed current commercial serological assays in detecting women with tubal pathology and chlamydial infertility. Additionally, women who tested positive for *C. trachomatis*-related infertility in this assay had equal likelihood of achieving successful outcome in an IVF procedure as those who were infertile due to other reasons. Thus, women tested positive in this assay could proceed directly for IVF treatment without undergoing additional tests and surgeries, therefore making it an ideal diagnostic tool for early infertility investigation in IVF clinics. The development of sequelae in some women is attributed by immune responses induced by host/pathogen factors that lead to pathology, ultimately resulting in infertility. Therefore, in order to understand the underlying mechanisms that lead to pathology in some *C. trachomatis*-infected women, this study also investigated and identified

immune markers that are associated with *C. trachomatis*-related infertility in women. Since the rate of *C. trachomatis*-related infertility in women is higher in low-resource, high prevalence settings, using commercial serological assays, this study determined the sero-prevalence and risk factors associated with *C. trachomatis*-related infertility in women from a developing country, Samoa. High prevalence of chlamydial infertility was detected in this population, hence, drawing attention to need for immediate screening and treatment strategies to control *C. trachomatis* infections and its associated sequelae in the population.

1.3 PURPOSES

The overall purpose of the study is to develop a highly sensitive and specific diagnostic assay for *C. trachomatis*-related infertility in women. However, the study also explored epidemiological and immunological aspects of *C. trachomatis*-related infertility. The three specific aims of this project were:

1) To develop a novel multi-antigen peptide ELISA for the diagnosis of *C. trachomatis*-related infertility in women.

2) To identify immune markers in mononuclear cells that is associated with chlamydial infertility in women.

3) To determine the sero-prevalence and risk factors associated with *C. trachomatis*-related infertility in women from Samoa.

1.4 THESIS OUTLINE

Chapter two is the literature review relevant to the project

Chapter three includes the general procedures used in this study

Chapter four aims to develop a multi-antigen peptide ELISA for the diagnosis of women with *C. trachomatis*-related infertility. The chapter pertains to identification of suitable peptides for the assay and its development. The diagnostic performance is assessed and compared with current commercial assays.

Chapter five identifies immune markers that are associated with *C. trachomatis*-related infertility. The regulation of immune genes was assessed in women with chlamydial infertility compared to women with other forms of infertility. The study also evaluated the level of cytokines secreted. Thus, these

markers reveal one of the underlying mechanisms associated with *C. trachomatis* pathogenesis and subsequent infertility in some women.

Chapter six estimates the sero-prevalence of chlamydial infertility in the female population in Samoa. The risk factors associated with this disease have also been identified.

Chapter 2: Literature Review

Chlamydia (C.) trachomatis is one of the most common bacterial sexually transmitted infections in the world, with WHO's most recent estimates indicating approximately 105.7 million new infections annually [3], and in the United States of America alone, the Centre for Disease Control estimated approximately 2.86 million new *Chlamydia* STI in 2008 [4]. The infection is widely reported to result in devastating reproductive consequences in a proportion of infected women, including pelvic inflammatory diseases (PID), ectopic pregnancy and infertility. Haggerty *et al*. [5] illustrated that 2%-5% of women with chlamydial infections develop PID within two weeks, and about 18% of women with PID develop infertility. *C. trachomatis* is a Gram Negative, obligate intracellular bacterial pathogen with a unique biphasic developmental cycle [6]. The organism has been characterized using biology and genomics as a highly evolved or ancient pathogen with evidence of a reduced and customized genome, specifically tailored for its human host and obligate intracellular niche [8]. In spite of the considerable worldwide burden of chlamydial disease it is still not well understood what host and pathogen factors are associated with infertility. The following review focuses on a range of topics such as the morphology and developmental cycle of *C. trachomatis*; factors that are associated with its ascension into the upper genital tract; the host/human factors and host genetic factors that contribute to chlamydia-related infertility; immunological response induced in infertile women; and diagnostic techniques used to detect women with chlamydia-related infertility.

2.1 DEVELOPMENTAL CYCLE AND MORPHOLOGY OF *C. TRACHOMATIS*

C. trachomatis has a unique biphasic developmental cycle characterized by functionally and morphologically distinct cell types that are adapted for intracellular multiplication and extracellular survival [9]. The bacteria comprises regularly-spaced dome shaped projections on its surface, followed by a peptidoglycan-free rigid cell wall, and an lipopolysaccharide outer cell membrane [10, 11]. Microscopic analysis and antibody reaction to *Chlamydiae* reveal that similar to organisms with peptidoglycan cell wall, the chlamydial cell envelope possess penicillin binding proteins that make the organism sensitive to peptidoglycan inhibiting drugs [11]. The peptidoglycan is a closed covalent polymer with glycan strands made of alternating N-acetylglucosamine and N-acetylmuramic acid residues of *C. trachomatis* that are

cross-linked by peptides [12]. The outer membrane proteins are cross-linked by disulfide bonds which are thought to maintain the structural integrity of the cell wall in reported absence of peptidoglycan [11]. Hence, it has been described as an anomaly. However, recent studies by Liechti *et al.* [13] have shown demonstrated the presence of peptidoglycan by using dipeptide probes which are incorporated into chlamydial peptidoglycan and captured via click chemistry reaction. Based on the characteristics of their outer membrane proteins, *C. trachomatis* are categorized into nineteen different serovars [14, 15].

The developmental cycle consists of infections and non-infectious stages of *Chlamydia* with unique morphological, biological and biochemical properties. The extracellular, infectious and non-dividing Elementary body (EB) is designed for passage between host cells; while the replicating, intracellular form of the bacteria is referred to as the reticulate body (RB) [6, 17]. Through ligand-receptor interaction, the small dense EB establishes contact with the host epithelial cell, and during the course of the infection they remain within a parasitophorous vacuole called the inclusion vacuole [18, 19]. Microscopic analysis revealed that EB differentiates into RB, which further replicates by binary fission [19]. The elementary body (EB) is a small (0.3μm), round, electron dense, infectious form of the organism [20]. The nucleoid of elementary body are highly compacted due to condensation of nuclear material by histone-like proteins HctA and HctB [21]. These morphologically different forms dictate the unique developmental cycle of the *Chlamydiae* species. The pregenomic investigations of metabolic functions of the bacteria had established that only RBs are metabolically active, and EBs were inert forms of the organism. The failure to detect ATPase and cytochrome c oxidase activities in EB indicated that the infectious form of the bacteria was metabolically dormant [22]. In the RBs, the reduction of cross-linked cysteine rich outer membrane of MOMP (Major outer membrane protein) by DTT (dithiothreitol) generates a channel for passage of nutrients and ATP. However, the treatment of EB with DTT revealed that although cysteine rich outer membrane of MOMP (Major outer membrane protein) complexes were reduced, it failed to stimulate ATP transport or incorporate methionine [23, 24]. Thus, this further demonstrates that EBs are metabolically inactive. The advent of genomic investigations revealed a wide range of genes with unknown functions that were involved in several metabolic pathways (as reviewed in [25]. Through the

whole proteome study, Skipp *et al.* [26] identified several proteins in EBs that are involved in metabolism and transcription. Quantitative proteomic analysis undertaken by Saka *et al.* [27] also identified proteins required for central metabolism and glucose catabolism in EBs. Additionally, Omsland *et al.* [28] measured de novo protein synthesis of *Chlamydia* intracellular phosphate -1 (CIP-1) in axenic medium, and showed that EBs preferentially used glucose-6-phosphate as an energy source, while RB required ATP. This proves that EBs are metabolically active. The developmental cycle is completed when an unidentified signal assumed to be triggered by depletion of nutrients and dearth of ATP induces asynchronous differentiation of RBs back into EBs [6, 29]. The EB is released from the inclusion through host cell lysis such that it can infect neighbouring epithelial cells to propagate the infectious process [6]. Depending on the species, the developmental cycle varies from 36 and 72 hours [17]. The developmental cycle is illustrated in Figure 2.1.

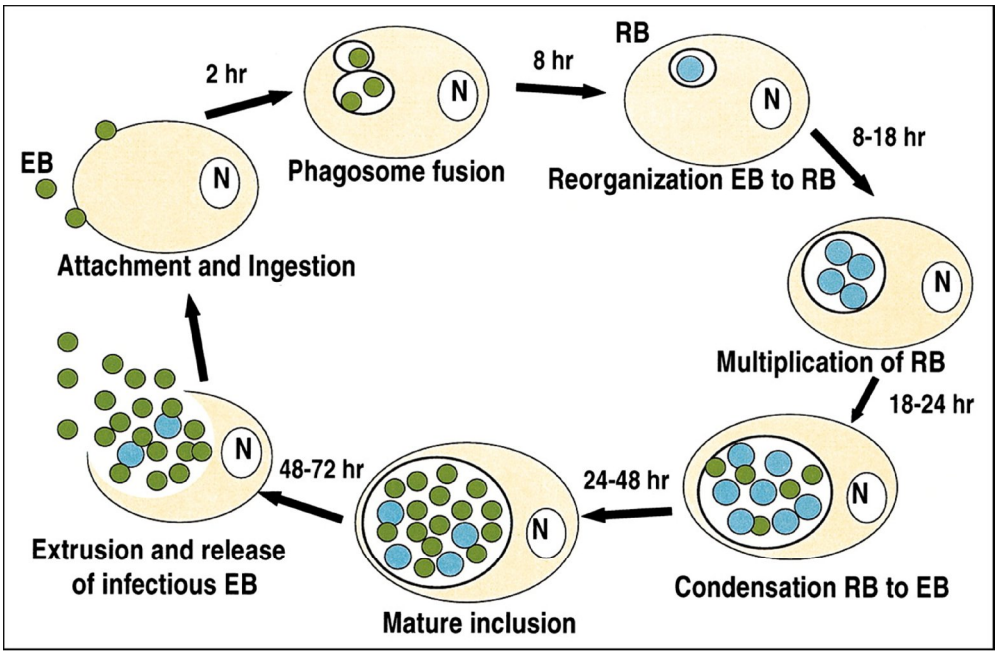

Figure.2.1:Diagram representing the various stages in the developmental cycle of *Chlamydia trachomatis*

The developmental cycle begins with attachment of EBs on the host cell facilitated by Type Three secretion system (TTSS). Once inside the host cell, it resides within a parasitophorous vacuole called inclusion. Within 24-30 hours post infection, the EBs differentiate into replicative RBs, which further replicate through binary fission. The RBs revert back to EBs through TTSS preloading and extruded from the host cell through lysis at 48-72 hours post infection**.** Figure copied directly from Hammerschlag [30].

2.1.1 Chlamydial entry into the host cell

C. trachomatis EB initiates infection by attachment and internalization into host epithelial cells through receptor-mediated endocytosis [31]. Chlamydial entry into host cell through adsorptive endocytosis and intracellular translocation is facilitated by proteins F- actin and Clathrin [32]. Glycosaminoglycans and outer membrane proteins act as adhesions to bridge unknown receptors for cell attachment [31, 33, 34]. Although there are numerous studies elucidating the role of clathrin-mediated endocytosis, ultrastructural and biochemical analysis conducted by Boleti *et al.* [35] demonstrated that *C. trachomatis* also utilizes clathrin-independent and actin-dependent pathway. This suggests that *C. trachomatis* enters a host cell through

a process similar to phagocytosis [35]. The role of actin-dependent mechanism in chlamydial internalization has been evaluated by addition of actin inhibiting agent, cytochalasin D, which resulted in significant reduction in internalization [36, 37]. Thus, actin plays an important role in chlamydial internalization by inducing changes in the microvillar structure [36]. This further supports the receptor-mediator phagocytic entry of *C. trachomatis* into the host cell.

In vitro inhibitor studies and *in vivo* CFTR deficient mutant mice showed that the bacteria also utilized a chloride channel located in the apical membrane of epithelial cells called cystic fibrosis transmembrane conductance regulator (CFTR) in its entry and internalization into the host cells [31]. Caveolae are glycosphingolipid are cholesterol-enriched invaginations in plasma membrane that are involved in endocytic pathways [38]. Although several studies provide evidence of *C. trachomatis* entry via caveolae –mediated endocytosis [31, 38, 39], Hybiske and Stephens [40] showed that targeted disruption of caveolae did not alter the internalization and infectivity significantly. However, caveolin plays an important role in the formation of inclusions by preventing chlamydial phagosome from fusing with lysosomes in most chlamydial species [38, 39]. In contrast, the entry of chlamydial species including *C. trachomatis* serovars A, 36B, and C, LGV serovar L2 and MoPn were not mediated by caveolin [39]. Tyrosine phosphorylation of host and bacterial proteins triggers a signalling pathway that induces actin mobilization for the entry of the organism [41]. The proteins that are phosphorylated are of chlamydial origin and are referred to as translocated actin-recruiting phosphoprotein (TARP) and it plays an important role in actin remodelling [42]. The TARP assists in translocation of tyrosine kinase substrates that stimulate actin-driven invasion by *C. trachomatis*, and subsequently subverts host cell functions [42]. Lane *et al.* [43] identified Rac-dependent signalling pathway (WAVE2 and Arp2/3) in facilitating TARP mediated actin recruitment for chlamydial entry and invasion. Interestingly, female hormones also play an important role in enhancing chlamydial attachment. It was reported that chlamydial attachment on oestrogen-dominant primary human cell was increased from 65% to 90%, while progesterone-dependent human epithelial cell decreased chlamydial attachment from 50% to 30% in a dose-dependent fashion [44, 45]. *In vitro* studies and co-culture models by Hall *et al.* [46] showed that exposure

to oestrogen activated the phosphatidylinositol-3 kinase (PI3K) pathway that aids in the entry of *C. trachomatis* into the host cell.

2.1.2 The nascent inclusion vacuole –trafficking & modification

After entering the host cell, the C*hlamydiae* reside inside an inclusion which is a parasitophorous vacuole that is nonfusogenic with endocytic pathway and intercepts exocytic vesicular traffic from Golgi apparatus [47]. The inclusion has been characterized as nonfusogenic vacuole with lysosomal and endosomal membranes due to the absence of the lysosomal (acid phosphatase) and endososomal markers (transferrin receptor and cation-independent mannose-6-phosphate receptor) [48]. Within 2 hours of entry into the host cells, chlamydial inclusions fuse with a subset of host vesicles containing sphingomyelin in the Golgi apparatus [20]. If the bacteria fail to modify the inclusion within the first 2 hours of infection, it could lead to its death by lysosomal fusion [49].

Organelle sequestration and subversion

The intracellular membrane trafficking by *C. trachomatis* is controlled by various host factors such as N-ethylmalemide-sensitive factor (NSF) attachment proteins (v-SNARE), small GTP binding proteins, ADP ribosylating factors and Rab GTPases [50]. Among these cellular factors, Rab GTPases play a primary role in SNARE recruitment, vesicle tethering and other distinct membrane trafficking process through specific recruitment of Rab effector molecules to *C. trachomatis* [50]. Moreover, the inability to detect specific associations between endosomal Rabs, GFP- Rab7, GFP- Rab9 and GFP-Rab5 through immunofluorescence microscopy, further support the theory that during the formation of inclusion, the bacteria bypasses the early endocytic pathway completely [50]. Some of the Rab GTPases that are actively involved in the biogenesis of chlamydial inclusion are Rab1, Rab4 and Rab11 that belong to endocytic/phagocytic pathway, and ER-Golgi related Rab 1, 6 and 10 [51, 52]. Rab 6, 11 and 14 were reported to be recruited in the absence of microtubules, and their presence has been detected throughout the developmental cycle [50]. They also play an important role in sphingomyelin delivery to the inclusion [49]. In addition to Rab GTPAses, the organelle identity is also governed by specific phosphoinositide (PI) species that are present on the organelle [53].

Phosphoinositides are short-lived phosphorylated derivatives of phosohatidylinositol that play an important role in vesicle –mediated trafficking by controlling subcellular localization, cellular signalling and activation of effector of PI binding proteins [54]. The Golgi-to-Plasma membrane trafficking is aided by PI4P, a product of Oculocerebral syndrome of Lowe protein 1 (OCRL1) gene that is localized in the Golgi complex [53]. Moorhead *et al.* (2010) proposed that unlike *Legionella pneumophila*, *C. trachomatis* does not secrete PI4P binding proteins. Instead, chlamydial maturation is inhibited by OCRL1 via endosomal pathway through PIP2 substrate removal. The protein further facilitates intracellular trafficking of clathrin-coated vesicles between early endosome and trans-Golgi network [53]. Through time course analysis of isoleucine incorporation into the phospholipids of *C. trachomatis* infected cells, Wylie *et al.* [55] demonstrated that infection by *C. trachomatis* is also associated with the bacterial acquisition of host-derived lipids. The trafficking of eukaryotic phospholipids is yet another way the bacteria uses to evade lysosomal fusion as the vacuole would resemble a cytoplasmic organelle.

Lipid trafficking

Sphingomyelin from Golgi apparatus is acquired early in the cycle and it is essential for chlamydial development [56]. Fluorescent microscopy analysis by Hackstadt *et al.* (1997) [57] indicated that sphingomyelin is localized to the luminal surface of exocytic vesicles which on fusion with chlamydial inclusion is adsorbed by the *Chlamydiae* into the inclusion. The study also reported that sphingomyelin prevents fusion of inclusions with lysosomes by modifying the inclusion membrane. Mittal and Hackstadt [58] reported that Src family tyrosine kinase Fyn plays an important role in sphingomyelin acquisition. Although, the sphingomyelin synthesis or trafficking was not altered by the siRNA mediated silencing of Fyn in the absence of chlamydial infection, it reduced the amount of sphingomyelin trafficked into the inclusion [58]. Scr family kinase phosphorylation of dynamin 2 also leads to Golgi fragmentation, and inhibition of Golgi fragmentation that results in the reduction of sphingomyelin acquisition [58]. Although, chlamydial requirement for sphingomeylin is low initially, the demand increases with increased bacterial replication and expansion of the inclusion, during which Golgi fragmentation also takes place [59]. This suggests that fragmentation of Golgi apparatus is a pathway for

lipid transport and acquisition. *C. trachomatis* also obtains its lipid requirement through lipid droplets (LD) which are translocated from the host cytoplasm to the inclusion membrane, as observed through fluorescence microscopy and electron microscopy. The translocation of LDs is facilitated by inclusion membrane protein IncA and chlamydial Lda3 protein that link cytoplasmic LDs to inclusion membrane [60]. Soupene *et al.* [61] established that human acyl-CoA carrier hACBD6 binds to LDs during development and expansion of *C. trachomatis* by modulating the acyltransferase activity of bacterial protein CT775 and formation of phoatidylcholine. Therefore by harnessing LD function, *C. trachomatis* may co-opt non-vesicle mediated phospholipids and cholesterol transport [62].

Inc Proteins

The Chlamydial inclusion membrane proteins (Inc) reside within the inclusion membrane and interact with host proteins. These proteins share a common bilobular hydrophobicity motif that spans the inclusion membrane, but only a limited number of proteins are conserved amongst themselves [20, 63]. Inc proteins were first identified in *C. psittaci* [64] and subsequently identified in *C. trachomatis* [20, 65]. By using fusion proteins and antifusion antibodies, Li *et al.* [66] reported that out of 50 Inc proteins in *C. trachomatis*, 22 were localized in the inclusion membrane, while 3 were detected in the inclusion and the location of remaining 21 were indeterminable. The study showed that most of the membrane localized proteins identified were immunogenic.

IncA, first identified in *C. psittaci*, regulates the fusion of inclusions and interaction between the inclusion and intracellular compartment [67-70]. In addition to IncA, Inc G and Inc F are exposed to the cytosol and undergo phosphorylation, an integral step in chlamydial entry and infection [70]. Src Kinase and Fyn co-localizes four inc proteins including Inc B, CT101, CT222 and CT850 in inclusion membrane microdomains where it affects the centrosome stability of the organism by interacting with the microtubule network [71]. Yeast two hybrid analysis suggests a guanine nucleotide-dependent interaction between *C. trachomatis* specific Inc CT229 and host Rab4, which subsequently promote interactions with host secretory pathways through recruitment of Rab GTPases [49, 72]. Inc D recruits CERT, a host factor that is involved in *C. trachomatis* infection through nonvesicular transfer of ceramide at ER-Golgi membrane contact sites (MCSs) and induces metabolism or signalling

events favourable to replication and development of the organism [73]. Several putative Inc proteins possess a high probability of coiled-coil conformation which is consistent with the hypothesis that proteins exposed to the cytosol regulate interaction between the inclusion and cellular compartments of the host [73]. About 90% of these proteins also possess Type III secretion signals which suggest that the proteins are secreted and translocated through the type III secretion system [74].

Type III secretion systems

Similar to other Gram-negative pathogens, the Type III secretion system (T3SS) serves as a conduit in *C. trachomatis* that connects bacterial cytosol and host cell cytoplasm, whereby the pathogen secretes proteins into the host cell or into inclusion membrane [75, 76]. Although the expression of T3SS apparatus occurs mid cycle, EBs contain the fully assembled apparatus that mediate secretion of substrates during initial stages of host cell infection and establishment [29]. One of the earliest roles of T3SS in chlamydial developmental cycle includes EB invasion by remodelling actin cytoskeleton through translocation of effector proteins translocated actin-recruiting protein (TARP) and CT694 [41, 75, 77]. Using bacterial two-hybrid and size exclusion chromatography, Brinkworth *et al.* [75] elucidated the role of putative type III secretion chaperone Slc1 (SycE-like chaperone 1) in enhancing TARP translocation for actin remodelling. Other extended apparatus of T3SS secretion system are *Chlamydia* outer proteins CopN and CopB and host interactive proteins such as *Chlamydia* protein associated with death domains (CADD) [77]. Chlamydial protease, chlamydial protease-like activity factor (CPAF) enters into the host cytoplasm and facilitate inclusion expansion through a type-III independent secretion mechanism independent mechanism by degrading a wide array of host proteins [77, 78]. The protein TepP (Translocated early phosphoprotein) facilitates early establishment of niche for chlamydial replication within the cell by recruitment of additional scaffolding proteins like Crk, which in turn recruit proteins to nascent inclusions [79]. Although, CPAF induces Golgi fragmentation, the fragmentation and sphingomyelin acquisition is not dependent on cytosolic CPAF [80]. The pivotal role of T3SS in manipulating host cell processes and replication has been elucidated with the help of chemical inhibitors such as compound 1 (C1), which inhibited the T3SS secretion system. This resulted in the suppression of multiple sigma factors, reduced amount of late cycle genes (*hctB*, *omcA* and *omCB*) required for differentiation of

RB to EB, low abundance of T3SS secreted apparatus components (CopN, CopB2) within bacteria and inhibition of IncA [78].

2.1.3 Exit from the inclusion

The EB accumulate within the inclusion until released by extrusion from the host cells or inclusions rupture inside host cytosol for lateral entry into uninfected host cells [77]. Extrusion includes a packaged release process by which chlamydial inclusion are released by membranous protrusion [81]. The asynchronous process of differentiation of RBs back to EB begins about 18 h after infection and continues upto 44 h in *C. trachomatis* L2 strain [82]. Using RT-PCR, Shaw *et al.* (2000) confirmed the role of OmcB (60kDa cysteine-rich outer membrane protein) and chlamydial nucleoprotein Hc1 (encoded by *hctA* gene) in the later stages of developmental cycle and are facilitate the production of EB from RB. Hc1 regulates the repression of transcription and condensation of nucleoid that is characteristic of differentiation of RB to EB [82, 83]. OmcB confers structural stability to EB as it forms a component of the disulphide-liked outer membrane protein complex [23]. Through microarray analysis, about 26 late genes were identified such *hctA* and *hctB* that encode for chlamydial histone-like proteins which facilitate nucleoid condensation [84]. Recruitment of actin and actin polymerization plays an integral role in chlamydial extrusion. The polymerization and the coat assembly have been driven by products of type III secretion, and at 68 hours post- infection, the chlamydia prepares for extrusion by increasing the actin recruitment and enwrapping the entire inclusion [85]. Treatment of *C. trachomatis* with pharmacological inhibitors revealed the involvement of neuronal Wiskott-Aldrich syndrome protein, myosin II and Rho GTPase in extrusion mechanism. [81]. GFP analysis and mutation studies showed that depletion of Septins that function in organizing actin coat for inclusion resulted in reduced extrusion [86]. The areas marked for imminent extrusion were those where the inclusion was distal to nucleus or bulged out of the cell [85].

2.1.4 Molecular mechanisms associated with chlamydial persistence

C. trachomatis enters an alternate morphological state called ‘persistence’, which is a viable, but unculturable long-term state. The state of persistence in *Chlamydiae* is driven by the presence of various stress conditions including; IFN-γ, antibiotics, and iron chelating agents [87]. Matsumoto and Manire [88] were one of

the first to characterize chlamydial persistence in *Chlamydia psittaci*. The study reported that the treatment with 200 units of penicillin prevented binary fission of RBs to EBs and resulted in large abnormal RB forms in *C. psittaci*. The study further revealed that after 36 hours post infection, masses of cytoplasmic membranes formed within RB and around it, resulted in the formation of immature nucleoids within the RBs. Skilton *et al.* [89] demonstrated the effect of penicillin (100units/mL) using high quality time lapse video analysis, and found that in addition to the morphological abnormalities, replication of chlamydial chromosomal and plasmid DNA remain unaffected. However, removal of penicillin did not induce complete transformation from aberrant RBs to normal RBs, and with the exception of few inclusions, the normal developmental cycle was re-established after 10-20 hours of removal. Confocal microscopy and immunocytochemistry of penicillin induced persistent *C. trachomatis* infection revealed high levels of HtrA at mid (20h PI) and later stages (44 hPI) of persistence [90]. This suggests that HtrA adopts a protective function during penicillin persistence against additional protein stress. Addition of an HtrA inhibitor, JO146, to *C. trachomatis* Hep-2 culture during recovery and reversion from penicillin persistence resulted in complete lethality for *C. trachomatis* [91]. Thus, confirming the functional role of HtrA in chlamydial persistence.

Persistence in *C. trachomatis* is also induced by inflammatory cytokine, IFN-γ, which induces cytosolic tryptophan degrading enzyme indoleamine 2,3-dioxygenase (IDO) that catabolizes L-tryptophan to N-formylkynurenine [92]. However, IDO-mediated tryptophan starvation leads to conversion of *C. trachomatis* into its persistent form [93]. Beatty *et al.* [93] elucidated this mechanism through HeLa cell models, and reported that IFN-γ reduced intracellular levels of tryptophan and increased the activity of IDO (measured by HPLC). The study further reported that IDO deficient cell line and tryptophan deficient studies implicated that tryptophan depletion is responsible for IFN-γ -mediated *C. trachomatis* persistence. These findings were further supported by Leonhardt *et al.* [94] who demonstrated that low exogenous tryptophan concentration prevented bacterial trafficking to the microtubule organizing centre (MTOC) and prevents lysosomal fusion. Thus, the organism remains in its aberrant form and exhibits low transcriptional activity, and despite replenishing the tryptophan levels, the rate of re-activation of *Chlamydia* remain slow. Transcriptomic analysis of IFN-γ -mediated *C. trachomatis* persistent

infection revealed an upregulation of genes required for tryptophan synthesis (*tprR*, *trpB* and *trpA*), phospholipid metabolism, and early cycle genes. The genes that were downregulated included genes that regulate RB to EB differentiation (*hctA*, *hctB*, *ompB*, and *ompC*). A *Chlamydia*- specific gene (Euo) that regulates late gene expression and facilitates complete silencing of these genes were also upregulated during persistence. Thus, during chlamydial persistence, genes pertaining to metabolic process were activated, whilst genes regulating late cycle genes were down regulated [95]. Akers *et al.* [96] reported that the aporepressor, TrpR regulates tryptophan biosynthesis genes in *C. trachomatis* by forming a complex with coreporessor tryptophan and subsequently activating the trpRBA operon. The study also showed that the TrpR gene also allows the bacteria to sense tryptophan limitation. IFN-γ treatment of *C. trachomatis* in HeLa cells downregulates chlamydial protease-like activity factor (CPAF) activity and prevents cleavage of proaptotic BH-3 only proteins, Bad and Puma. The study further reported increased production of high mobility group box 1 (HMGB1) protein following prolonged IFN-γ treatment. This suggests that although persistent *C. trachomatis* infections are apoptosis-resistant, but they contribute to chronic inflammation and disease through HMGB1 release [97].

Iron is an essential co-factor that is critical in several biochemical reactions and is highly regulated in most organisms [98]. Iron-chelating agents such as deferoxamine mesylate (DFO) deplete iron, resulting *C. trachomatis* persistence. The effectiveness of DFO in depleting iron or ferritin content from infected cells was demonstrated by Dill *et al.* [99], who reported a significant drop in ferritin level (28.2 ng/mL to 3.2ng/ml) by DFO as compared to transfected cell line with inducible over-expression of eukaryotic iron efflux protein ferroportin (49.3 ng/mL to 38.2 ng/mL) that facilitates iron starvation in *C. trachomatis* E. Addition of DFO also induced enhanced expression of cHSP60 in *C. trachomatis* E infected cells [100]. This suggests that cHSP60 facilitates persistence mediated by iron starvation in *C. trachomatis*. Thompson *et al.* [98] identified another iron chelator, 2-2'-bipyridyl (Bpdl), which is membrane permeable and exhibited a greater degree of chlamydial inhibition and induction of aberrant morphology compared to DFO. The study showed that 100 μM of Bpdl retarded chlamydial DNA replication and upregulated chlamydial iron responsive genes, *ahpC* and *devB*, in contrast to DFO which

upregulated only one gene, *devB*. The effectiveness of Bpdl in inducing iron starvation-mediated chlamydial persistence over DFO was further validated by the upregulation of *ytgA* gene, an iron-response gene that is involved in iron acquisition/ transport into *Chlamydia*.

2.2 SEQUELAE ASSOCIATED WITH *C. TRACHOMATIS* INFECTIONS IN WOMEN

Despite enhanced screening efforts and control programs, the number of genital *C. trachomatis* infections have been reported to have increased significantly over the last decade in Australia, United Kingdom, North America and Europe [101]. Furthermore, long-term *C trachomatis* infections pose a major problem in women's health care as the disease can lead to reproductive morbidity [102]. Acute diseases caused by *C. trachomatis* include urethritis and cervicitis, while the *C. trachomatis* - related infertility / chronic form of the infection include ectopic pregnancy, pelvic inflammatory disease and tubal infertility [102]. *C. trachomatis* infections are responsible for about 45% of reported cases of tubal factor infertility (TFI) in women [103]. The recurrence of chlamydial infection have been reported to be about 30-50% in young adolescents, and repeated exposure to the bacteria by either persistence or recurrent infection induces cell mediated immunological reactions and delayed hypersensitivity response which lead to fibrosis, scarring and tubal factor subfertility [7]. In the United States itself, tubal factor infertility accounts for about 10% of assisted reproductive technology cycles and the average cost of an *in vitro* cycle is upto $12,000 [104]. In Europe, annually about 600,000 cases of salpingitis (inflammation of fallopian tubes) caused by *C. trachomatis* is reported, of which 12,000 cases resulted in mechanical infertility [105].

2.2.1 Pelvic Inflammatory Disease

Pelvic inflammatory disease is characterized by the infection of fallopian tubes, uterus and adjacent pelvic structures that are not associated with surgery or pregnancy [106]. About 10,000 women in Australia are treated for PID each year, and the incidence of the disease is highest in women between the ages 20 to 29 [107]. It encompasses a wide range of upper genital tract conditions such as endometritis, tuboovarian abscess, salpingitis, periappendicits, perihepatitis and pelvic peritonitis [108]. When the bacteria ascends from the vagina and endocervix to the endometrium, fallopian tubes and adjacent structures, it results in acute PID [108].

PID is treated by administering antibiotics such as parenteral cephalosporin and doxycycline [109]. PID can result in serious *C. trachomatis* -related infertility such as chronic pelvic pain, ectopic pregnancy and infertility [109]. Each year 750,000 women experience acute PID in the United States, of which about 10%-15% of these women become infertile when left untreated [110]. Davies *et al.* [111] reported that in 307 sex workers recruited from Genito- Urinary Medicine (London), the rate of PID in women with recent chlamydial infection was 27.4 per 100 person-years as compared to the 11.2 in women with previous chlamydial infection as estimated by direct immunofluorescence. Scholes *et al.* [112] showed that with the increase in *Chlamydia* diagnosis and subsequent treatment rates from 449 cases/10,000 per year in 1997 to 806/ 10,000 per year in 2007 resulted in a decrease in the PID rates from 823 cases/100,000 per year in 1997 to 473/100,000 per in 2007 in USA ($P<0.01$). These findings were further supported by a mathematical modelling study, wherein an implementation of hypothetical screening intervention resulted in prevention of 187 PID cases per 100,000 women in 5 years and 956 PID cases per 100,000 women in 10 years [113]. Thus, increasing the screening strategies and intervention for *C. trachomatis* could significantly reduce the incidence of PID in women.

2.2.2 Acute Salpingitis

Acute salpingitis is caused by an ascending infection from the lower genital tract and it may result in peritubal adhesions, post- inflammatory fibrosis and tubal restriction [114]. It is diagnosed laproscopically and it is characterized by the presence of erythema and odema in the fallopian tubes [115]. The odema associated with salpingitis augments intraluminal agglutination which leads to clubbing of fallopian tubes that result in dysfunctional, partially or completely obstructed fallopian tubes [116]. The agglutination may develop into a filmy, thick pelvic adhesive disease resulting in pelvic pain [116]. Sweet *et al.* [117] established a relationship between the menstrual cycle and the onset of acute salpingitis caused by *C. trachomatis*. The study highlighted the importance of hormonal changes in chlamydial pathology, which explains the increased prevalence of acute salpingitis during the beginning of menses rather than other times in the menstrual cycle. This has been further supported by Rank *et al.* [118], in which treatment with hormones such estradiol on a guinea pig enhanced infection in the cervix and resulted in hydrosalpinx. Hydrosalpinx is characterized by occlusion of fimbriated end of the

fallopian tube and distension of the distal end with fluid [119]. Treharne *et al.* [120] reported that *C. trachomatis* antibody titers were found in two-thirds of women with acute salpingits (n=143), while only two tested positive in women without salpingitis (n=19). Based on the histopathology, the study also showed that salpingitis is often accompanied by endometritis.

2.2.3 *C. trachomatis*-related infertility in women

Subclinical PID is clinically recognized as acute PID (caused by ascending spread of organisms from lower genital tract to upper genital tract) and it stimulates the development of PID-related *C. trachomatis*-related infertility [121].The disease is often referred to as "silent epidemic" as it is asymptomatic and hence goes unnoticed. Malik *et al.* [122] showed that the predominant cause of infertility were asymptomatic *C. trachomatis* infections. *Chlamydia* positive cases could be characterized by clinical presentations such as bleeding per vaginum (on touch) and vaginal discharge. Prolonged infection in either of these tracts may result in subfertility, or infertility. Subfertility generally describes any form of reduced fertility for long periods of time of unwanted non-conception [123]. Infertility is similar to subfertility, however subfertility is characterized by sporadic occurring spontaneous pregnancies [123].

Long term *C. trachomatis* -related infertility or chronic infection develops due to reinfection, persistence or treatment failure [124]. It was reported that Australian women show a re-infection rate of 22.3 per 100 person-years and it is more prevalent in women between the ages of 16-20years [124]. In developing countries such as Nigeria, the overall prevalence rate of asymptomatic *C. trachomatis* infection estimated by PCR in 132 infertile women attending Gynaecological clinic was 20.5% as opposed to 10.4% in the UK and other developed countries [125, 126]. In Brazil, the prevalence of IgG antibodies against *C. trachomatis* were significantly higher in subfertile group (n=55) as compared to parous women (n=55) (56.4% vs 31%; $P<0.01$)[127]. The prevalence of *C. trachomatis* in infertile women attending gynecological clinic in South West Nigeria was 20.5% (n=130)[125]. The infection often results in recurrent spontaneous abortion [129]. The infection is also transferred to the fetus as evident from the presence of IgM antibodies to *C. trachomatis* in the cord blood of infants of mothers who delivered preterm [130]. This results in stillbirth, which occurs in 20-30 gestational weeks.

Ectopic pregnancy is one of the major causes of maternal mortality in the first trimester in developing countries [102]. Coste *et al.* [131] reported that *C. trachomatis* was the strongly associated (25.2%) with ectopic pregnancy in women (n=624) recruited from 15 maternity hospitals in France. Svensson *et al.* [132] showed that out of 112 women with ectopic pregnancy, 75% showed the presence of IgG antibody to *C. trachomatis* as compared to 21% of the controls. In addition to the IgG antibodies, cHSP60 antibodies have also been found in abundance in the sera of infected females [133]. In Nigeria, a greater number of women with ectopic pregnancy (confirmed by laparoscopy) (n=98) were seropositive for *C. trachomatis* as compared to age matched pregnant controls (n=98) (48% vs 16%; P<0.001)[134]. A prospective study by Sziller *et al.* [135] reported that the antibody responses to cHSP60 in women with ectopic pregnancy correlated with decreased subsequent spontaneous fertility and repeated ectopic pregnancy.

Thus, the prevalence of *C. trachomatis*-induced sequelae could be reduced with routine testing and timely treatment. The mechanism of reproductive sequelae of *C. trachomatis* infections in certain women is yet to be understood.

2.3 PROPOSED MODELS FOR THE DEVELOPMENT OF INFERTILITY FOLLOWING *C. TRACHOMATIS* INFECTION IN WOMEN

This review outlines four models that are most commonly referred to within the field to explain the process that underlies the development of infertility following *Chlamydia* infection. The models are not exclusive of each other and there is evidence for and against each model. Figure 2.2 summarises the factors associated with chlamydial infertility.

2.3.1 Ascension model

This model suggests that the infection ascends to the upper reproductive tract in only some women, and this ascension is usually typified by the presence of symptoms, development of PID and if unchecked consequent development of tubal pathology. There is considerable direct evidence that the chlamydial infection can ascend beyond the cervix, however, the proportion of cases in which this ascension occurs is unknown. The following studies provide evidence to the ascension model of *C. trachomatis* pathology. In a study by Lan *et al.* [136] , salpingentomy tissues (n=48 from 37 women) were tested for *C. trachomatis* DNA by PCR and it was

reported that *Chlamydia* DNA was detected in the salpingectomy (removal of fallopian tubes [137]) specimens of one woman with ectopic pregnancy.While six of the 37 women tested positive for *C. trachomatis* DNA in endometrial and cervical specimens. A hospital- based study in Myanmar comprising of 113 women with ectopic pregnancies, showed that higher *C. trachomatis* DNA was detected in tubal specimens (15%) as compared to cervical specimens (8%) from women with ectopic pregnancy [138]. The presence of *C. trachomatis* in upper genital tract and the affected organs were further elucidated in Barlow *et al.* [139], where women recruited from hospitals in UK with ectopic pregnancy (n=33) and tubal factor infertility (n=14) were tested by PCR and *In situ* hybridisation (ISH). The study reported that *C. trachomatis* was detected in 44% of TFI patients (ISH), of which five patients had *C. trachomatis* DNA in the fallopian tube while two patients were positive in the endometrium. Using PCR, *C. trachomatis* DNA was detected in 71% of the TFI patients, of which *C. trachomatis* DNA was present in the endometrium of seven patients, while five patients showed positivity in the fallopian tube and five others in the ovary [139]. *C. trachomatis* organism presence was associated with endometritis (plasma cell influx in the endometrium) [140, 141]. A small study in the UK identified the presence of *C. trachomatis* in the upper genital tract from 4 out of 10 women all of whom had no symptoms suggesting that is it possible to have asymptomatic ascension and that ascension may not occur in all women [142].

This ascension model is also supported by substantial evidence of chlamydial inducing a pathological immune response in human cell and tissues and is mutually inclusive with the Persistence and/or Cellular Paradigm models. However, there is only limited evidence to support that; 1. Ascension of the infection occurs in some but not all women or that; 2. that lower genital tract symptoms and/or signs typify upper reproductive infection because mucopurulent cervicitis and other cervical/genital symptoms/signs have been reported in both the presence and absence of upper reproductive tract symptoms or with laparoscopic evidence of salpingitis (ascending infections) [143, 144]. However, upper genital tract infection does not happen in the absence of cervicitis/lower genital tract symptoms of inflammation. This was supported by Westrom and PEACH PID studies [145, 146] whose diagnostic and inclusion criteria included women with mucopurulent cervicitis.

It seems possible or even likely that ascension may be independently moderated by numerous other factors that could explain the low frequency of pathology development, supporting that it only occurs in some women. These moderating factors could include host factors such as menstrual cycle stage or hormone status at time of infection, as chlamydial or gonococcal endometritis was found to be more likely to be detected in women in the proliferative phase [147]. Equally important moderating factors could include immune status, genotypic factors, other co-infections, or reproductive tract microbiome composition. However, there is no published evidence that associate these factors with ascension of *C. trachomatis* infection into the upper genital tract. Furthermore, pathogen factors could also moderate the likelihood of ascension such as infectious burden (although chlamydial serovar alone has not been able to be significantly associated with pathogenic potential or clinical manifestations [143]).

2.3.2 Persistence model

Chlamydia persistence, or the presence of viable but non-culturable chlamydial organisms, develops in response to adverse conditions, and can remain dormant but present for considerable lengths of time (as reviewed in [18]). *C. trachomatis* are tryptophan auxotrophs which they obtain from their host and the host cell intracellular pools of tryptophan are inhibited by IFN-γ induced tryptophan catabolizing enzyme (indoleamine 2,3-dioxygenase) [18]. IFN-γ mediated persistent *C. trachomatis* infection results in the absence of EBs, and they are typically in the form of larger RBs with altered morphologies with fewer cells in each inclusion [18].

C. trachomatis persistence is mediated by virulence factors, environmental factors and host immune factors [148]. It was reported that following treatment for chlamydial infection, LCR (Ligase Chain Reaction) testing of urine showed that about 13.4% of women had persistent infection after a median of 4.3 months [149]. Ligase Chain Reaction detects *C. trachomatis* infection in urogenital speicmens and urine, and targets nucleotide sequences of chlamydial cryptic plasmid [102]. The study diagnosed women with persistent *C. trachomatis* infection if they were still positive for the infection, despite abstaining from sexual activity during the first and second follow-up. In contrast, women werer diagnosed with recurrent infection if they had resumed sexual activity or had multiple sexual partners, and tested positive for the infection in their first and second follow-up. Persistence can also be induced

by antibiotics such as ciprofloxacin and ofloxacin that restrict productive infection but cause the bacteria to go into a viable, metabolically inactive period during the course of the treatment [150]. Removal of the antibiotic allows the organism to resume its normal growth, reactivating the infection [150]. *In vitro*, the persistent stage can be induced by treatment with IFN-γ [151-154], penicillin and nutrient starvation [154, 155]. IFN-γ secreting CD4+ and CD8+ T cells induce the production of indoleamine 2,3-dioxygenase (IDO) which mediates tryptophan starvation by catabolising it to kynurenine [156-158]. This induces persistence of *C. trachomatis* in the host as it interferes with the growth of the bacteria, a tryptophan auxotroph [156]. [158]. The IFN-γ induced persistent *C. trachomatis* could be eradicated by azithromycin, which is also effective in treating chronic infections at MBC_{100} (2.5-5μg/mL)[159]. *In vitro* studies have demonstrated the effect of different classes of antibiotics such as tetracyclin, penicillin, cephalosporin and β-lactamase based drugs induced persistence (characterized by abberrant RBs and immediate recovery on removal of antibiotics) at physiologically relevant conditions [160]. Removal of tryptophan prompts the bacteria to import it from the neighbouring cells and use it to overcome IFN-γ induced persistence [157]. Low intracellular amino acid concentrations give rise to abnormal organism development with reduced infectivity yields [155]. A similar effect was observed when the media was deprived of glucose [155]. Infected cells treated with IFN-γ was found to have increased levels of cHSP60 [161], major outer membrane protein (MOMP) (omp1)[161, 162] and LPS [153, 163].

Persistence *in vivo* has been proposed as a model where this on-going presence of the organism may drive a long-term pathological immune response resulting in the tissue damage to the fallopian tubes (certainly the high expression of the dominant antigen cHSP60 has been detected in laboratory persistence models) (reviewed, [164]). There are studies that have presented evidence of *C. trachomatis* DNA or antigens being present in the tubal material of women with tubal infertility or ectopic pregnancy or salpingitis, supporting that organism persistence in some form occurs *in vivo* [139, 166-168]. Recently, morphologies consistent with laboratory models of persistence were observed in endocervix samples associated with IFN-γ, and transcriptomic analysis revealed down-regulation of genes associated with RB-EB differentiation and reactivation of *euo* and *omcb* genes that are differentially

expressed in active and IFN-γ associated persistent growth [169]. Thus, further supporting the likely *in vivo* relevance of laboratory models of persistence. It is difficult to determine if chlamydial persistence results in pathology. Persistent infection may resolve at any time after pathology develops and prior to detection, given the difficulties in specimen collection from the upper genital tract the evidence is reasonably compelling that chlamydial persistence (either consistent with the lab models or a distinct *in vivo* form) could be a relevant factor in pathology. It is important to highlight a recent review that suggested that some forms of persistence may be 'colonisation' and in fact not associated with 'pathogenesis' and disease [170]. The evidence that chlamydial persistence is induced by a range of conditions *in vitro* (reviewed, [18, 164]), and persistence like morphology and/or chlamydial antigen/DNA detection in the absence of active infection has been observed in clinical samples, suggests that persistence may be a relevant physiological status *in vivo* particularly in the case of ascension to the upper reproductive tract tissues, and therefore likely a determinant of pathological outcome of infection.

2.3.3 Cellular paradigm model

The cellular paradigm model proposes that the chlamydial infected epithelium immediately responds to the presence of the *Chlamydia* or specific antigens from *Chlamydia* in a pro-inflammatory manner, typified by pro-inflammatory cytokines and growth factors which in turn induces a pro-inflammatory secondary immune cell activation and migration to form a local lymphoid follicle resulting in cell damage and fibrosis or scarring (comprehensively presented and reviewed elsewhere [171]).

Kinnunen *et al.* [172] showed that human leukocyte antigen (HLA) class II molecules regulates the production of interleukin 10 (IL-10) at inflammation site, and it acts as a substantial suppressor of cellular immunity by enhancing Th2-type immune response and suppressing Th1- type immune response. The study also shows that cHSP60 is a marker for TFI in women, as it was associated with enhanced IL-10 production in women with TFI. Interleukin-1 production results in destruction of ciliated cells and subsequent scarring of the fallopian tubes by triggering and priming superoxide production [173]. IL-10 is an anti-inflammatory cytokine that decreases tissue damage significantly by inhibiting IL-1β and IL-8 and subsequently generate a weakened inflammatory response that accounts for the asymptomatic nature of infection in women [173].

This model is widely supported by the published evidence of human epithelia and immune cell responses to *Chlamydia* and a primary tissue *ex vivo* model. This model also presumes that chlamydial ascension is associated with pathology and also mutually allows for persistence of *Chlamydia* in the upper reproductive tract [171]. A pathological adaptive memory response proposed as the secondary component of this model is also supported by the existing data that repeat infections increase the likelihood of pathological outcomes in women [174], and by numerous immunological results.

2.3.4 cHSP60 induced delayed hypersensitivity model

There has been considerable focus on a pathology model that involves delayed hypersensitivity and/or molecular mimicry in response to specific chlamydial antigens (reviewed [175]). This model has largely resulted from a body of literature demonstrating high titre human antibody response to cHSP60 in participants with tubal pathology and additionally that in several animal models tissue pathology developed after repeated inoculations with cHSP60 protein (reviewed [171]). In humans, delayed hypersensitivity (immune responses mediated by $CD4^{+}$T cells) could be induced either at the conjunctival mucosal surface or the vaginal mucosal surface [176]. Both murine and human models have revealed that the correlation between host resistance and chlamydial infection and the role of DTH could contribute to mucosal scarring [176, 177]. In one study, using IL-10 KO mouse, it was reported that IL-10 inhibits Th-1 like immune responses via inhibition of IL-12 and TNF-α and thereby inhibit DTH following chlamydial infection [177].

However, this model is subject to considerable controversy due to; inconsistencies between the different published findings; problems with protein preparations that involved a hypersensitive detergent being used in some animal models; and cross reactivity or poor specificity for some of the antibody tests [178-182]. On the other hand there is no doubt that cHSP60 is a predominant chlamydial antigen that does frequently elicit an immune response. Furthermore, in human trachoma cases it is frequently possible to observe ongoing follicles present and disease pathology in the absence of PCR detectable *Chlamydia* suggesting that there is continuing immune activity in the absence of organism during pathology development [183].

Interestingly, one group proposed that cHSP60 antibodies detected in the follicular fluid of female IVF patients may induce early destruction of the embryo by cross reacting against embryo human HSP60 and result in lower transfer success [184]. They observed a 74.1% cHSP60 seropositivity in women without embryo implantation (n=47) and 47.9% seropositivity in women with successful embryo implantation (n=91) (p=0.0004). However, this observation directly contradicts a finding in separate and much larger study (n=1279) that cumulative IVF cycle pregnancy rates are the same for women who are chlamydial seropositive compared to those who are seronegative [185]. A study in France also contradicted the possible role of adverse embryo implantation impact of anti-chlamydial immunity as it that for found for both men and women with PCR or serological evidence of *Chlamydia* (n=52) the IVF pregnancy rate and semen characteristics were not different compared to controls that were negative for chlamydial testing (n=119) [186].

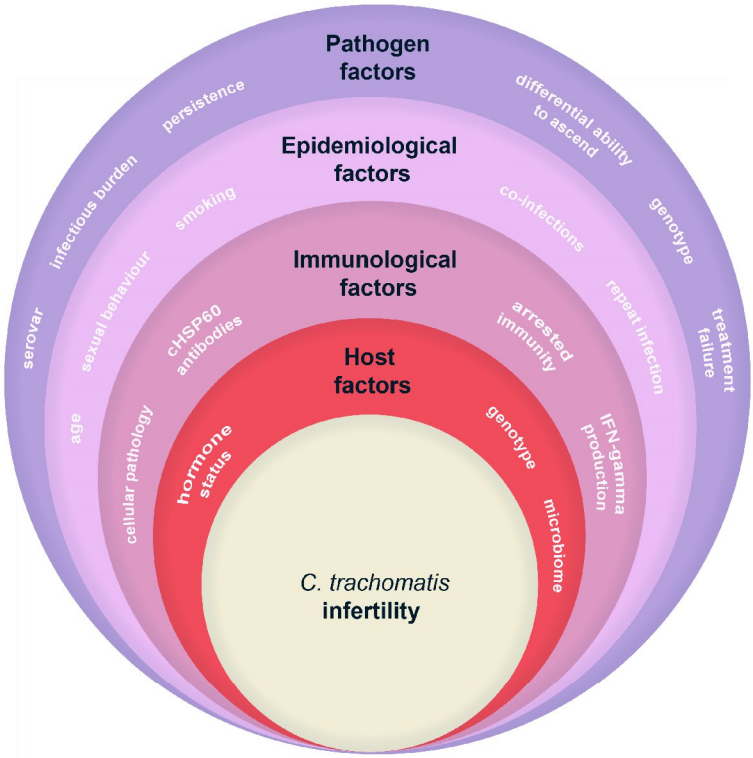

Figure 2.2: Summary of host and pathogen factors that are associated with the development of chlamydial infertility in women

The factors that contribute to chlamydial infertility at a broader level include pathogen factors that depend solely on the serovar, infectious burden, sensitivity to antibiotics and persistence. The epidemiological factors affect a particular population or cohort, characterized by their age, sexual behaviour, smoking status, co-infections and repeat infections. Although immunological factors are shared between individual, depending on their immune system the sequelae may develop due to cellular pathology, cHSP60 antibodies, arrested immunity and IFN-γ mediated protection. The host factors such as hormone status, genotype and microbiome vary between individuals.

2.4 HOST/HUMAN FACTORS ASSOCIATED WITH CHLAMYDIAL INFERTILITY IN WOMEN

There are several human/ host factors that increase the risk of *C. trachomatis* –related infertility in women. The factors may be attributed to repeat infections, treatment failure, co-infections with other STIs, hormonal status or sexual lifestyle. Although these factors are not consistent among different populations, they play an integral role in the increasing rate of *C. trachomatis* –related infertility in women.

2.4.1 Repeat infection and infertility

Repeat infection represents a substantial proportion of chlamydial infections detected annually (re-infection review; [187]). It is likely that these repeat infections are made up of reinfection, treatment failure, and persistent infections [188]. Batteiger *et al.*, [188] conducted a longitudinal cohort of 210 adolescent women (14-17 years old) participants (USA) and identified that 121 experienced a repeat infection. In a longitudinal cohort in Australia, 1116 women (16-25 year old) were followed, and 14 re-infections were observed from a total of 81 women (three of these had two episodes of re-infection) (cumulative risk over 12 months of 20.3%, 95% CI: 13.2-37.6%) [124]. The infection was classified as re-infection, if the genotype of *C. trachomatis* in the second infection was different from the first infection. Walker *et al*. [124] used the same criteria to characterize women with repeat infection; the difference in serovars between the first and second infections, and it was further confirmed by negative tests between two positive tests. Interestingly, in this study in Australia organism load was lower in re-infections compared to prevalent infections detected at the study baseline, but there were no associations between participant characteristics and re-infection [124] (although this was possibly a statistical power issue). However, careful consideration into study design is needed when evaluating repeat infection, the Batteiger and Walker studies tested quarterly for *Chlamydia*, and PCR may detect residual DNA from dead organisms causing a false positive. A study conducted in Vancouver, showed that the repeat infection occurred in 8 of 42 participants in a longitudinal study (both genders) indicating a cumulative incidence of 29% (95% CI: 12-46%), although the study was confounded by re-testing immediately being conducted in some participants [189]. A study in the UK showed re-infection was 29.9 (19.7-45.4) per

100 person years from a GP clinic setting [190]. Infection and repeat infection significantly associates with sexual behaviour risk factors such as new partners or failure to use condoms [124]. These repeat infection rates appear to be higher than the reported infertility outcome rates in women who have had a positive *Chlamydia* test in the Low *et al.* [191] retrospective cohort study, suggesting that repeat infection alone is not the sole determinant of infertility or pathology. However, there is some evidence to suggest that repeat infection increases the risk of developing infertility. A retrospective cohort study by Hillis and co-workers in Wisconsin examined the risks of hospitalisation for ectopic pregnancy or PID in 11000 Wisconsin women who had one or more chlamydial infections. They identified elevated risks of ectopic pregnancy in women who had two (OR 2.1; 95% CI: 1.3-3.4), and three or more infections (OR 4.5; 95% CI: 1.8-5.3) [174]. Pelvic inflammatory disease risk was also increased for women who had two (OR 4.0; 95% CI: 1.6 - 9.9) and three or more chlamydial infections (OR 6.4 95% CI 2.2-18.4) [174]. Therefore, repeat infection is likely to be a significant contributing factor in at least some cases of chlamydial infertility.

Treatment failures or persistence induced by antibiotics have also been proposed to be contributing to repeat infection rates (reviewed [192]). Batteiger *et al.* [188] showed that repeat infections in 13.7% of the patients were due to possible or probable treatment failures. Additionally*, in vitro* studies have shown that azithromycin induces chlamydial persistence [193]. Therefore, treatment failure or persistence (presenting as treatment failure) could be a contributing factor to development of infertility in some women via all four of the proposed pathology models.

The rise of repeat infections detected over time has resulted in the proposal that increased public health investment into chlamydial screening and treatment is preventing the development of natural immunity to the infection (arrested immunity hypothesis) [194, 195]. These repeat infections may also be the source of pathological sequelae that may not occur in women who have a protective immunity from a naturally resolved infection [194, 195].

2.4.2 Role of co-infections or microbiome in *C. trachomatis* pathology

The interactions of *Chlamydia trachomatis* with microbiome in the human genital tract are likely to contribute to the progression of the disease to sequelae. Co-infections with *Neisseria gonorrhoea* and *Mycoplasma genitalium* increase the risk of developing PID and tubal factor infertility. Co-infections with other STIs are not uncommon, for example 46% of female patients positive for *N. gonorrhoeae* were also be positive for *Chlamydia* in a family planning clinic in the USA [196]. However, a serology study in Zimbabwe, demonstrated that women with antibodies to either *C. trachomatis* of *N. gonorrhoeae* were significantly more at risk of developing PID, ectopic pregnancy, or abnormal fallopian tubes than those women with antibodies to both pathogens [197], suggesting that it is not necessary to have had infection with both pathogens to develop reproductive pathology. Patients with bacterial vaginosis who were a recent contact of a male with chlamydial urethritis had an odds ratio of 3.4 (95 % CI: 1.5-7.8) to test positive for chlamydial infection. The presence of H_2O_2 producing lactobacilli in the vagina was protective against acquisition of infection (odds ratio 0.4, 95% CI: 0.2-0.8) [198]. Recently an *in vitro* model was used to demonstrate that it is actually the acid or pH lowering impact of the lactobacilli that confers the anti-chlamydial activity [199]. It has been hypothesized that indole producing organisms that increase in the microbiome during or subsequent to bacterial vaginosis enable *C. trachomatis* to synthesize tryptophan from indole [200], consequently evading the activity of interferon gamma at the infected site and thus may facilitate the infection [200, 201].

Bacterial vaginosis (BV) during *C. trachomatis* or *N. gonorrhoeae* infection was recently found to be associated with risk of PID, although the authors conclude that difficult to determine if the bacteria underlying the vaginosis facilitated ascension and PID caused by the STIs or alternatively if the bacteria responsible for vaginosis cause the PID [202]. Patients with BV were about 3.4 times more likely to test positive for *C. trachomatis*, as it alters the vaginal flora and the succinate produced during the infection alters leukocyte function and compromises host defences, thereby increasing susceptibility to *C. trachomatis* infection [198, 203]. Although several studies have implicated the role of BV in tubal diseases by encouraging ascension of *C. trachomatis* into upper genital tract [203], Gaudoin *et al.* [204] found that they were independent of each other and found no association

between active BV or past chlamydial infection in the development of PID. However, due to the low power of the study, co-infection with BV might still result in the increased risk of tubal infertility in women. Clearly, there is increased chlamydial infection risk associated with co-infection or bacterial vaginosis, and also evidence for an association with PID, supporting those co-infections could therefore result in an increased likelihood of development of infertility.

2.4.3 Sexual behaviour and age of sexual contact

Age is a risk factor in likelihood of contracting chlamydial infection [205, 206]. Cross national studies from 1999-2008 in Denmark, Australia and Sweden showed that increasing *C. trachomatis*-related PID rates were associated with increasing age (30-34 years), with the exception of New Zealand, where the rates were higher in women of ages 15-19 [207]. This could be because New Zealand has the highest Chlamydia testing rates compared to any of the countries included in the study, and this has led to early detection of *Chlamydia*-related PID. Consistent with previous findings, the highest incidence of *C. trachomatis* infections were reported in women between the ages 16-24 [124]. These studies support the finding that the pathology associated with *C. trachomatis* is more predominant in younger women. Consistent with these studies, being under 20, having cervical symptoms were all risk factors for endocervical chlamydial infection in women, and the organism burden was also higher in younger women when measured by viable culture counts [208]. Potentially the organism burden may influence the ability to ascend to the upper reproductive tract and ability to maintain a longer or persistent infection, therefore even though there is no direct evidence, there seems to be the potential for younger age of acquisition of chlamydial infection to increase the risk of pathological outcomes.

Sexual behaviour (new partners or higher number of partners) is often significantly associated with increased risk of *C. trachomatis* infection [209, 210]. As shown in the study by Skjeldestad *et al.*, 2009 [211], there was a 37% probability of a woman acquiring *C. trachomatis* infection if she has had 3 more partners within 42 months. A younger age at first coitus, higher number of sexual partners, and self-reported history of medically diagnosed sexually transmitted infection were all significantly associated with tubal infertility compared to fertile controls ($p<0.01$) [212]. However, as these are also known to be significant factors for increased

likelihood of acquisition of chlamydial infection it is not certain if they directly influence the likelihood of pathology outcomes from the infection.

2.4.4 Confounding variables and covariates associated with chlamydial infertility in women

Several environmental and lifestyle factors may contribute to the development of female infertility, that may present as potential confounders to *C. trachomatis*-induced infertility.

Age is considered an important predictor of female infertility. Women of advanced age (above 36 years) have 41% chances of conceiving, which is much lower than women who are below 30 years, as the likelihood of conceiving could be as high as 71% [213]. In Spain, a cross-sectional population based study of women between the ages of 30 and 49 (n=443) revealed that 17.5% of women could not achieve pregnancy within 1 year, and the prevalence of primary and secondary infertility was 6.12% and 11.2% respectively [214]. A cross-sectional based survey of 623 women seeking infertility evaluation in USA reported that 30% of women between the ages of 37-40 years have undergone infertility evaluation and treatments as compared to 8% women between the ages of 25-28 years and 17% of women between the ages of 29-32 years [215]. Several studies have demonstrated that infertility is more predominant at advanced age due to the decreased levels of ATP in oocytes that result in aneuploidy and chromosomal misalignment that cause implantation failures and miscarriages [216, 217]. Thus, age has an adverse effect on infertility and therefore considered an important covariate with regards to chlamydial infertility in women.

Body mass index (BMI) has a negative impact on the reproductive outcomes, as overweight women (BMI≥ 25kg/m^2) take longer to conceive and have lower spontaneous conception [218]. A retrospective study of 52 women seeking infertility treatments at Stanford University Medical Centre reported that a meaningful weight loss (10% of their maximum weight) yielded significantly higher conception (88% vs 54%) and live birth (71% vs 37%) rates than those who did not lose weight [218]. Although not significant, the spontaneous conception rate was also higher amongst women who had lost ≥10% of their body weight (35% vs 17%). A similar study conducted by Clark *et al.* [219] reported that 67 anovulatory women who lost 10.2 kg/m^2 after 6 months, resumed ovulation (>66%) of which 77.6% and 67% had

conceived and attained live births. Based on the hospital records of 6305 subfertile couples obtained from 24 hospitals in Netherlands, a lower probability of spontaneous pregnancy was observed in women with BMI over 29 kg/m^2 (hazard ratio: 0.96; 95% CI: 0.91-0.99), and with a unit increase in kg/m^2, the probability to conceive reduced by 4% [220]. Obese women are at a higher risk of infertility due to lower levels of Leptin (product of *ob* gene) that not only regulates adipose mass levels but also embryo implantation [221]. Thus, BMI is an important risk factor for infertility.

Amongst lifestyle factors, smoking and alcohol have been implicated in reducing fertility in women. Casreta *et al.* [222] reported that among 296 infertile women, the antra follicle count (AFC), a significant predictor of ovarian response was significantly higher in non-smokers (n=194) than current smokers (n=102). The study also reported higher levels of follicle stimulating hormone (FSH) in smokers as compared to non-smokers. Thus, reduction of AFC and elevation of FSH as a result of smoking could lead to follicle atresia and decline in ovarian reserve and oocyte quality [222]. Heavy smokers had a higher proportion of immature diploid oocytes as compared to non-smokers (21.2% (n=102) vs 5.1% (n=14)), which suggest that smoking impedes meiotic division in oocytes and reduces the function and viability of the oocytes [223]. Due to its effect on oocytes, smoking also has a negative effect on the fertility outcome of women undergoing IVF. Van Voohris *et al.* [224] reported that the adverse effect of smoking on ovarian function was dose-dependent and prolonged, as women who quit smoking before an IVF procedure (33.3%, n=111) were as likely to have the same ongoing pregnancy rate as non-smokers (35.3%, n=351), and they were significantly higher than current smokers (16.2%, n=37). Interestingly, several studies have a established a strong correlation between BMI and smoking status [225, 226], which in turn may have a detrimental effect on the fertility in women. A cross-sectional study encompassing 4305 women from Germany, Denmark, Sweden, France and Italy reported that women with higher BMI (<30 kg/m^2) showed strong correlation with infertility only in smokers and not in non-smokers (odds ratio (95% CI): 11.54 (3.8, 36.15) [227]. This suggests that the risk of infertility may be higher in obese women smokers as compared to obese non-smokers. The leptin levels in current smokers (19.7ng/mL) were reported to be significantly lower than in non-smokers (24.5ng/mL) in a cross-sectional study of

Mexican Americans and non-Hispanic white population in San Antonio (n=91)[225]. Since smoking reduces the leptin levels, it positively correlates with obesity and therefore has a negative impact on fertility.

Alcohol consumption has also been reported to impact female fertility negatively. A prospective observational study of 124 women between the ages of 23-41, revealed that the conception rate was highest in women who did not consume alcohol (24.5%, n=28) and the lowest levels were reported in heavy drinkers (>91g/week; 8.3%, n=13)[228]. Additionally, alcohol consumption negatively impacts the success rate of IVF treatments. The number of oocytes aspirated was reported to be 13% more in women who consumed less than 12g of alcohol per week (1 year before the procedure) as compared to those who consumed more than 12g of alcohol per week (n=148), and the miscarriage rates were 2.2times higher in the latter a week before the procedure (1 week before the IVF treatment; n=14) [229]. Wdowiack *et al.* [230] reported that amongst 54 women between the ages 25-39 years who underwent IVF, the embryo with the best reproductive potential (based on embryo implantation capability, same size of blastomeres and symmetry in positioning) were the lowest amongst those who consumed alcohol (4.35% (n=23) vs 41.94% (n=31), particularly those who consumed more than 25g of alcohol daily (4.55% (n=19) vs 70% (n=10)) as compared to those who did not consume alcohol. As expected, the embryos with abnormalities and lowest reproductive function were reported to be highest in women who consumed more than 25g of ethanol daily (22.73% (n=19) vs 0% (n=10)) and no abnormalities were reported in those who did not consume alcohol. Additionally, women undergoing IVF (n=250) who reduced their alcohol consumption (20.4%) or abstained (24.8%) before the treatment, were twice as likely to be pregnant as compared to those who continued their drinking habits (OR (95% CI): 2.27 (1.01-5.15); p=0.049)[231]. This suggests that by adopting healthy lifestyle choices, the risk of infertility in women could be diminished.

2.4.5 Hormonal status at time of infection

Oral contraceptive use was found to be a risk factor for *C. trachomatis* infection (p=0.006), and the organism burden was found to be higher in oral contraceptive users (p<0.001) [208]. Prospective recruitment of young female participants (>17) attending genitourinary medicine clinics in the UK identified that

those who had a *Chlamydia* infection were also more likely to have elevated progesterone concentrations (p=0.05) [232]. In contrast, oral contraceptive usage has been linked to protection against subsequent infertility or conception problems by two different studies [146, 233]. However, it would be extremely difficult to link hormone status at time of infection with subsequent pathological outcomes such as infertility, even in a longitudinal study, given the high numbers of oral contraceptive users, and the independent importance of these factors in the risk of acquiring an infection.

Following infection, *C. trachomatis* infects trophoblast cells which play an important role in implantation and placentation, and reduces chorionic gonadotropin expression and depletes oestrogen and progesterone synthesis which impairs trophoblast functions of implantation and placentation, resulting in infertility [234]. Higher prevalence of serum IgG to *C. trachomatis* in patients whose ovarian response was poor to gondatropin suggests that *C. trachomatis* impairs ovarian function [235]. Progesterone is reported to peak during the third week of menses and inhibit cell-mediated immunity in several *in vitro* models. Moreover, it has been shown to enhance chlamydial growth in uterine epithelial of several species [236].

2.5 HUMAN GENOTYPIC FACTORS ASSOCIATED WITH *C. TRACHOMATIS* INFERTILITY IN WOMEN

It is interesting to note that among women with asymptomatic infections, 8%-10% of women infected by *C. trachomatis* develop sequelae, while 20% resolve the infection [203, 237]. *C. trachomatis* infection and ascension into the upper genital tract is greatly influenced by the host genetic factors. The development of *C. trachomatis* related infertility in only some women may be a consequence of a genotypic pre-disposition (reviewed comprehensively [238]).

Toll-like receptors (TLRs) recognize and bind to antigens of pathogen, and signal to upregulate an immune response. There are 10 Toll like receptors (TLR) identified in the human body, and TLR 1-4 are expressed in the female genital tract [239]. TLR2 and TLR4 are also expressed on macrophages, dendritic cells and epithelial cells and they bind chlamydial lipopolysaccharide [240]. In a prospective genotypic study of female participants attending a sexual health clinic and a fertility clinic in Amsterdam, Karimi *et al.* (2009) [241] demonstrated a possible protective role of a TLR2 haplotype. The haplotype consisting of two distinct single nucleotide

polymorphisms (SNPS) was found to be present in a significantly higher proportion of women with a history of *Chlamydia* who did not have tubal pathology and in sexual health clinic attendees was present in a higher proportion of women who were asymptomatic [241]. In the PEACH PID study TLR1 and TLR4 polymorphisms were found to significantly associate with chlamydial PID [242], whilst SNPS from TLR2 (different SNPs from those included in the Karimi study), TLR6, Myd88, and TIRAP did not significantly associate with chlamydial PID [242].

Den Hartog *et al.* [243] identified that the risk of tubal pathology was higher in *C. trachomatis* IgG positive women with multiple TLR polymorphisms. The study also showed a trend towards association of various individual polymorphisms in TLR9 and TLR4 with increased risk of tubal pathology in *C. trachomatis* IgG positive women [243]. However, the small sample size in the study makes it difficult to draw a correlation between the polymorphisms and level of risk for female infertility. NLRP3, a component of the inflammasome that activates a pro-inflammatory response has been identified to have polymorphisms reported to result in altered IL-1β secretion (a pro-inflammatory cytokine) [244]. Several NLRP3 polymorphisms were tested in women prospectively recruited at a sexual health clinic and fertility clinics in Amsterdam, women heterozygous or homozygous with the one of the NLRP3 polymorphisms were at significant risk of developing abdominal pain during *C. trachomatis* infection [244].

Mannose-binding lectin (MBL) is a pro-inflammatory protein that activates complement and is locally synthesised in the vagina [245]. MBL and MBL promoter polymorphisms have been previously demonstrated to alter the levels of MBL produced [245]. It was identified that women with tubal factor infertility who were chlamydial seropositive more frequently had the low MBL producing genotypes compared to healthy controls [245]. However, this was not the case in an ectopic pregnancy group who were less likely to have the MBL-deficient genotypes [245]. This later finding sheds some doubt on the role of MBL genotypes, given that it seems unlikely that the pathology associate with ectopic pregnancy is distinct from tubal infertility.

Human leukocyte antigen (HLA) class II molecules (DR, DQ and DP) present peptides to CD4 T cells and induce adaptive immunity. DQ polymorphisms were more frequent in women with tubal infertility who were also *C. trachomatis*

seropositive [246].Whilst another polymorphism was negatively associated with *C. trachomatis* positive tubal infertility [246]. Wang *et al.*, [247] showed that in adolescent females with recurrent chlamydial infections, certain HLA polymorphisms were significantly associated with recurrent infections. A recent study attempted to link different immune genotypes to immunological outcome by testing a functional role for HLA DQ alleles combined with an IL-10 allele for both frequency in a tubal factor infertility (*Chlamydia* positive) cohort compared to controls and also for lymphocyte proliferative response to cHSP60 [172]. The HLA alleles and IL-10 allele were more frequently identified in the chlamydial tubal factor cohort compared to the control, however the lymphocyte proliferation in response to cHSP60 was not significantly different in relation to the alleles [172]. However, in a different study, distinct IL-10 and IFN-γ polymorphisms were found to significantly impact on lymphocyte proliferation in response to chlamydial antigens (cHSP60) [248].

A range of cytokine polymorphisms have been shown to significantly associate with women with *C. trachomatis* tubal infertility, including: IL-10 [249], TNF- α [249] and IL-12 [250]. Polymorphism in IL-1β and receptor genes were not associated with *C. trachomatis* related tubal pathology [251]. Adolescent females with recurrent chlamydial infections had a lower frequency of three IL-10 promoter polymorphisms [247]. Eng *et al.* [252] demonstrated that a CD14 allele (TLR-4 co-receptor) was associated with increased TNF-α production when whole blood was stimulated with *C. trachomatis* and *C. pneumoniae* [253].

Whilst none of these studies were able to account for all chlamydial tubal infertility cases, it is a convincing body of evidence that human genotype is a contributing factor in pathology development. It is interesting that to date the vast majority of studies have focussed on immune factors, yet as an obligate intracellular pathogen *Chlamydia* has key host nutritional requirements, inclusion vacuole requirements, and therefore the ability to ascend and cause pathology may in fact be independently determined by a as yet unknown non-immune host genotype.

2.6 IMMUNE RESPONSES ASSOCIATED WITH *CHLAMYDIA TRACHOMATIS* IN WOMEN

Insight into the immunological factors that may be involved in *C. trachomatis* infertility in women has been obtained through various human studies, including investigation of; local secretions (cervical and vaginal lavages), human tissues, peripheral blood mononuclear cells (PBMC), as well as through *in vitro* cell culture models. A commonly used framework to categorize the immune response is the Th1:Th2 paradigm (reviewed [254]). This paradigm suggests that the immune response can polarize towards a cytotoxic pathological response (Th1) or a humoral antibody mediated response (Th2). The profiles of these responses include Th1 subset of T helper lymphocytes (Th) and cytokine production such as: IL-2, IL-6, tumor necrosis factor (TNF-α), and interferon-gamma (IFN-γ) [255, 256]; and Th2 responses involve IL-6, IL-4, IL-10, IL-5 and IL-13 [255]. In addition to Th1:Th2 regulatory immune profiles typified by IL-17 or IL-23 have also recently been described (reviewed [257]). The immune responses in different models reviewed below and summarised in Table 2.1 enable a better understanding of the chlamydial pathology resulting in female infertility.

2.6.1 Immune responses from reproductive sites and tissues

Genital secretions have been used to reveal the immunological responses that occur at the site of infection, although it is not possible to link these with subsequent pathology. IFN-γ levels were found to be five times higher in the endocervical secretions of women with *C. trachomatis* infection detected by culture (n=47) compared to uninfected women (n=52) [258]. Analysis of immune factors using multiplex immunoassay in cervical-vaginal lavages of women attending a STD clinic with acute *C. trachomatis* infection (n=5) compared to controls with no infections (n=13), revealed significantly higher levels of IL-1β, lactoferrin, TNF-α, IL-8, VEGF, G-CSF, IL-10, IL-3, IL-7, IL-12 and IL-6 [259]. However, in a separate study in India, IFN-γ levels were only significantly higher in women with recurrent *C. trachomatis* infections compared to controls but not higher than those with primary infection [260]. IL-17 and IL-22 were 5 times and 3 times higher in the cervical secretion of *C. trachomatis* positive women (n= 27) compared to negative controls (n=17) [261]. IgG and IgA antibodies to *C. trachomatis* EBs were higher in the cervical washes of *C. trachomatis* positive fertile women during primary

infection compared to recurrent infection [260]. However, cervical IgG to specific chlamydial antigens (cHSP60 and cHSP10) was higher in recurrent infection than compared to primary infection, which is what would be expected [260]. Stimulation of cervical monocytes with chlamydial EBs showed an increased expression of Toll-like receptors (TLRs) TLR2 and 4, and both receptors were also expressed at higher levels in cervical cells from women with infection compared to uninfected women [263]. The levels of IL-1β, IL-4, IL-5, IL-6 and IL-10 were reported to be significantly higher from enriched cervical T cells stimulated with chlamydial inclusion membrane (Inc) proteins from *C. trachomatis* related infertile women (n=18) as compared to *C. trachomatis* positive fertile women (n=14) [264]. Whilst not always a *Chlamydia* related pathology, a study by Balasubramaniam *et al.* [265] which found that level of cytokines IL-8 and IL-6 were significantly upregulated in the fallopian tube immediately in the location of implantation of ectopic pregnancy, but not in the fallopian tubes of women undergoing benign hysterectomy. A very recent study identified that differences in IFN-γ levels correlated with differences in the chlamydial cellular morphologies at the cervix when comparing two patients [169]. These local responses all support that immune mediated pathology and key chlamydial antigens are likely involved in the development of infertility.

The effects of chlamydial infection in human tissue *ex vivo* studies provided a controlled insight into the cellular responses that likely occur *in vivo* during the immediate primary infection. Though *ex vivo* fallopian tube studies, Hvid *et al.* [266] showed that addition of IL-1RA receptor antagonists, blocked IL-1β and IL-8 production preventing the pathology from chlamydial infection [266]. This confirms that IL-1β along with IL-8 are likely major factors for tubal pathology [266], supporting the cellular paradigm of pathology development and not supporting the hypersensitivity model. Fallopian tube explants from ectopic pregnancy cases that were *C. trachomatis* seropositive were used to demonstrate that *C. trachomatis* induced higher expression of a prokineticin receptor via TLR2 binding [267]. The prokineticin pathway impacts on smooth muscle contraction and intrauterine implantation as well as angiogenesis, which may imply a mechanism for *C. trachomatis* damage leading to ectopic pregnancy [267]. T lymphocytes from endometrial and salpingeal tissues cultured *ex vivo* in the presence of *Chlamydia* or cHSP60 showed induction of lymphocyte proliferation and IFN-γ secretion from PID

or ectopic pregnancy cases [268]. In the same participants, higher levels of IFN-γ mRNA expression and lower levels of IL-5 in fallopian tube and peritoneal cavity specimens of PID patients who had T cells proliferating in response to the organism *ex vivo* further implicated Th1 cytokine production in *C. trachomatis* related pathology [268]. Primary endocervical cell cultures infected with *C. trachomatis* also demonstrated a pro-inflammatory cytokine response with abundant production of IL-8, IL-1α and TNFα, all of which were maximally detected during live infection with active bacterial protein synthesis [269]. Combined these data predominantly support the cellular paradigm and ascending infection models of pathology and link to a Th1 profile of immunity.

2.6.2 Immune responses in peripheral blood mononuclear cells (PBMC) in women

Peripheral blood mononuclear cells (PBMC) are often used to provide insight into immune cell responses to *Chlamydia* from different participant cohorts. PBMC proliferation induced by purified *C. trachomatis* EBs showed a prominent secretion of IFN-γ in women with genital infection [270]. PBMC from women at high risk of acquiring an infection (commercial sex workers) were stimulated with cHSP60 and the production of IFN-γ strongly correlated with protection against incident *C. trachomatis* infection [271]. In a separate study PBMC from healthy donors were incubated with *C. trachomatis* serovar K, leading to the finding that pro-inflammatory gene expression (measured by microarray and validated by qRT-PCR) was sustained for up to seven days, especially for IFN-γ, and IL-2receptor [272]. However in a separate study, cHSP60 PBMC production of IFN-γ was lower in women with PID or repeat infections compared to women with current infections [273, 274]. Human dendritic cells prepared from human PBMC were infected with *C. trachomatis* serovar E or L2 that induced production of IL-1β, IL-12p70, IL-6, IL-8, IL-18 and TNF-α [275]. A separate study also detected production of IL-12 and TNF-α by human PBMC sourced dendritic cells infected with *C. trachomatis* L2 [276]. Hook *et al.* [277], showed that stimulation of PBMC with chlamydial EBs elicited IFN-γ production by natural killer cells. Cohen *et al.* [271] showed IFN-γ production from PBMC stimulated with cHSP60 correlated with participants who were protected from acquisition of infection in a longitudinal study of 143 female participants [271]. This study also showed the PBMC production of IL-13 in

response to chlamydial EBs also correlated with a reduced risk of infection [271]. IL-6 production was reported from endometrial and endocervical primary *ex vivo* cultures from live infections with *C. trachomatis* [278].

2.6.3 Immune pathway elucidated by *in vitro* culture models

Epithelial cell culture are most commonly used for *in vitro* growth and characterization for *C. trachomatis* infection [44]. *C. trachomatis* infection in HeLa, SiHa (human cervical carcinoma epithelial cell) and HEp2 (human epithelial cell) were used to demonstrate that IL-1α regulated IL-8 production from these cell lines during infection [279]. Similarly, mRNA measurement of cytokine gene expression after *C. trachomatis* infection of HeLa, SiHa cervix squamous carcinoma cells and HT-29 colon adenocarcinoma cells showed increased levels of IL-8, GROα, GM-CSF, IL-6 and IL-1α levels. These cytokines are activators of neutrophils, monocytes and T lymphocytes, and potent chemoattractants [269]. A HeLa/THP-1 co-culture model attempting to re-capitulate the *in vivo* cellular cross-talk also identified sustained IL-6 and IL-8 cytokine secretion during *C. trachomatis* infection with IL-1β transiently detected [280]. IL-6 and IL-8 were also detected in a separate study using infections of cervical and epithelial cell models [269]. A HeLa and THP-1 (mono-nuclear cell) co-cultures model identified that there are distinct profiles of innate immune responses to *C. trachomatis* serovar E compared to L2. The results also showed that the monocyte released innate cytokines (such as TNF-α) had different effectiveness in controlling the infection in infected epithelia model cells by the two serovars [281]. Using a similar co-culture model as Rasmussen *et al.* [269], Cunningham *et al*. [278] also demonstrated a sustained IL-6 production in response to *C. trachomatis* L2 infection. A RNAseq- based examination of host and chlamydial gene expression enabled investigators to identify that a fibrotic profile of gene expression being induced in infected HEP-2 cells [282]. The role of TNF-α in inhibiting chlamydial development was evaluated by addition of recombinant TNF-α to Hep2 cells prior to infection which resulted in smaller inclusion bodies [283]. Furthermore, addition of IFN-γ to the cell line showed that in combination with TNF-α, chlamydial replication was inhibited [283]. Lu *et al.*, [284] showed that chlamydial infection of several epithelial cell lines resulted in the secretion of mature IL-18. IL-18 is known to potentiate the Th1 immune response and enhance production of IFN-γ [284].

Table 2.1: Immune responses associated with *C. trachomatis*-related infertility in women

Type of model	Sample	Immune Response	References
Reproductive sites and Tissues	Cervical secretions from women with acute *C. trachomatis* infection (n=5)	Increased expression of IL-1β, lactoferrin, TNF-α, IL-8, VEGF, G-CSF, IL-10, IL-3, IL-7, IL-12 and IL-6	[259]
	Cervical washes stimulated with *C.trachomatis* HSP60 and HSP10	High IFN-γ production in women with recurrent *C. trachomatis* infections (n=197)	[260]
	Cervical secretions from *C. trachomatis* positive infertile women (n=17)	High IFN-γ, TNFa, IL-10, IL-12 levels in infertile patients	[285]
	Cervical secretions stimulated with IncB, Inc C and *C. trachomatis* EBs	Increased expression of IL-1β, IL-4, IL-5, IL-6 and IL-10 in women with C. *trachomatis*-related infertility.	[264]
	Tissue sections of fallopian tube stimulated with *C. trachomatis* serovar D	High levels of IL-1β and IL-8 associated with tubal scarring and pathology.	[266]
	Fallopian tube specimens from women with past *C. trachomatis* infection (n=14)	High expression of prokineticin (PROKR2) receptor via TLR2 binding. PROK signalling reduces embryo implantation and play an active role in tubal ectopic pregnancy.	[267]
	Salpingeal tissues stimulated with cHSP60	High IFN-γ levels in women with PID (n=14) and TFI (n=22) may be associated with tubal infertility.	[268]
	Primary endocervical epithelial cell stimulated with *C. trachomatis* L2	IL-8 and IL-1α induce epithelial cytokine production and proinflammatory cytokines	[269]
	PBMC stimulated with cHSP60 and a mixture of *C. trachomatis* serovars (E, F, K, L2) EB	Increased production of IFN-γ and IL-13 conferred a protective effect against *C. trachomatis* infection in commercial sex workers (n=18).	[271]
	PBMC stimulated with cHSP60	High IL-10 and low IFN-γ and IL-12 production in women with PID (n=9)	[273]

Peripheral Blood Mononuclear Cells (PBMC)	Dendritic cells stimulated with viable and heat-inactivated *C. trachomatis* serovar E or L2	Increased production of IL-1β, IL-12p70, IL-6, IL-8, IL-18 and TNF-α. These pro-inflammatory cytokines activate Th1 T cell responses (resolve infection).	[275]
	Dendritic cells stimulated with viable or UV inactivated *C. trachomatis* L2	High levels of pro-inflammatory cytokines, IL-12 and TNF-α.	[276]
	PBMC stimulated with viable *C. trachomatis* L2	High levels of IL-6 in women with chlamydial infertility (n=3)	[278]
In vitro cell culture	SiHa (human cervical carcinoma epithelial cell) and HEp2 cells stimulated with *C. trachomatis* L2	High levels of IL-1α and IL-8.	[279]
	HeLa and SiHa cells infected with *C. trachomatis* L2	High levels of chemoattractants and pro-inflammatory cytokines IL-8, GROα, GM-CSF, IL-6 and IL-1α levels.	[269]
	Co-culture of HeLa and THP-1 cells infected with CtTsp, CtHtrA, and UV-killed *C. trachomatis* L2 infection	High levels of IL-6. The cytokine may be associated with disease pathology.	[278]
	Infection of HeLa , A549 airway epithelial (CCL185) and HT-29 colonic epithelial (HTB38) cells with *C. trachomatis* serovars L2, E, F, and K	Increased production of IL-18 through caspase-1 mediated pathway, which facilitates immune evasion of *C. trachomatis*.	[284]
	HeLa and SiHa cells infected with *C. trachomatis* L2	Downregulation of major histocompatibility complex (MHC) molecules.	[277]

2.7 CHLAMYDIAL FACTORS THAT MAY INFLUENCE THE DEVELOPMENT OF PATHOLOGY

Although host/human factors and the host immune response influence the outcome of the *C. trachomatis* infection, the pathogenesis of the bacteria is also influenced by characteristics inherent to the bacteria. These factors include chlamydial serovars, chlamydial genotypes and virulence factors.

2.7.1 Association of chlamydial serovars with repeat infection and symptoms that may link to a propensity to cause pathology and infertility

Chlamydial serovars are assigned based on the outer membrane proteins, typically by sequencing so studies that refer to serovars are actually frequently referring to genovars [286]. Serovar E and serovar F are generally found to be predominant in most countries including: Australia, Netherlands, and Sweden [287-291]. Using *omp*1 PCR based RFLP genotyping Lan *et al.* [292] reported that in women less than 30 years old, serovars D (4/21 asymptomatic women) and I (4/21 asymptomatic women) were associated with asymptomatic infection, while serovar G was associated with symptomatic infections (4/30 symptomatic cases). Similarly, Gao *et al.* [289] also reported the predominance of serovar G in symptomatic infection, particularly lower abdominal pain (18/33 patients) as compared to asymptomatic patients ($P<0.001$) amongst female sex workers and STD patients in China. 47.5% (28/59) asymptomatic patients had serovar E [289]. Verweij *et al.*, (2014) reported that different serovars induced differential serological responses in 510 women with PCR confirmed *C. trachomatis* infection [293]. The study showed that serovar D (n=45) and E (n=217) from serogroup B elicited the highest IgG response (Median IgG titre of 200) followed by serovar H (n=20) [293]. Women with persisting infections were found to have twice as may serovar E infections (67%, 8/12) compared to women who naturally cleared infections (33% 3/9) during a longitudinal study of women with asymptomatic *C. trachomatis* infections, however this was not significantly different [294]. Consistent with previous findings, Workowski *et al*. [295] also showed that while serovars F, B and C showed no apparent association with infertility in women with PID, serovar F exhibited different symptoms compared to B and C in women with lower genital tract infection (n=99), and it was accompanied by fewer clinical manifestations of mucopurulent

endocervical. However, several study design flaws have been raised and therefore it may not be relevant to include these data to make conclusions about the effect of serovars on resolution of infection [296, 297]). Whilst not linked directly to infertility, the majority of evidence from these studies support that different serovars do have different associations with symptoms, re-infection, or infection duration, all of which may contribute to pathology development.

2.7.2 Chlamydial genotypes can mediate tissue tropism and may be a factor in pathology development

There are several published examples of *C. trachomatis* genotypic changes associating with tissue tropism, however, no reports yet aligning chlamydial genotype with pathology development and infertility. The most well know genotype that mediates tissue tropism is actually polymorphisms in the *trpAB* operon and the ability to synthesis tryptophan from indole between *C. trachomatis* ocular and genital strains [201, 298] which we will mention here given the significance of this finding to the field even though this review is focussed on infertility. There is also evidence of positively selected genotypes that align with tissue trophism (ocular, genital, mononuclear/invasive), for example in a review of 59 *C. trachomatis* genome sequences, it was reported that *tarp*, and the *pmp* genes have positively selected polymorphisms that significantly cluster with tissue niche [299]. Polymorphisms in three distinct open reading frames have been found to associate with rectal but not cervical serovar G isolates, and two further open reading frame polymorphisms were found to associate with rectal and cervical tropism of serovars E, F and J [300]. These studies do not identify pathology-associated polymorphisms, however they do support the theoretical potential for chlamydial genotypes to exist that either improved ascension, or upper reproductive tract survival or polymorphisms that alter the immune response. Any association a chlamydial genotype has with pathology development would only be possible by a longitudinal study of a large group of women throughout their reproductive years with continual sampling and analysis of infecting chlamydial strains.

2.7.3 Virulence factors associated with chlamydial pathology in women

Secretion of immunomodulatory antigens is one of the principle virulence mechanisms of *Chlamydia trachomatis*. These proteins enable organisms to evade

host immune system thus leading to the development of serious sequelae such as Tubal factor infertility and ectopic pregnancy.

Heat shock proteins (HSPs) are chaperones that aid in correct folding of intracellular proteins [126]. Chlamydial heat shock proteins (cHSP60) are strong B and T cell antigens that play an important role in the immunopathology of sequelae of chlamydial infections [172, 301]. Kinnunen *et al.* (2003) [270] showed that when PBMCs were stimulated with cHSP60, a higher level of IFN-γ, IL-2 and IL-10 was produced, as opposed to weaker IL-10 production by the *C. trachomatis* Elementary bodies. Since, protective immunity and chlamydial clearance is dependent on the mutual interplay between IL-12 and IFN-γ, any imbalance could result in the development of TFI. Additionally, IL-10 does not support protective immunity and control of infection, thus leading to pathology [270]. The development of sequelae is highly dependent on the balance Th1 and Th2 immunity. Based on the cytokine expression obtained from salpingeal tissues and PBMC of patients with endometritis, PID and multiple infections, it was reported that cHSP60 induced a dominant Th2 response, which leads to chronic inflammation [273, 302]. Th1 immunity is associated with clearance of infection, while Th2 induces pathology. Hence, based on his findings, Debattista *et al.* (2002) [273] proposed that during chronic inflammation, a premature switching from Th1 to Th2 occurs. In addition to cHSP60, cHSP10 has also been shown to elicit T and B cell responses that contribute to pathology [303, 304]. cHSP10 is genetically linked to cHSP60 and perform similar roles as cHSP60 in protein folding and denaturation. On comparing acute infection to TFI, Srivastava *et al.* (2008)[303] found that in conjunction with higher antibody titres, both cHSPs induced higher production of IL-10 and IFN-γ in the latter. TNF-α was not found to be significant between patients with acute infection and patients with *C. trachomatis*-related infertility. The higher antibody titres for cHSP10 in the previous study is concurrent to the study Betsou *et al.* [304] which showed that anti-cHSP10 IgG antibodies are associated with chronicity.

Chlamydial protease like activity factor (CPAF) is a major [305] virulence factor that is secreted into host cytosol and has been reported to manipulate host immune system [305-307]. It is an atypical serine protease conserved within the *chlamydiales* [305]. The protease plays an important role in the developmental cycle of *C. trachomatis* through expansion of inclusion [307]. CPAF degrades host

transcription factors USF-1 and RXF-5 that are required for MHC Class I and II gene activation [308]. In women with cervicitis, antibody response to CPAF was found to be higher than MOMP and cHSP60 [306]. Downregulation of chlamydial protease-like activity factor (CPAF) and upregulation of high mobility group box 1 (HMGB1) protein renders the bacteria apoptosis-resistant and pro-inflammatory, thus promoting chronic inflammation [309].

The inclusion membrane proteins (Inc) are the only known proteins that reside in the inclusion membrane that interact with host proteins. These proteins share a common bilobular hydrophobicity motif that spans the inclusion membrane, but the common amino acid sequences among themselves are limited [20, 63]. Inc proteins were first identified in *C. psittaci* [64] and subsequently identified in *C. trachomatis* [20, 65]. IncA, first identified in *C. psittaci*, regulates the fusion of inclusions and interaction between the inclusion and intracellular compartment [67-70]. The SNARE-like fusion protein IncA facilitates fusion between endoplasmic reticulum and inclusion membrane, and the ER is essential in antigen processing and presentation [310]. IncB and IncC were reported to be higher in the cervical cells of infertile women (*C. trachomatis* infection) than women with acute infection. They also induce higher levels of IL-12, IFN-γ, TNF-α, IL-6, IL-4 and GMCSF in the PBMC of *C. trachomatis* infected fertile women [311]. The expression of these cytokines indicates that InC and IncB induce strong Th1 immune response in the genital tract.

2.8 DIAGNOSIS OF *CHLAMYDIA TRACHOMATIS*

Historically, cell culture was the diagnostic technique for *C. trachomatis* infections. This was followed by the DFA (direct fluorescent antibody) techniques, and today the most widely used techniques are the NAATs (nucleic acid amplification tests). However, the asymptomatic nature of the disease means many people do not get tested. Table 2.1 illustrates the sensitivity, specificity and the characteristics of the various diagnostic techniques used to detect *C. trachomatis* [312].

Table 2.2:A brief summary of the various diagnostic techniques for *C. trachomatis* infections

The table lists the specificity and sensitivity, advantages and disadvantages associated with cell culture, antigen testing, enzyme immunoassay, nucleic acid amplification testing and point of care tests. (Table directly adapted from Mylonas *et al.* [286].

Test procedure	Sensitivity	Specificity	Advantages	Disadvantages
DNA amplification	80-90%	>98%	Sensitive test Non-invasive specimen collection	Cost Appropriate material storage and processing
Cell culture	60-80%	>99%	Appropriate for medical-legal issues Specific strain	Low sensitivity Labour intensive
Direct fluorescence of antibodies (DFA)	65-75%	97-99%	Simple test Unit test	Low sensitivity Labour intensive Subjective reading
Enzyme immunoassay (EIA)	60-75%	97-99%	Automation	Low sensitivity Verification test recommended
Rapid point of care test (POC)	25-67%	>97%	Low cost Unit test	Very low sensitivity Verification test recommended

2.8.1 Cell culture

Tang *et al.* [313] were the first to successfully isolate and culture a trachoma biovar strains of *C. trachomatis* from embryonated chicken eggs. *C. trachomatis* strains infect only certain cell lines such as HeLa-229, McCoy, Buffalo green monkey kidney and BHK-21 [314]. Studies by Hodinka *et al.* [315] suggested that cell line infection by *C. trachomatis is* enhanced by centrifugation which promotes phagocytic entry and prevents phagosome-lysosome fusion. The chlamydial inclusion bodies are visualised with the help of immunofluorescence and iodine or Giesma stain [316]. Despite isolating the bacteria from specialized culture medium, and with the assistance of skilled staff, cell culture techniques achieves a sensitivity of around 70-80% for detecting *C. trachomatis* [314]. Additionally, the technique is difficult, time consuming and costly [314].

2.8.2 Non-Culture Techniques (Non-nucleic amplification tests)

The non-culture techniques involve direct visualization of the chlamydial organism through cytologic examination, immunohistochemical detection of antigen and hybridization to DNA probe (EIA and DFA) [317]. Unlike culture techniques, non-culture techniques are standardized test that are rapid and less labour intensive [317].

Direct immunofluorescence assay involves the use of fluorescein-tagged monoclonal antibody specific for *C. trachomatis* to detect the infection [318]. Another technique that employs the use of fluorescein tagged molecule is the commercially available MicroTrak test which detects individual elementary bodies of *C. trachomatis* using fluorescein-labelled monoclonal antibodies [319]. Using this technique, the sensitivity and specificity of detecting *C. trachomatis* antigen in endocervical swabs of infertile women (n=200) was reported as 66.7% and 100% respectively, which was much higher than EIA (sensitivity=48.1%; specificity =100%)[320]. DFA is a rapid technique that has a higher specificity. However, its broad range of sensitivity suggests that it is highly subjective and is not suitable for large number of samples [321]. A study conducted by Rahm *et al.* [321] showed that the sensitivity of DFA was only about 85% among asymptomatic patients, thus it is not the preferred diagnostic technique for asymptomatic patients. Thejls *et al.* [322]

reported that *C. trachomatis* elementary bodies were detected in 8.2% (n=256)of women with infertility using DFA and it was comparable to EIA. EIA. is prone to false positives due to cross-reactions with lipopolysaccharide (LPS) of other microorganisms [323].

2.8.3 Nucleic acid amplification tests

Nucleic acid amplification tests are highly accurate techniques that are widely used in *C. trachomatis* diagnosis [318]. They detect small amounts of chlamydial nucleic acids, transcripts from genital specimens, and non-invasive samples such urine specimens and self -obtained vaginal swabs [318]. NAATs have a sensitivity of 95% and specificity of 100%, hence they are considered the "gold standard" for chlamydia diagnosis. The amplification techniques include PCR, real-time PCR, strand displacement amplification, transcription-mediated amplification and nucleic acid sequence based amplification [323]. NAAT is particularly useful in asymptomatic women because it is capable of detecting *C. trachomatis* at low IFU (inclusion forming units) counts which is a direct correlation to the endocervical discharge and other anatomical changes associated with chlamydial infection [324]. Although, NAAT is capable of detecting *C. trachomatis* in FCU (First- catch urine) from women, vaginal swabs specimens are preferred over FCU due to the presence of inhibitors in urine such as phosphate that inhibit LCR reactions [325] . The evaluation of the sensitivity and specificity of NAAT techniques such as strand displacement amplification (SDA), PCR and Abott ligase reaction (LCR) to *C. trachomatis* showed that the three techniques performed similarly with regards to sensitivity and specificity [326]. However, when compared to the performance of EIA, the detection rate by PCR was 62% higher than that of EIA [327]. The commercial NAATs for *C. trachomatis* that are widely used due to their high specificities and sensitivities are COBAS Amplicor test (Roche systems; 16S rRNA PCR-based NAAT), Abbott m2000 (Abbott systems; LCR-based NAAT), APTIMA *C. trachomatis* (Gen-Probe; Transcription-mediated NAAT) and BD ProbeTec (Becton Dickson, USA; SDA-based NAAT). The sensitivities for detecting *C. trachomatis* in vaginal swabs of 575 women recruited from sexual health clinics for APTIMA (Gen-Probe), Abbott m2000 and ProbeTec were 96.2%, 98% and 90.6% respectively, while in urine samples the sensitivities were reduced to 88%, 76.9% and 75.5% respectively [328]. The specificities all the assays ranged between 98.4%

to 100%. Among self-collected vaginal swabs (n=675), COBAS ® 4800 (Roche systems) and Abbott m2000 (Abbott systems) had sensitivities of 97.1% and 100% respectively, and specificities of 99.7% and 99.5% respectively, in detecting *C. trachomatis* infections [329]. Despite their high specificities and sensitivities, in Sweden, the NAATs yielded a high number of false negatives owing to the lack of detection of a new variant of *C. trachomatis* (nvCT) that was discovered in the country in 2006 [330]. This 377-bp deletion in the cryptic plasmid of the bacteria is often the target sequence for most NAATs and thus the bacteria was not detected. Moller *et al.* [331] compared the performance of Roche COBAS Amplicor CT (RCA), Abbott m2000 (Abbott systems), Gen-probe Aptima combo 2 assay (targets 23S rRNA) and Aptima *C. trachomatis* assay (targets 16S rRNA) in detecting nvCT in first catch urine samples and vaginal swabs from female participants in Sweden (n=1231). The study reported that both Gen-probe NAATs (specificity=100%; sensitivity =99.6%-99.9%) had higher sensitivity and specificity in detecting nvCT than m2000 (sensitivity= 68.7%; specificity= 99.9%) and RCA (sensitivity = 63.8%; specificity= 99.9%) in detecting all mutant strains characterized by *in-house* PCR [331]. Klint *et al.* [330] reported that in 2007 counties in Sweden that used Abbot/Roche systems had high proportion of nvCT, while counties using BD Probe Tec had lower proportion of nvCT, but after rountine testing the proportion declined in 2009 (65% to 48% with Abbot/Roche, 24% to 18% in Uppsala with BD). A 5-year follow-up revealed that in 2011 the proportion of the new variant had reduced to one-fifth of the absolute and relative numbers, while the proportion of wild type remained the same in Skane (Sweden) [332]. Thus, this highlights the importance of appropriate and routine screening of chlamydial strains in order to control their rate of transmission.

The disadvantages of NAAT is that they require sophisticated laboratory conditions [333, 334]. Several studies have used PCR testing to determine the prevalence of *C. trachomatis* infections in a population [320, 335-337], the organism load [338-341] and rapid detection of multiplex detection of *C. trachomatis* along with other microorganisms such *N. gonorrohoeae [342-346]*. In Iran, PCR testing of 150 women with infertility and 200 fertile healthy women revealed that *C. trachomatis* was detected in 32% of women with infertility as compared to 8% of healthy fertile women [347]. In another study, the prevalence of *C. trachomatis*

among infertile women (n=106) recruited from fertility clinic in Brazil was determined as 58% using PCR amplifying *C. trachomatis* DNA plasmid [348]. In-house real time PCR targeting cryptic plasmid and omp1 gene in endocervical swabs collected from 200 infertile participants in an fertility clinic in India, showed that *C. trachomatis* was present in 13.5% infertile women.

2.8.4 Point of Care tests to detect *C. trachomatis* infections

Point of care tests (POC) are assays that are affordable, sensitive, specific, user friendly, rapid and robust, equipment free and deliverable (ASSURED) [349]. There are several commercial POC tests such as *Chlamydia* Rapid Test, QucikVue test and Clearview *Chlamydia* for chlamydial diagnosis. Although, they are cost-effective and rapid, their sensitivity and specificity falls short in comparison to the current "gold standard"; nucleic acid amplification tests. Alarmingly poor performance has been reported by Dommelen *et al.* [333] which revealed that the sensitivities of *Chlamydia* Ag test, QuickVue *Chlamydia* test and Handilab-C were extremely poor and were prone to false positive results. Similar results were obtained in the study conducted by van der Helm *et al.* [334] who reported poor sensitivities (41.2%) for *Chlamydia* Rapid test (CRT). However, continued efforts have been undertaken to improve the performance of POC tests and some of the techniques developed recently have demonstrated improved diagnostic performance. Cepheid GenXpert CT/NG test is the first genetic POC that amplifies one chromosomal target for *C. trachomatis* detection and it can detect 15 serovars of *C. trachomatis* including nvCT [349]. A field evaluation trial of POCs GenXpert and Diaquick CT for *C. trachomatis* detection in remote Aboriginal communities of Australia reported that GenXpert CT had a sensitivity of 100% and a specificity of 99.5% (n=198), while Diquick CT had a sensitivity of 27.3% and a specificity of 66.7%[350]. Similar sensitivities and specificities for GenXpert CT were also reported by Gaydos *et al.* [344], wherein testing of endocervical, vaginal and urine samples from 1722 females yielded sensitivities between 97.4% -98.7%, while maintaining a specificity of 99%. Several novel POC techniques with superior sensitivities and specificities have been developed, but they are yet to be used widely. Some of the techniques include Velox technology [351] that utilizes novel electrochemical detection principle and reported a sensitivity of 98.1% and specificity of 98% in a pre-clinical validation of 306 clinical samples. aQcare *Chlamydia* TRF kit which is based on the newly based

lateral flow immunoassay that uses europium (Eu) (III) chelated nanoparticles as a labeling substances also yielded a high sensitivity of 93% and a specificity of 98% (n=441) [352]. Thus, quick, user friendly and cost-effective POC could improve screening strategies and effectively control *C. trachomatis* infection and prevalence.

2.8.5 Serological diagnosis

Serological assays for *C. trachomatis* involve detection of antichlamydial antibodies [2]. It is a useful technique in diagnosing ectopic pregnancies, pelvic inflammatory diseases and recurrent miscarriages [2]. Serological diagnosis overcomes the problem of invasive sampling using techniques in upper genital tract infection in women, such as needle aspiration of the affected fallopian tubes in women with salpingitis [353]. The type of antibody and the specific antigen/epitope in the serum may also aid in differentiating between past and present infection. For instance, IgA and IgM antibodies have a half-life of five to six days, hence they are markers of active chlamydial infection [355], while IgG are markers of previous or long-term infections [356].

Van den Broek *et al.* [357] analyzed the association of serum IgG, serum IgA, mucosal IgG and mucosal IgA with infertility and active *C. trachomatis* infection. The anti-chlamydial antibodies obtained from the sera and vaginal swabs of infertile women attending fertility clinics (n=85) and women attending STI clinic (n=117) demonstrated strongest correlations between infertile participants and serum IgG and mucosal IgA ($P<0.001$), but not mucosal IgG. Similar results were reported for women recruited from STI clinics with active infection (PCR positive), who showed strong associations to serum IgG, IgA and mucosal IgA, but not mucosal IgG. This shows that serum IgG had stronger associations with current or past infections, and local IgA had a better diagnostic predictive value than mucosal IgG in detecting tubal pathology. In women with a history of PID, immunofluorescence test revealed that although the proportion of serum IgG was higher than serum IgA and cervical IgA, there was a correlation between both serum antibodies [358]. Although IgA antibodies have also been implicated in chronic infection as reliable markers of persistent infection [356], the findings by Muvunyi *et al.* [359] contradict previous studies by showing that the role of IgA is negligible in chronic *C. trachomatis* infection in women. IgG antibodies were also so found to be higher in women with tubal occlusion or with prior ectopic pregnancy [360]. Additionally, In sub-fertile

women, the IgG seroprevalence ranged between 17.3% to 18.8% and IgA seroprevalence rates was 7.4% [359].

Regardless of test performance, numerous studies have identified a correlation of *Chlamydia* serology positivity (*Chlamydia* antibody testing: CAT) with diagnosed tubal factor infertility (typically using a case: control design of tubal infertility compared to other causes of infertility). These studies include the following: a significantly higher percentage of women with tubal pathology were CAT positive (50.2%, n=141) compared to infertile for other reasons (29.6%, n= 326, $p<0.001$) using microimmunoflourescence (MIF) [361]. In a separate study high CAT titres using MIF correlated with presence of tubal damage (n=434) compared to all women with infertility n=1006 ($p<0.001$), as did pelvic adhesions [362]. The study further demonstrated that participants with tubal damage (median 1:1024) had significantly higher titers than participants with endometriosis (median <1:64) ($P<0.001$). The degree of tubal adhesion also positively correlated with *C. trachomatis* seropositivity [363]. Thus, the level of antibody titers is instrumental in diagnosing the type of infertility. Another study reported that CAT (MIF) was 74% sensitive and 93% specific for detection of tubal disease (total 210 patients) $p<0.001$ [364]. In contrast, a study by Muvunyi *et al.* [359] reported that the sensitivity and specificity of three different chlamydial ELISAs in detecting *C. trachomatis* infection in subfertile women from Rwanda was low compared to the fertile post-natal controls (n=312). The study concluded that *Chlamydia* is not a major cause of tubal pathology in this population because despite having high proportion of tubal pathology among infertile participants (185 of 303 infertile participants), the prevalence of *C. trachomatis* infections were reported to be low in this cohort.

The Microimmunofluorescence (MIF) test developed by Wang and Grayston [366] showed a high sensitivity and it is still considered the serologic "gold standard". However, it is labour intensive and highly subjective [2]. Enzyme linked immunosorbent assay (ELISA), overcomes the problems posed by MIF and further enhances the specificity to *C. trachomatis* with the help of recombinant chlamydia antigens [367]. ELISA-based serological tests are capable of detecting *C. trachomatis* antibodies in a large number of samples, and are less expensive, rapid and less labour intensive than MIF [2]. Serological assays are instrumental in identifying novel antigens that are specific to *C. trachomatis.*

The commercial ELISAs are generally based on recombinant peptide antigens derived from immunodominant antigens such as cHSP60 and MOMP (major outer membrane protein). The MEDAC pELISA (MOMP peptides) in the Netherlands was able to predict tubal disease in infertile women (n=40 CAT positive, compared to n=167 CAT negative) with a sensitivity of 0.37 (95% CI: 0.26-0.49) and specificity of 0.88 (95% CI 0.82-0.92) [368]. IgA reactive with MOMP has been detected in the urogential tract, which suggest that MOMP might confer protective immunity and maybe associated with host clinical outcomes [369]. Among the serological diagnostic techniques, the majority of the studies have demonstrated that ELISA had a higher sensitivity and specificity compared to MIF [370, 371]. Furthermore, it was also reported that in asymptomatic infertile women, a decline of IgG antibody titers were observed after a 4-7 year interval by MIF, however, it was not reflected in the ELISA [372]. Thus, it highlights the credibility of MIF in detecting tubal infertility after a long-term persistent infection. Additionally, comparison of MIF positive subfertile participants with MIF negative subfertile participants revealed a significant difference in the prevalence of *C. pneumoniae* antibodies ($P<0.0005$), which indicates a high rate of cross-reactivity between *C. trachomatis* and *C.pneumoniae* [373]. However, a meta-analysis of individual patient data found that the diagnostic accuracy of MIF in diagnosing tubal pathology was significantly better than accuracy of ELISA and IF alone [374]. In another study, the performance of six enzyme immunoassays were compared on women with ectopic pregnancy (n=90), infertility (n=187), and PID (n=33), and MEDAC MOMP ELISA was reported to have highest specificity (97.2%) and showed a high correlation to whole inclusion immunofluorescence (correlation coefficient =0.7)[375]. This was supported by another study that showed that the MEDAC MOMP pELISA was the best of three ELISAs (compared with Savyon and Labsystems) for detection of tubal pathology in infertile women at a fertility treatment clinic with 55% sensitivity and 87% specificity [376]. The same study compared two MIF tests (n=315, with 51 tubal pathology cases) reported that with a cut-off titre of 1/64 the Biomerieux test had 71% sensitivity and 74% specificity, compared to 47% sensitivity and 95% specificity for the Labsystems test [376]. Bax *et al.* [377] compared the performance of a *Chlamydia* enzyme immunoassay (EIA) (ANILabsystems), MEDAC MOMP pELISA and MIF in women with subfertility (n=76)and reported that despite its low sensitivity (36.4%), MEDAC MOMP had the highest specificity (85.7%). Therefore,

making MEDAC MOMP a good alternative for MIF in detection of chlamydial infertility. However, a meta-analysis conducted in 2011 compared MIF and several ELISA and found that MIF had the highest accuracy (area under the curve) for detection of tubal pathology P<0.001, and P=0.01 (for bilateral occlusion) [374]. Although, MIF is still considered a 'gold standard', ELISAs also show great promise as a diagnostic tool for *C. trachomatis*- related infertility due to its ease of use and its ability to exploit a wide range of antigens to achieve high sensitivity and specificity.

One of the important markers for *C. trachomatis* sequelae is the *Chlamydia* heat shock protein 60 (cHSP60) [378]. Antibody responses to cHSP60 was detected in women with *Chlamydia*-associated tubal infertility (n= 72) with a sensitivity and specificity of 81.3%, and 97.5% respectively. However, these women were first tested by laparoscopy for tubal damage and for a positive result in the MIF serological assay to assign case group before testing the ELISA so this study has likely over-reported the test performance [379]. In a subsequent study of a further 77 participants this group further reported that cHSP60 antibodies were 92% specific and 44% sensitive for tubal factor infertility [380]. cHSP60 ELISA positivity was also found to be significantly more frequent in women with tubal factor (n=88) compared to controls of infertile women with other know factors for infertility or healthy blood donors (n=163) (43.2% vs 13.5%) in a study conducted in Helsinki [381]. Although, antibody response to cHSP60 is expected to be abundant in the serum of *C. trachomatis* infected women, it was found that contrary to expectations, anti-cHSP60 antibodies were not detected in women infected less than four months and a very small proportion (18%) were found in patients with PID, multiple infection and post infection [382]. In addition to cHSP60, antibody response to cHSP10 exhibited a strong correlation to women with TFI (n=33) when compared to women with acute *C. trachomatis* infections (n=139) (P<0.001) [383]. Serological analysis of infertile women before their initial IVF cycle revealed that antibodies to cHSP10 was more predominant in women with hydrosalpinx (46.8%) than in women with tubal occlusion (P=0.0009; 15.5%)[179]. Antibodies to cHSP10 had a sensitivity of 41.7% and a specificity of 86.1% in detecting women with ectopic pregnancy (n=72), however it was reported to have high cross-reactivity with *C. pneumoniae* (78.4% identity) and 14.5% of the participants showed cross-reactivity [304]. Persson *et al.* [384] reported that in addition to correlating with women with

TFI (n=163; rs = 0.46, P <0.001), cHSP60 antibodies showed no correlation with *C. pneumoniae* (rs = 0.17, P =0.03).Women with secondary tubal infertility were reported to have a higher number of anti-cHSP60 (54.8%, 30%) or cHSP10 (40.5%, 26.7%) than women with primary infertility, of which higher specificity was obtained for cHSP60 [378]. Hence, a commercial ELISA screening test has been developed using cHSP60 (Medac) to facilitate easy diagnosis of *C. trachomatis.* Thus, the Medac-cHSP60 assay is not the preferred diagnostic technique as it has a sensitivity of only 69% and specificity of 93% [126].

Identification of novel antigens associated with chlamydial infertility

In addition to cHSP60, several new antigens have been discovered that could improve the serological approach for diagnosing tubal pathology associated with *C. trachomatis* in women. Antigens used in combination have proved to be more specific and sensitive than those used alone in serological diagnosis, at least in research studies, although large scale commercial or clinical trials have not yet been reported with many of those discussed here. Rodgers *et al.* [385], conducted a genome wide identification of antigens, using a proteome array containing individual *chlamydial* GST (glutathione-S transferase) fusion proteins, and the serological response in women with TFI (n=31) and infertile women with no TFI (n=25) was estimated. The study reported that a high antibody titer to *C. trachomatis* (P<0.001) as compared to *C. pneumoniae* (P=0.269) was obtained in participants with TFI, the study further identified 10 antigens specific only to the participants with TFI (CT110 (HSP60), CT322, CT376, CT381, CT414, CT443, CT681, CT795, CT798, and CT813). While these antigens maintained 100% specificity, combination of all 10 antigens yielded a sensitivity of 67.7% (n=21). However, the sensitivity was also maintained with the combination of four antigens (HSP60, CT376, CT381 and CT798) and two antigens (CT443 and CT381). Interestingly, Combination of CT443 and CT381 yielded higher specificity and sensitivity than other techniques used to diagnose tubal infertility, such as hysterosalpinogram (sensitivity=65%; specificity =83%) and Hsp60 (sensitivity=35.5%; specificity =100%)[386]. Using the same glutathione-S-transferase fusion protein microplate ELISA as in the previous study, Budrys *et al.* [387] reported that combination of HSP60, CT376, CT557 and CT443 identified women with TFI from fertile controls with a sensitivity of 63% and specificity of 100%. Furthermore, the study also reported that a combination of

CT875 and CT147 identified women with acute *C. trachomatis* infection from women with *C. trachomatis* indued TFI with a sensitivity of 63% and specificity of 100%. Other immunodominant antigens include OmcB [388] [identified through fusion protein microplate ELISA]; CT795 [369, 389], CT089[369], CT694 [369] [identified using microplate array with 156 chlamydial fusion proteins] and Pgp3 [390] [identified through western blotting and indirect ELISA]. CT147 and CT314 found in female patients are associated with multiple *C. trachomatis* infections [382]. CT396 antigen is conserved between *C. trachomatis* and *C. pneumoniae*, while CT157 is the least conserved antigen between the chlamydial species and it is greatly decreased in *C. pneumoniae* [382]. Using two-dimensional proteomic analysis and a novel line immunoassay, Forsbach-Birk *et al.* [353] identified proteins such as PmpD (polymorphic membrane protein D) (sensitivity =71%; specificity=82%) and OMP2 (outer membrane protein) (sensitivity=94%; specificity =100%) that had high sensitivity and specificity in PCR-confirmed women with upper genital tract infection (n=20). Another immunodominant antigen is Pgp3, which is a plasmid encoded, membrane associated protein that is found within the chlamydial inclusion [391]. Among the antigens (OMP2, LPS, cHSP60) analysed using serum ELISA, pgp3 exhibited the highest specificity (89%) for *C. trachomatis* infection [391]. The IgG pgp3 antibody responses were evident in patients seropositive for *C. trachomatis*, but not in patients whose serum contained *C. pneumoniae* antibodies. This suggests that pgp3 antigen is specific for *C. trachomatis* infection in the human genital tract [391]. Furthermore, the immunization of mice with pgp3 inhibited the spread of *C. trachomatis* infection from LGT to UGT in C3H/ HeN mice [392]. Although the pgp3 antibody response is reported to be specific to *C. trachomatis* in humans, it is also evident in majority of chlamydial infected animals, hence the degree of cross-reactivity between different species need to be evaluated [370].

Proteomic techniques have been widely used to identify novel immunodominant antigens. Protein microarray technology is valuable in serological diagnosis because it is a rapid technique that involves a semi-quantitative antibody detection and requires low volume of sera [2]. In addition to protein microarray techniques, a general proteomic approach to identifying novel antigens include protein solubilisation and digestion, isolation of immunoreactive proteins using western blot or two-dimensional blots, identification of proteins by Mass

spectrometry analysis followed by protein expression, purification and testing [353, 393, 394]. Using this technique, several antigens such as MOMP, OMP2, cHSP60, PmpD, TARP, TSAP, RpsA and HP 17 have been identified and evaluated for diagnostic purposes [353]. Therefore, by identifying novel antigens and developing specific-peptide based serological assays, the sensitivity and specificity of the chlamydial diagnosis could be enhanced.

The value of serological diagnosis in C. trachomatis-related tubal factor infertility

Infertility is estimated to affect 8%-12% of the population worldwide, and in high prevalence regions, the rates of infertility could reach upto 30% [395]. Tubal pathology are among the most common causes of infertility and it affects 30%-35% of couples [396]. A mathematical model, including a number of assumptions, and adjusting for serology test sensitivity and specificity estimated that 45% of tubal factor infertility episodes are likely to be attributable to *C. trachomatis* (CI: 28-62%) [103]. Currently, the techniques that are used to diagnose *C. trachomatis*-related tubal infertility are laparoscopy and hysterosalpinography (HSG). Laparoscopy is the gold standard for diagnosing tubal infertility, however it is invasive, expensive, can lead to major surgical complications [1] and are prone to misdiagnosis [397]. Hysterosalpinography is commonly used in the detecting tubal pathology [364] and based on the meta-analysis of seven primary articles (n=4521), its sensitivity and specificity in detecting tubal occlusion was 53% and 87% respectively [398]. However, several studies [396, 399] have demonstrated that HSG has lower diagnostic accuracy than laparoscopy. Additionally, they cannot detect adenxal and peritubular adhesion [399] and may not be effective in detecting bilateral tubal occlusion.

As desribed previously, CAT has also emerged as one of the techniques that can effectively diagnose tubal infertility in women. Thomas *et al.* [400] demonstrated that CAT could potentially replace laparoscopy in routine testing as it is non-invasive, cost-effective and was able to identify women with tubal damage from those without ($P<0.001$). It was also reported that HSG followed by CAT did not provide additional diagnostic value in detecting tubal pathology [401]. Additionally, The receiver operating (ROC) curves obtained from meta-analyses of 188 studies revealed that the discriminative capacity of CAT using MIF, immunofluorescence

and ELISA were comparable to that of HSG [402]. Dabekausen *et al.* [403] showed that in 112 infertile women, CAT had a higher positive likelihood ratio as compared to HSG in detecting TFI (9.1 vs 2.6). Johnson *et al.* [404] used ELISA for CAT and validated with MIF and reported that in women with tubal disease (n=36), a negative predictive value of 81% was obtained for CAT which was significantly higher than when clinical features were analyzed (61%).

The diagnostic performance of CAT could be augmented by complementing the assay with medical history. Coppus *et al.* [368] demonstrated that using CAT or medical history alone in 207 subfertile women, yielded low sensitivities of 37% and 38% respectively, but on combination, the sensitivity increased to 54%. In another study, the seropositivity of *C. trachomatis* (ELISA) in women with a history of infertility (n=50) and women with more than three abortions (bad obstetric history) (n=80) was 35% and 62.5% respectively [405]. While healthy fertile women (n=50) and women with a history of fewer than two abortions showed low seropositivity of 4% and 7.3% respectively [405]. In addtion to gynecological history, risk factors such as smoking can be identified through medical history. Smoking was identified as a risk factor in *C. trachomatis*-induced tubal infertility ($P<0.001$) [362].

Although, serological diagnosis could identify tubal pathology in women and recommend IVF treatment, they may not be able to predict the outcome of the treatment. A prospective observational study at a reproductive health centre in New York used MIF serological prediction of previous *C. trachomatis* infection to compare with infertility diagnosis and treatment outcomes. A total of 1279 participants were screened and 70 (5.5%) were positive for *C. trachomatis* [185]. Of those selected for investigation of tubal disease seropositive women were significantly more likely to have hysterosalpingography detected tubal blockage (37.5% compared to 10.1%; $P= 0.001$) or laparoscopically diagnosed tubal damage (85.7% compared to 48.9%, $P= 0.002$) [185]. In women with secondary infertility (n=40), 63% of women seropositive for *C. trachomatis* had tubal occlusion [406]. Seropositive women also had 57% less pregnancy rates than seronegative women prior to IVF but once IVF treatment commenced they were equally likely to conceive by IVF as women with other causes of infertility [185]. Concurrent with previous findings, Barberyrac *et al.* [186] showed that the presence of past or current *C. trachomatis* did not affect the IVF outcome of subfertile couples as the number of

oocytes retrieved, fertilization rate, the mean embryos transferred and transfer rate was not different between *C. trachomatis*- positive and *C. trachomatis*- negative women. Combined, these studies provide substantial evidence that a past history of chlamydial infection is significantly associated with tubal pathology and infertility in women, regardless of serological assay performance. However, they do not allow us to determine how frequently this tubal infertility is an outcome of primary infection, and therefore the attributable risk of *Chlamydia* for infertility is not known.

In a 2002 survey, Collins [407] estimated that the cost of a single IVF cycle varied between countries, the most expensive being USA with each cycle costing upto $9547 and the average cost from 25 countries were estimated at $3518. As the costs associated with Assisted reproductive technologies (ART) are high and a pose a significant economic burder on the health care systems, it is important to ensure that the diagnostic techniques used to evaluate tubal infertility are highly sensitive and do not yield false positives. Thus, there is a need to develop a serological diagnostic technique that could detect *C. trachomatis*-related infertility that would result in recommendation for progress directly to IVF, without additional diagnosis by laparoscopy/dye studies. Additionally a serological assay would be non-invasive, cost-effective and that could be widely applied in early infertility investigation in IVF clinics.

Chapter 3: Materials and Methods

3.1 GENERAL STOCKS AND SOLUTIONS

3.1.1 10X Phosphate buffered saline

10X Phosphate buffered saline was prepared by a combination of 0.007M of disodium hydrogen phosphate ($Na_2HPO_4.H_2O$), 0.027M sodium hydrogen phosphate ($NaH_2PO_4.H_2O$), 1.37M of sodium chloride (NaCl) and 800mL of distilled water. The pH was adjusted to 6.6 and the final volume was topped up to 1L.

3.1.2 Phosphate Buffered saline (PBS)

Phosphate Buffer Saline (PBS) was prepared from 10X stock by adding 100ml of 10X PBS to 900mL of distilled water. The pH was adjusted to 7.3. For cell culture, PBS was autoclaved at 121°C for 15 minutes prior to use.

3.1.3 1X PBS-Tween (PBST)

PBST was prepared by adding 0.1% Tween 20 to 1XPBS. The solution was stored at room temperature.

3.1.4 2X PBS-Tween (PBST)

2XPST was prepared by diluting 10XPBS stock in the ratio 1:5 with distilled water (200mL of 10 X PBS in 800 mL of distilled water) and 0.1% Tween 20.

3.1.5 Sucrose Phosphate Glutamate (SPG)

SPG was prepared by adding 10mM sodium phosphate (Na_3PO_4), 250mM sucrose and 5mM L-glutamine. The pH of the solution was maintained at 7.2 by addition of 1M sodium hydroxide (NaOH). The solution was filter sterilized and stored at 4°C.

3.1.6 3,3,5,5- Tetramethyl benzadine (TMB)

TMB stocks were prepared by dissolving 0.1g of TMB in 10mL Dimethyl sulfoxide (DMSO). The solution was stored at room temperature and it is light sensitive.

3.1.7 Red blood cell (RBC) lysis buffer

RBC lysis buffer was prepared by adding 155mM Ammonium chloride (NH_4Cl), 10mM Sodium bicarbonate ($NaHCO_3$) and 0.1mM of

Ethylenediaminetetraacetic acid (EDTA). The solution was prepared by adding 1L of milli-Q water and the pH was adjusted to 7.4. The solution was filter sterilized (0.22 μm filter) and stored at 4°C.

3.2 HUMAN CELL LINE CULTURE AND MAINTENANCE

3.2.1 Fetal Calf serum (FCS)

Heat inactivated Fetal calf serum (Lonza, Mt. Waverly, Victoria) was added to cell culture media. FCS was heat inactivated by placing the bottle in water bath at 56 °C for 42 minutes.

3.2.2 Growth Media

Dulbecco's Modified Eagle Medium (DMEM) was used as a culture medium for McCoy and HeLa cells. The media was supplemented with 10% Heat inactivated Fetal calf serum (HI-FCS) (Lonza, Mt Waverly Australia), 25μg mL^{-1} Gentamycin (Invitrogen) and 100μg mL^{-1}streptomycin sulphate (Sigma-Aldrich).

3.2.3 Cell lines

Cell lines used in this project were obtained from American Type Culture Collection (ATCC). Hep-2 (ATCC CCL-23), human adherent epithelial cells and McCoy B (ATCC CRL-1696), mouse adherent fibroblast cells, THP-1 (ATCC TIB-202), human peripheral blood monocyte cells were used in this study. For McCoy and HeLa cells, DMEM supplemented with FCS, gentamycin and streptomycin were used. THP-1 cells were grown in RPMI 1640 medium with 2mM L-glutamine and 10% FCS. THP-1 cells were made adherent using PMA (phorbol myristate acetate) treatment. All three cell lines were cultured in 175cm^2 tissue culture flasks (BD Bioscience, North Ryde, Australia) with their appropriate growth media at 37°C and 5% CO_2 . They were periodically tested for *Mycoplasma spp*. contamination using PCR in section 3.2.4.

3.2.4 Testing of *Mycoplasma spp*. in human cell lines

All cell lines were tested for *Mycoplasma spp*. contamination by PCR periodically. The cells were cultured in 175cm^2 tissue culture flasks (BD Bioscience, North Ryde, Australia) for 24 hours until they were confluent. On achieving the confluency, the cells were dislodged by adding 1mL Trypsin (Invitrogen) and incubating for 5 minutes at 37°C. The DNA was extracted using the QIAamp DNA

mini kit (Qiagen, Victoria, Australia). The primers for PCR was a universal primer targeting the conserved 16s rRNA gene of *Mycoplasma spp*. Cycling conditions was initiated at 94°C for 3 minutes, followed by denaturation at 94°C for 60 seconds, annealing at 55°C for 30 seconds and extension at 72 °C for 60 seconds repeated for a total of 32 cycles. The amplicon size was 425bp and it was verified on an agarose gel electrophoresis.

3.3 *CHLAMYDIA* STRAIN CULTURE

C. trachomatis D (ATCC VR-885) and F strain (ATCC VR-346) were cultured in McCoy cells. Confluent cells were infected with the strains and incubated at 37°C for 44 hours. The infection was enhanced by centrifugation at 500 × g at 25°C for 60 minutes. The media was changed after four hours of infection and 1mg mL^{-1} cycloheximide was added along with the fresh media to control further propagation of McCoy B cells. Following infection, the strains were harvested and semi-purified or ultra-purified as described in section 3.3.2.

3.3.1 Semi-purification of *Chlamydia* strains

The infection was stopped by removing the media and adding cold SPG. The cells were dislodged using cell scraper and homogenized using glass beads. The samples were centrifuged at 300 × g for 5minutes at 4°C to pellet the cell debris. The supernatant was removed and centrifuged at 18,000 × g for 30 minutes at 4°C to pellet the semi-purified chlamydial strains. The pellet was resuspended in SPG and stored at -80°C. The semi-purified *Chlamydia* strains may contain host cells, which was removed through ultra-purification.

3.3.2 Ultra-purification of *Chlamydia* strains

The semi-purified *Chlamydia* strains were further purified through Ultra-purification. 500U/mL of Heparin, 10mM/mL $MgCl_2$, 40U/mL DNase I were added to the semi-purified *Chlamydia* strains and incubated at 37°C for 30 minutes. A density gradient was prepared by adding 29% v/v urografin Ultravist® (Bayer, USA) in ultracentrifuge tubes Optima® L-90K ultracentrifuge (Beckman Coulter, Gladsville, Australia). The strains were centrifuged at 335 × g for 5 minutes and the supernatant was layered on top of the 29% v/v Ultravist solution and the tube was topped up with 1xPBS. The solution was centrifuged at 70,000 × g (20,000 rpm

using SW-40) for 35 minutes at 4°C. The *Chlamydia* that has been pelleted was re-suspended in SPG and it is devoid of host debris.

3.3.3 Quantification of *C. trachomatis*

To quantify the amount of *C. trachomatis* yielded during an infection, 96-well plates were seeded with Hep-2 cells and incubated for 24 hours at 37°C. After obtaining 90% confluency, the cells were infected with several dilutions (ten-fold dilutions) of *C. trachomatis* stored in SPG. The plates were centrifuged at 500 × g for 30 minutes at 25°C to enhance the infection and the media was replenished after 4 hours of infection with 1mg mL^{-1} cycloheximide. After 24 hours of incubation, the media was removed and cells were washed with sterile PBS. The cells were fixed with 100% methanol for 10 minutes and subsequently washed with PBS. The plates were then permeablised with 0.5% Triton-X and blocked overnight with 1% Bovine serum albumin (BSA). The cells were incubated at room temperature for 1 hour with 1:500 of primary antibody anti-HtrA rabbit sera and 125ng/mL of 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen). The cells were washed with PBST (0.2% v/v Tween-20) and treated with 1:600 dilution of secondary antibody goat anti-rabbit IgG (H+L)- Alexa Flour 488 (Invitrogen), incubated at room temperature for 1 hour. The plates were washed thoroughly with PBS, and stored in PBS solution at 4°C in the dark.

The plates were viewed under a Nikon Eclipse TE2000-U fluorescent inverted microscope with Nikon Digital Eclipse DXM 1200C camera (Nikon Pty Ltd, Lidcombe, Australia). The chlamydial inclusion- forming units (IFU) were determined using a Metamorph Imaging series 7.6 software (Molecular Devices, Sunnyvale, USA). The IFU/well was determined by multiplying the average IFU/FOV (field of view) x Total FOV/well. The IFU/mL was determined by multiplying the IFU/well with the dilution factor of 1000, and dividing it by the volume of strain aliquoted in each well.

3.4 SEROLOGICAL DIAGNOSIS OF *C.TRACHOMATIS* AND *C.PNEUMONIAE* INFECTIONS USING COMMERCIAL KITS

Serological assays are a useful technique in diagnosing ectopic pregnancies, pelvic inflammatory diseases and recurrent miscarriages [2]. The presence of *C. trachomatis* specific antibodies in the participant sera was tested using commercial

serological assays such as MEDAC *C. trachomatis* IgG p-ELISA (Medac GmbH, Wedel, Germany), MEDAC cHSP60 IgG p-ELISA (Medac GmbH, Wedel, Germany), ANILabsystems *C. trachomatis* IgG ELISA and Microimmunofluorescence (MIF) IgG assay. The presence of IgG antibodies against *C. trachomatis* indicates previous exposure to *C. trachomatis*. Since MIF is considered a gold standard among chlamydial serological assays, the assay was included in cohort definition and participants positive for *C. trachomatis* MIF assay were considered positive for *C. trachomatis* infections. The diagnostic performance of the in-house peptide ELISA on the cohorts was compared to existing commercial chlamydial serological assays.

3.4.1 MEDAC serological assays

The MEDAC serological assays used in this study were *C. trachomatis* IgG p-ELISA and MEDAC cHSP60 IgG p-ELISA. The MEDAC *C. trachomatis* IgG p-ELISA is based on the peptides derived from immunodominant antigen MOMP (major outer membrane protein). MEDAC cHSP60 IgG p-ELISA is based on cHSP60, an immunodominant antigen associated with chlamydial infertility [270, 380].

According to the recommended guidelines by The Dutch Society for Obstetrics and Gynaecology, the absorbance values of MEDAC *C. trachomatis* IgG p-ELISA greater than 1.1 could be used to determine the potential risk of infertility and could be used as a first line of investigation for subfertile couples [409]. Additionally, guidelines by MEDAC manufacturer's highlight that positive outcomes in both MEDAC assays in participant sera indicate a high risk of *C. trachomatis* induced infertility (Referred to as MEDAC infertile in this study). Therefore, the sera from all cohorts were tested with *Chlamydia trachomatis*-IgG ELISA-plus MEDAC (specificity=87%; sensitivity=55%; determined against women with infertility (n=315)) [410] and cHSP60-IgG ELISA MEDAC (cHSP60 protein) (specificity = 93%; sensitivity=69% determined against women with tubal factor infertility (n=70)) [126].

The assay was validated by the mean absorbance values of negative (<0.1) and positive control (<0.8). The cut off was determined by adding the mean absorbance value of negative control to a previously established value of 0.34 and 0.35 for MEDAC IgG *C. trachomatis* pELISA (MEDAC MOMP) and cHSP60 IgG ELISA

(MEDAC cHSP60) respectively. The absorbance values of participant sera that were above the cut-off value was considered positive for *C. trachomatis* infection, while those whose absorbance values were below the cut-off value were considered negative for *C. trachomatis* infections. The absorbance values of those participants that were within 10% less than and more than the cut-off value were considered to be in the grey zone. The samples that were in the grey zone were considered as equivocal (neither positive nor negative) for the test and the testing was repeated on the sera.

C. pneumoniae in the serum was tested using *Chlamydia pneumoniae* IgG ELISA plus MEDAC. The assay uses a purified *C. pneumoniae* antigen. The assay was validated by the absorbance value of a negative (<0.1) control and the lot–specific absorbance value of positive control. The mean OD value of calibrator was also lot-specific and the absorbance was corrected by dividing the nominal absorbance value of the calibrator (lot-specific) by the measured absorbance value of the calibrator. The concentration was determined using the formula: concentration [AU/mL]= $b/(a/OD_{corrected}-1)$; a and b were arbitrary values specific to the lot that was used for the assay. The cut-off value was set at 25 AU/mL and the absorbance values of sera that were above the cut off value was considered positive for *C. pneumoniae* infection and those that were below the cut-off value were considered negative for the infection.

3.4.2 ANILabsystems *C. trachomatis* serology kits

The sera was also tested with another commercial serological assay, ANILabsystems *C. trachomatis* IgG ELISA, which is based on synthetic peptides from *C. trachomatis* -specific variable domain of MOMP (major outer membrane protein). The specificity and sensitivity of the assay was estimated to be 84% and 86% respectively, by screening the sera of 330 women with infertility and PCR diagnosed *C. trachomatis* infection [365]. The assay was considered valid if the mean absorbance value of negative control was < 0.2 and the mean absorbance of the positive control was between 0.7 and 2, and the calibrator values lay between 0.4 and 1.2. The participants were considered negative for *C. trachomatis* infection if the Signal (S)/ Cut-off (CO) was <1 and positive for the infection if the ratio was above 1.4.

3.4.3 *C.trachomatis* IgG Microimmunofluorescence assay (MIF)

Microimmunofluorescence assay was used to characterize the participants as serologically negative or positive for *C. trachomatis* IgG antibodies. The Focus Diagnostics *Chlamydia* MIF IgG assay (mdi Europa GmnH, Langenhagener, Germany) comprised of a slide with twelve wells. Each well constitutes elementary bodies of a single strain of *C. pneumoniae*, 2 strains of *C. psittaci* and 8 serotypes (D-K) of *C. trachomatis*. Chlamydial elementary bodies devoid of interfering LPS were diluted in 3% yolk sac served as a control for background fluorescence. The detectable control served as the positive control while non-detectable control was the negative control. The sera were loaded onto the slide and incubated at 37°C for 30 minutes. The plates were then rinsed with PBS and treated with IgG conjugate for 30 minutes at 37°C. The slides were rinsed again with PBS and mounting medium was added onto the slide and covered with 24x50mm coverslip. The slides were subsequently viewed under a fluorescence microscope at a final magnification of 400X.

3.5 IN-HOUSE PEPTIDE ELISA

The in-house peptide ELISA developed through this study was based on peptides selected through bioinformatics analysis from a series of 55 unique peptides that showed serological response to *C. trachomatis.* Stansfield *et al.* [411] developed and designed the peptides that was capable of distinguishing women with *C. trachomatis*-related infertility from those without. The methodology is outlined as follows.

3.5.1 Peptide identification and design for in-house ELISA

The peptides were derived from immunodominant proteins such as HtrA (High temperature requirement protein), cHSP60 (chlamydial heat shock protein 60) and CT443 (outer membrane chlamydial protein). The B cell epitopes, antigenicity and hydrophilic domains were identified using software such as BepiPred algorithm software, antigenicity prediction software respectively [412]. The specificity of the linear B cell epitopes were tested using BLAST and the peptides were selected if their E values were <0.0004 for *C. trachomatis* and >0.1 for *C. pneumoniae*. The epitopes were used to design a series of peptides. The peptide epitopes were synthesized commercially using solid phase synthesis onto a Biotin-SGSG motif

(Mimotopes, Melbourne, Australia) and solubilized in 50% isopropanol overnight to make up a concentration of 1mg ml^{-1}.

The serological responses of the peptides to *C. trachomatis*-related infertility were tested using an ELISA format and screened against 39 participant sera belonging to five cohorts. The five cohorts included sequelae (tubal factor infertility, ectopic pregnancy, PID), acute, multiple, negative (infertile but *C. pneumoniae* seropositive) and negative (seronegative for *C. trachomatis* and *C. pneumoniae*, infertile but no tubal damage). The peptides that obtained a higher absorbance in the sequelae cohort as compared to negative and acute cohort (>0.015), and obtained an absorbance <0.015 between the two negative cohorts were chosen to design the in-house ELISA. Based on these criteria, peptides HSP60-E2 [SGSGVFSSPPFSNKPP-NH2](derived from chlamydial heat shock protein 60), 443-N2 [SGSGPVSFSGPTKGTIT-NH2] (derived from outer membrane protein Ct443) and 443-A3 [SGSG VDRKEVAPVHES –NH2](derived from outer membrane protein Ct443) were selected for in-house peptide ELISA. One of the higher performing peptide, peptide 11 (ADTRGILVVAVE) (derived from HtrA) was tested on 129 women with primary and secondary infertility, and a specificity of 95% and sensitivity of 46% were obtained on this cohort. In order to improve the sensitivity of the assay, peptide based ELISA was designed with peptide 11 in combination with peptides from other immunogenic peptide antigens such as Hsp60-E2, 443-N2 and 443-a3.

3.5.2 ELISA based on peptide 11 in combination with other immunogenic peptides

The biotinylated peptides were coated on high binding capacity streptavidin plates (Thermofisher Scientific, Victoria, Australia). Peptide 11, Hsp60-E2, 443-N2 and 443-A3 at concentration of 1mgmL^{-1} (0.25µg $well^{-1}$) was diluted in 1XPBST (1:100) and coated separately on each well. The peptides were incubated for 1 hour at room temperature. The plates were then blocked overnight with SuperBlock PBS (Thermofisher Scientific, Victoria, Australia) at 4°C. The sera was diluted in superblock blocking buffer PBS with 0.1% Tween20 at 1:200 dilutions, and incubated for 1 hour at 37°C. The plates were rinsed thoroughly with 2X PBST. The secondary antibody, goat anti-human IgG HRP was then coated at a dilution of 1/15,000 in superblock blocking buffer PBS with 0.1% Tween 20. The plate was

developed by addition of 100μg/mL of 3,3,5,5-Tetramethyl benzadine (TMB) in DMSO dissolved in phosphate citrate buffer with sodium perborate and incubated at 10 minutes at room temperature. The reaction was stopped with 1M H_2SO_4 and the plates were read on Bio-Rad xMark microplate spectrophotometer at 450 nm. The optimization of the assay for superior sensitivity and specificity included assessment of the influence of several parameters such as peptide concentrations and combinations, dilution of secondary antibodies and peptide solubility.

3.6 PERIPHERAL BLOOD MONONUCLEAR CELLS ISOLATION (PBMC)

The PBMC were processed from the whole blood from patients as soon they were obtained. The blood was centrifuged at 78 × g for 10 minutes to remove the plasma layer. The removal of plasma layer was followed by dilution of the blood with 2mL DMEM media. The blood was then layered onto a tube containing 2mL Ficoll-Plaque™ Premium reagent (GE Healthcare). The solution was centrifuged at 754 × g for 25 minutes at 4°C (deceleration set to zero). The centrifugation separated the solution into three layers, of which the middle layer that comprised of the lymphocytes was transferred to a tube containing the media. To the solution, 5mL of RBC lysis buffer was added to remove the red blood cells. The solution was centrifuged at 754 × g for 10 minutes at 4°C, and the pellet was resuspended in DMEM. The pellet was washed until it appears white and resuspended in 1mL of media. The cells were counted using a hemocytometer and the desired number of cells were added to the wells and stimulated with the antigen. The cells were incubated for 15 hours at 37°C.

3.7 STATISTICAL ANALYSIS

All statistics (relative risk, sensitivity, specificity, chi squared statistics and forest plots) were calculated in the R statistical environment (3.0.1) using the 'EpiR' (0.9-48) and 'metafor' package (1.9-1) for conducting meta-analyses in R (Viechtbauer 2010). Mixed-effects models were used to combine cohort data using a fixed-effect model with a restricted maximum likelihood estimate of the log odds ratio [413]. All calculations were performed in the R statistical environment (v 3.0.1) using the Linear and Nonlinear Mixed Effects Models package 'nlme' (v 3.1-111) [414] .

Chapter 4: Development and evaluation of a novel multi-antigen peptide ELISA for the diagnosis of *C. trachomatis*-related infertility in women

4.1 INTRODUCTION

Sexually transmitted *C. trachomatis* infections in women can have adverse effects on their reproductive health [195]. The infection can manifest as mucopurulent cervicitis and urethritis in the lower genital tract. However, it can progress to pelvic inflammatory disease (PID), ectopic pregnancy (EP) and tubal factor infertility (TFI) in the upper reproductive tract [102]. The risk of developing PID after a lower genital tract infection is reported to be 9.5% in untreated women (n=1275) [415], and in about 20% of cases, PID leads to tubal pathology [416]. The prevalence of tubal factor infertility in sub-fertile couples is reported to be between 10%-30% [417], and analysis of published literature indicate that 45% of TFI is caused by *C. trachomatis* infection [103]. Heinonen *et al.* [418] reported that *C. trachomatis* was the primary pathogenic agent in 44% of women with acute PID (n=72) (laparoscopy confirmed). The study further reported that *C. trachomatis* was more predominant in women with severe PID (n=35) as compared to other pathogenic agents such as *Neisseria gonorrhea*. *C. trachomatis* was also more predominant in women with endometritis (n=26) ($p<0.001$) than in women without endometritis or salpingitis (n=83) [419].

The techniques that are used to diagnose tubal fertility are typically hysterosalpinography and laparoscopy. Hysterosalpinography (HSG) detects tubal patency using oil or water based contrast medium in the uterine cavity and fallopian tubes [398]. In a study by Lavy *et al.* [420], among 23 women who were HSG positive for bilateral tubal occlusion, 30% were found to have patent tubes by laparoscopy. While in another study, omission of tubal patency verification by laparoscopy in 41% (n=126) of participants who were diagnosed for bilateral occlusion by HSG, would have resulted in 60% of the participants being misdiagnosed [421]. Thus, laparoscopy is recommended for diagnosis of tubal pathologies even after HSG treatment.

Although laparoscopy is considered a gold standard for diagnosing tubal pathologies, the invasive nature of the technique increases the risk of complications [422]. In addition, the high costs associated with laparoscopy (upto $976) limits its availability to women in developing nations and low resource settings [423].

Chlamydia antibody testing (CAT) has been widely employed during the infertility investigation to identify women who may have tubal damage [362, 402]. Jones *et al.* [424] established the earliest association of *C. trachomatis* in TFI women, by reporting that antichlamydial antibodies were detected in 35% of infertile women (n=172) and 75% of women with tubal infertility. These findings were supported by Sellors *et al.* [425] who reported significantly higher levels of anti-chlamydial IgG and IgM ($p<0.0001$) antibodies in participants with TFI (n=52; 79.1%) as compared to participants who are sterile due to tubal ligation (n=114) and have had a hysterectomy as a result of PID (n=99). Mol *et al* [402] showed that although the ability of CAT to diagnose tubal pathology did not vary significantly between laparoscopically verified tubal pathology and verification of tubal pathology using a combination of HSG and laparoscopy ($p=0.8$). Thus, the diagnostic performance of CAT is comparable to both laparoscopy and HSG.

The two main CAT methods are MIF and ELISA. The IgG titers in MIF is instrumental in determining the risk of developing tubal damage, as low titers (1:32) indicated 5% incidence of developing tubal damage, while titers greater than 1:32 were associated with tubal pathology (n=57) [400]. In Brazil, MIF assay on a relatively small cohort of infertile women (n=33), revealed that 42% of the participants had a MIF IgG titer greater than 1:64 and the IgG titer was greater than 1:128 in women with tubal occlusion (42.4%) and ectopic pregnancy (40.9%) [360]. Gijsen *et al.* [372] compared the MIF test results with ELISA (ANILabsystems IgG ELISA) in women with TFI (n=39) and, and reported a decline in MIF IgG titers from an initial titer of >1:64 after 4-7 years in 18% of the participants, however these changes were not reflected in the ELISA and the signal remained the same. Thus, ELISAs are capable of detecting past *C. trachomatis* infection long after the infection. Although, MIF is considered the "gold standard", it is labor intensive, time consuming and shows high cross-reactivity between different chlamydial species

Meta analysis of 14 primary studies containing data of 3453 women by Broeze *et al.* [374] showed that the sensitivity and specificity for ELISA, MIF and IF assays for any tubal pathology ranged between 23%-91% and 41%-100% respectively, while for bilateral tubal pathology the sensitivities and specificities ranged between 31%-70% and 52%-86% respectively. Thus, this suggests that in order for serological

assays to be used for early infertility investigations, the sensitivity and specificity for diagnosing tubal pathology need to be improved.

Although most commercial serological assays are often based on antigens such as chlamydial elementary bodies, variable regions of outer membrane protein (MOMP) and chlamydial LPS, the sensitivity and specificity of the assays needs to be improved. Hence, several novel antigens that are immunodominant in women with TFI have been identified that may have diagnostic value. Chlamydial heat shock protein has been widely reported to be immunodominant in women with chlamydial infertility [126, 179, 380, 387]. Serological analysis of infertile women before their initial IVF cycle revealed that antibodies to cHSP10 was more predominant in women with hydrosalpinx (46.8%) than in women with tubal occlusion (p=0.0009; 15.5%)[179].

Shaw *et al.* [426] identified 21 proteins through 2-D electrophoresis and MS/MS, and with immunoblotting with the sera from participants with PID, Omp-2, HtrA, HSP70 was identified [427]. Forsbach-Birk *et al.* [428] used two- dimensional immunoblot analysis of chlamydial proteins and electrospray ionization MS/MS identified MOMP, OMP2 and CPAF to have a specificity of 100% and sensitivities of 87%, 90% and 94% respectively in diagnosing women with upper genital tract infections (n=33).

Development of a peptide ELISA involves the following steps; identification of immunodominant antigen through proteomic [371, 389, 390] or *in silico* methods [429]; evaluation of immunogenicity of the antigen through western blot [390, 429, 430] analysis against participant sera from well- defined cohorts; determine the performance of the assay by estimating it's sensitivity and specificity; comparison of diagnostic performance of the assay with existing commercial assays [389, 390, 430]; and validation of the assay by estimating its reproducibility and testing against a separate validation participant set [390, 429, 430]. The diagnostic performance is determined by constructing a receiver operating characteristic (ROC) curve which establishes a cut-off value based on sensitivity or specificity, and the accuracy is determined by the area under the curve (AUC) that usually ranges from 0.5 (least accurate) to 1 (perfect test) [431]. Sensitivity is defined as the ability of test to correctly classify an individual as diseased (function of true positive and false

negative), while specificity is the ability of a test to correctly classify an individual as non-diseased (function of true negative and false positive) [432].

In this study, an *in silico* approach was applied to identify and design novel peptide antigens that are specific to women with *C. trachomatis*-related infertility, and develop a peptide ELISA that could potentially replace invasive techniques such as laparoscopy for the infertility investigation. This is a follow up to the study conducted by Stansfield *et al.* [411] wherein, using an *in silico* approach, peptide 11 was identified and its sensitivity and specificity in diagnosing *C. trachomatis* associated tubal factor infertility was estimated as 47% and 95% respectively. Through this study, the diagnostic performance of peptide 11 ELISA has been improved by combination of other *C. trachomatis*-specific immunodominant peptide antigens.

4.2 METHODS AND MATERIALS

4.2.1 Identification of immunodominant peptides specific to *C. trachomatis* – related infertility

The 'initial proof of concept' of peptide ELISA has been detailed in Materials and Methods 3.5. The peptides identified by Stansfield *et al.* [411] through *in silico* analysis were used in this study. The peptides were derived from proteins the targets HtrA [433], cHSP60 [387] and CT443 (Omcb) [385, 387] which have been shown to be immunodominant in women with tubal factor infertility. The peptides were identified from a series of 55 unique peptides that were selected through bioinformatic analyses. The B cell epitopes, antigenicity and hydrophilic domains were identified using software such as BepiPred algorithm software, antigenicity prediction software respectively [412].The peptides selected for diagnostic purposes were chosen based on the criteria as outlined in section 3.5.1. The peptides chosen were Peptide 11 (ADTRGILVVAVE), Hsp60-E2 (VFSSPPFSNKPP) and 443-N2 (VSFSGPTKGTIT). The peptides were screened for homology between other chlamydial species and the BLAST E values for *C. trachomatis* were peptide 11, HSP60-E2 and 443-N2 were 0.001, 0.0003 and 0.0005 respectively. The BLAST E values for *C. pneumoniae* were 0.11, 0.86 and 12 respectively. The peptides were considered specific for *C. trachomatis* if the E values < 0.004 for *C. trachomatis* and >0.1 for *C. pneumoniae.* Since all the peptides met these conditions, they were selected based on their specificity and antigenicity. The peptide epitopes were

synthesized commercially using solid phase synthesis onto a Biotin-SGSG motif (Mimotopes, Melbourne, Australia). These peptides were used in combination in single-well and in multi-well ELISA formats to optimize the assay for the best diagnostic performance in identifying participants with tubal factor infertility.

4.2.2 Development of *In-house* peptide ELISA

The peptide ELISA involves coating of high binding streptavidin plates with biotinylated peptides. These peptides were coated individually in separate wells (multi-well) and in combination in one well (single well). The specificity and sensitivity of both multi-well and single well formats were assessed. The secondary antibody used in this study was goat anti-human IgG HRP (1:15,000) (Invitrogen). The combination and the multi-well format that had the diagnostic performance was designated the QUT *Chlamydia* infertility test. The peptide ELISA was performed as described in 3.5.2.

4.2.3 Commercial serological assays used to detect acute *C. trachomatis* infections and chlamydial infertility

The commercial assays used in the study have been detailed in 3.4.4. The commercial assays include Focus Diagnostics Microimmunofluorescence (MIF) IgG assay (described in 3.4.3) (mdi Europa GmnH, Germany), which is considered to be gold standard among serological assays in detecting chlamydial infertility. Among ELISAs, MEDAC IgG ELISA (MEDAC MOMP) (Medac GmbH, Wedel, Germany) and cHSP60 IgG ELISA MEDAC (MEDAC cHSP60) (Medac GmbH, Wedel, Germany) was used. A positive result in both MEDAC MOMP and MEDAC CHSP60 would indicate that the participants are at a risk of developing TFI and was referred to as MEDAC Infertile assay as recommended by manufacturer. ANILabsystems *C. trachomatis* IgG ELISA was also used in the study to determine the presence of past *C. trachomatis* infection. The participants were also tested for *C. pneumoniae* with *Chlamydia pneumoniae* IgG ELISA plus MEDAC) (MEDAC GmbH, Wedel, Germany).

4.2.4 Characterization of participants based on gynecological and *C. trachomatis* infection history

The participant recruitment process and the Human research ethics committee approval numbers are detailed below. The participants recruited for this study were characterized into cohorts based on their gynecological history. Figure 4.1 shows

experimental design for the development and validation of QUT *Chlamydia* infertility test.

Determination of sample size of women recruited for development of multi-antigen peptide ELISA

The required sample size was estimated based on the method outlined by Buderer *et al.* [434]. Based on a conservative estimate of 7% prevalence chlamydial infertility in Australian IVF clinics, the sample size required to yield 98% specificity (with 1% precision) would be 120 participants. In order to obtain a 70% sensitivity (95% C.I of 58-82%) with 0.12 precision, a sample size of 809 participants would be needed. However, despite having several collaborations with hospitals and fertility clinics, only 354 participants were included in the study.

Recruitment of women with Tubal Factor Infertility and healthy controls

Human Research Ethics Committee approval was granted for these, including Sexual Health Clinics (Prince Charles Human Research Ethics Committee approval number EC2809), Ipswich and West Moreton Health Services District (Human Research Ethics Committee approval number (10-09)), Gold Coast Hospital District (Human Research Ethics Committee approval number (200893)), Cairns Sexual Health Clinic (HREC/09/QCH/4-554), and Ethical approval for the study was obtained via Queensland University of Technology Human Research Ethics (1300000003 and 080000268). Participants were also recruited from several Australian fertility clinics including IVF clinics based in Brisbane (UC Health Human Research Ethics Committee approval number 1314) and Melbourne (Human Research Ethics Committee approval number 12099).

The target cohort included women with tubal factory infertility and a history of *C. trachomatis* infections. Women attending IVF clinics who were trying for more than a year to conceive were invited to participate in the study. Participants were included in the study only after providing written informed and complete a questionnaire regarding their history, including factors such as age of the patient, number of sexual partners, previous *C. trachomatis* infections, previous sexually transmitted infections, smoking habits and previous record of infertility such as ectopic pregnancy and tubal factor infertility.

Cohort Characterization

The participants were characterized into cohorts based on their disease state- infertile women recruited from IVF clinics with a history *C. trachomatis* infections; infertile women recruited from IVF clinics with no history of *C. trachomatis* infections; women with acute *C. trachomatis* infections (women recruited from sexual health clinics with current *C. trachomatis* infections as diagnosed by PCR); and fertile healthy controls (women recruited from obstetrics clinics with current spontaneous pregnancies).

Infertile cohort: women with infertility (n=97)

Infertility was defined as the inability to conceive after trying for a period of 1 year or greater. Participants in this cohort were recruited from an IVF clinic based in Melbourne (n=97). They were given a unique ID and were informed written consent was an inclusion criteria. These participants provided a blood specimen for the study. The participants were also requested to complete a questionnaire with information regarding their fertility and gynecological history. Their questionnaire included questions regarding history of gynecological surgeries such as endometrial polyp, fibroids, endometriosis, ectopic pregnancy, miscarriages, polycystic ovaries and patent tubes. The descriptive analyses of demographic characteristics of participants are enlisted in Appendix 8.1.

The participants were divided into TFI cohort and non-TFI cohort based on their tubal patency. The tubal patency was determined using techniques such as laparoscopy, hysterosalpinography and hysteroscopy. Participants with unilateral or bilateral tubal blockage were characterized as TFI positive (n=45). Polycystic ovary syndrome and fibroids were common among patients in TFI cohort. The TFI cohort comprised of women with partial tubal patency (n=3), bilateral occlusion (n=21) and unilateral occlusion (n=21). The non-TFI cohort (n=52) comprised of women with infertility (infertility associated with other factors and not tubal damage) caused due to endometriosis, idiopathic PCO (polycystic ovary syndrome), fibroids or due to unknown reasons.

TFI cohort was further characterized as CT TFI cohort and negatives cohort based on the *C. trachomatis* infection status. The positive cohort (n=11) was defined by the presence of *C. trachomatis* specific IgG antibodies in the sera from TFI participants as determined by micro immunofluorescence (Focus Diagnostic, USA).

The negative cohort (n=251) comprised of TFI participants who were seronegative in MIF, and non-TFI infertile participants who were negative all the serological tests.

Acute infection cohort: women with acute C.trachomatis infections (n=112)

The acute infections cohort comprised of participants with clinically and PCR diagnosed acute *C. trachomatis* infections, with no or minor symptoms reported. A total of 112 women between the ages of 22- 53 years with acute *C. trachomatis* infections were included in this study. 112 participant sera and their corresponding medical history report enlisting their fertility and disease states were collected from sexual health clinics and hospitals.

Healthy cohort: fertile women infections (n=53)

The healthy control cohort comprised of fertile women between the ages of 28-42 years attending a tertiary care hospital (Brisbane) who have current spontaneous pregnancies and were able to get pregnant within less than a year of trying (n=53). The participants were requested to provide a written consent to participate in the study and had to complete a questionnaire with questions pertaining to their gynecological history and history of sexually transmitted infections.

Collection and storage of sera from patients

Sera were collected from patients from IVF clinics, sexual health clinics and hospitals around Queensland. Each sera and the corresponding patient history sheet had a unique numbered-identification code for the study. 200µL of the serum was aliquoted into cryovials labeled with the unique code and stored in -80° C freezers.

4.2.5 Characterization of cohorts for small –scale evaluation of peptide ELISA

A 'triage' process used a small sample size of participants to optimize the ELISA conditions worthy of larger scale testing. For this small- scale evaluation of peptide assay, the CT infertile cohort included participants recruited from IVF clinics who were seropositive in MEDAC MOMP and MEDAC cHSP60 (n=9). The acute infections cohort included participants recruited from sexual health clinics who were diagnosed with active *C. trachomatis* infection by PCR, and were positive in MEDAC MOMP (n=9). The Negative cohort (n=9) included participants who were fertile (able to get pregnant within a year of trying) (n=7); infertile participants who were seronegative for *C. trachomatis* in MEDAC MOMP or MEDAC cHSP60 (n=2).

4.2.6 Characterization of cohorts for large-scale evaluation of peptide ELISA (development cohort)

The performance of QUT *Chlamydia* infertility test was evaluated on 262 participants (development cohort) and an absorbance threshold was established and the specificity and sensitivity was evaluated in this cohort (Figure 4.1). Infertile participants were women who required assisted reproductive technologies (ART) were recruited from IVF clinic based in Melbourne (n=97) (cohort characterization outlined in section 4.2.4); participants with PCR diagnosed acute *C. trachomatis* infections were recruited from sexual health clinics and GP clinics (n=112); and participants with current spontaneous pregnancies were recruited from a tertiary hospital antenatal clinic (n=53). The acute infections cohort (described in detail in 4.2.4) included participants who were diagnosed with acute *C. trachomatis* infections by PCR (n=112) and the fertile controls (described in detail in 4.2.4) included participants who were pregnant within a year of trying (n=53). Based on the *C. trachomatis* infection status as determined by microimmunofluorescence (MIF) assay, the participants were characterized as positive for *C. trachomatis*-related tubal infertility (CT TFI cohort) (n=11). The sera were also tested with commercial assays outlined in 4.2.3. An absorbance threshold was determined on this evaluation group when the assay was controlled for high specificity for both single-well and multi-well peptide ELISA formats. The peptide format that was most specific and sensitive to *C. trachomatis*-related TFI in this cohort was designated the QUT *Chlamydia* infertility assay and it was subsequently validated on the separate validation group.

4.2.7 Characterization of cohort for validation of the peptide ELISA (validation cohort)

The validation cohort included participants retrospectively and prospectively recruited from an IVF clinic based in Brisbane (Australia) (n=73) (Figure 4.1). The women who have completed their IVF cycle (retrospective) and those who are yet to complete their treatment (prospective) were included in this cohort. The participants were infertile. Their gynecological history consisted of information pertaining to tubal patency, parity and previous gynecological surgeries and diseases (PID, endometriosis). There were 19 participants with tubal pathology, 23 participants with unknown etiology and 32 participants with PCOS, salpingitis, PID and endometritis (Table 8.3 (Appendix)). The QUT *Chlamydia* infertility assay was validated on this cohort using the cut-off obtained in the development cohort. The specificity,

sensitivity, positive predictive value and the negative predictive of the assay were determined on this cohort.

4.2.8 Identification and evaluation of diagnostic performance of peptide variants of peptide 11

Peptide 11 was less soluble in 50% isopropanol solvent compared to HSP60-E2 8% hydrophilic, 58% hydrophobic, basic) and 443-N2 (8% hydrophilic, 38% hydrophobic, basic). Peptide 11 has a net acidic charge and contains 25% hydrophilic residue and 58% hydrophobic residue. Since it is an acidic peptide, it should be readily soluble in aqueous solutions; however, high proportion of hydrophobic residues hinders its solubility. Although, peptide 11 was soluble in 0.001M NaOH, several smaller variants of peptide 11 were manufactured with varied antigenic epitopes. The peptide variants were DTRGI (20% hydrophobic, TRGILVV (neutral, 57.14% hydrophobic), ILVVAVEAGSPA (66.7% hydrophobic, neutral) and ILLVVAVEAGSP (neutral, 63.64% hydrophobic). Since most of these peptides are neutral, they were soluble in 50% isopropanol solution. The ability of assays based on peptide 11 variants and their combination with HSP60-E2 and 443-N2 to identify *C. trachomatis*-related TFI in women was evaluated on the development cohort.

4.2.9 Antibody production in rabbits immunized with peptides from QUT *Chlamydia* infertility assay

Sera from rabbits immunized with *In-house* peptides (peptide 11, HSP60-E2 and 443-N2) developed for QUT *Chlamydia* infertility assay were used as controls for inter and intra assay variability studies. Prior to immunization, sera was extracted from the animals which served as a negative control for inter and intra assay variability assays. Each animal was dosed four times with 0.2-0.5mg of the peptide for 9 weeks. The peptides were conjugated with keyhole limpet hemocyanin (KLH) for HSP60-E2 and 443-N2, while peptide 11 was conjugated with cysteine at the C terminal. After the final dosage, the sera was collected and showed a strong serological response to *C. trachomatis* and hence they were used as positive controls for inter and intra assay variability tests.

The animal work and immunizations were undertaken at South Australian Health and Medical Research Institute (SAHMRI) (Adelaide).

4.2.10 Inter and intra assay variability of QUT *Chlamydia* infertility assay

The diagnostic accuracy and reproducibility of the QUT *Chlamydia* infertility test was further evaluated by determining inter and intra-assay variability. The intra assay variability was determined by testing the six samples from each cohort (CT TFI cohort, non-TFI cohort, acute infection and healthy cohort) six times on a single plate. Inter assay variability was measured by testing the same six samples on 6 different plates at different times. The mean, the standard deviation and coefficient of variation (%CV= 100 x standard deviation/mean) was calculated to evaluate variability.

In addition to participant sera, rabbit sera immunized with peptide 11, HSP60-E2 and 443-N2 were also tested for intra and inter assay variability. The pre-bleed that was extracted from the rabbit before exposure to the antigens served as a negative control.

4.2.11 Determination of diagnostic performance of QUT *Chlamydia* infertility test and statistical analysis

The performance of the QUT *Chlamydia* infertility test was evaluated on the development cohort. The absorbance threshold was determined from Area Under the Curve analysis (AUC). The sensitivity and specificity of the assay as a function of the cut- off value was determined using the receiver operating characteristic curve (ROC). All statistics (relative risk, sensitivity, specificity, chi squared statistics and correlation) were calculated in the R statistical environment (3.0.1) using the 'EpiR' (0.9-48) and 'metafor' package (1.9-1) for conducting meta-analyses in R [413]. In addition to sensitivity and specificity, positive predictive value (PPV) and negative predictive value (NPV) were determined. Positive predictive value is the probability that those patients positive for the infection and truly infected, and negative predictive value is the probability that those patients negative for the infection are truly negative [435]. Diagnostic odds ratio unlike the conventional odds ratio measures the test performance in diagnosing the disease by combing the strengths of sensitivity and specificity as prevalence independent indicators [436]. Mixed-effects models were used to combine cohort data using a fixed-effect model with a restricted maximum likelihood estimate of the log odds ratio. Inter and intra assay variation was estimated using linear-mixed effects models which were calculated for the different assays taking into account fixed differences in sample absorbance with

plate-to-plate differences as a separate stochastic variable. The resulting inter and intra assay standard errors were calculated as restricted maximum likelihood estimates. All calculations were performed in the R statistical environment (v 3.0.1) using the Linear and Nonlinear Mixed Effects Models package ‘nlme’ (v 3.1-111).

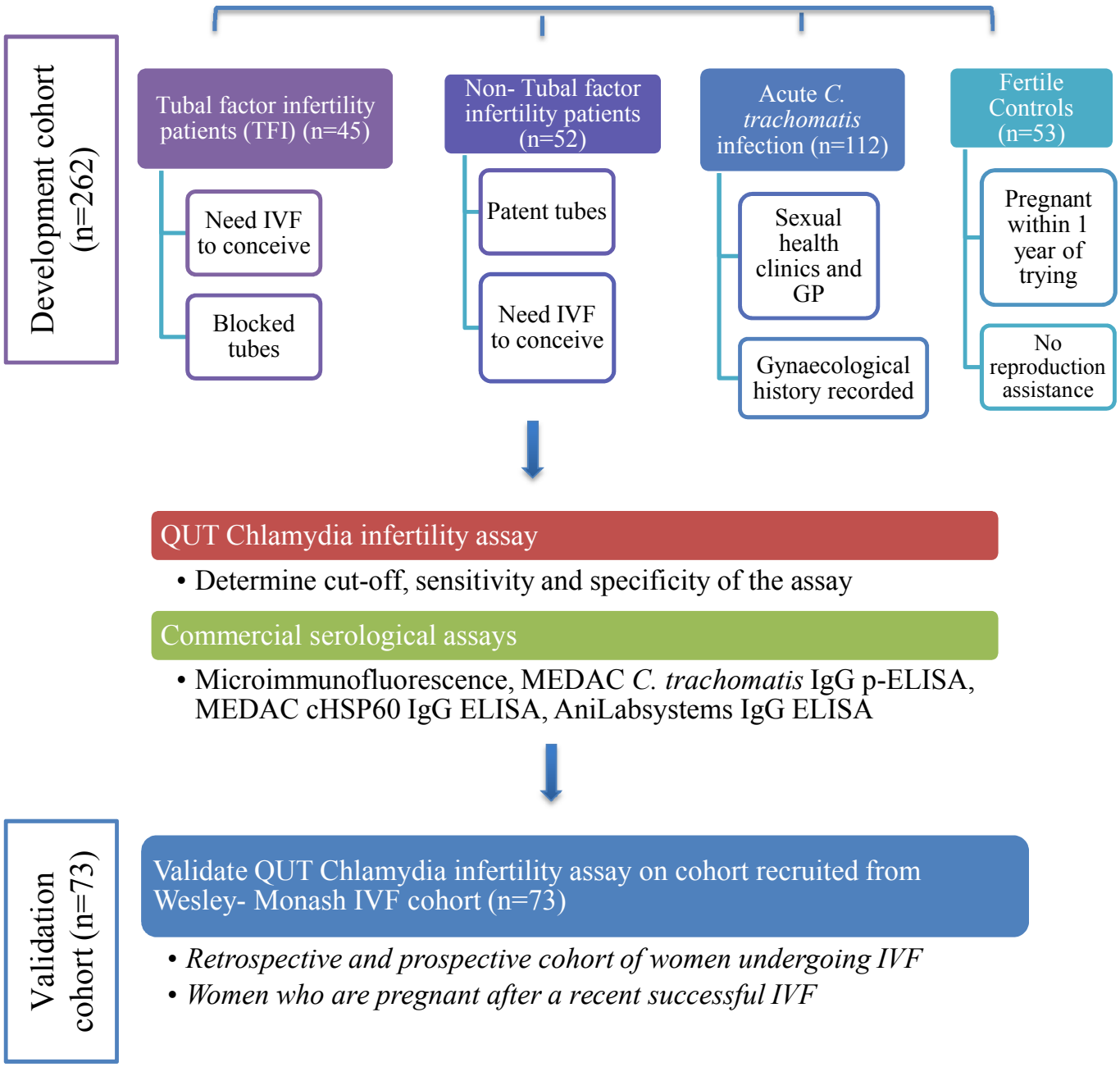

Figure 4.1: **Flow chart illustrating the experimental design for the development of QUT *Chlamydia* infertility test for diagnosing chlamydial infertility in women in the development cohort (n=262) and the validation cohort (n=73).**

4.3 RESULTS

4.3.1 Small-scale analysis reveal multi-well peptide ELISA format had the best potential for diagnostic performance

An initial triage using a small sample size was conducted to compare multi-well and combined well formats for the different peptides to determine which formats should be more rigorously tested and developed using larger sample size. The diagnostic performance of peptide 11, HSP60-E2, 443-N2 and 443-A3 were evaluated against women with *C. trachomatis*-related infertility (n=9) and also against the Negative cohort (participants with acute *C. trachomatis* infection (n=9) and healthy fertile participants (n=9). Table 4.1 lists the sensitivities and specificities of individual peptides, double combination of peptides in a single well and multi-well format, triple combinations of peptides in a single well and multi-well format.

The absorbance threshold for maximum sensitivity and specificity for each individual peptide assay and its combination was determined by receiver operator characteristic analysis (ROC). Although individual peptide assays exhibited high specificity ranging from 83% to 89% in detecting CT infertile cohort from negative cohort, peptide combinations in single well and multi-well format improved the sensitivity of the assay. While triple combination of peptides still demonstrated lower specificity (78%) and sensitivity (83%), double combination of peptide 11/ HSP60-E2 yielded a high specificity of 100% and sensitivity of 94%. The sensitivities were maintained at 100% when using the multi-well format for peptide11 and HSP60-E2; Peptide 11 and 443-N2; HSP60-E2 and 443-N2; and peptide 11, HSP60-E2 and 443-N2. However, compared to single-well peptide ELISA the specificity of the assay ranged from 78% and 89% depending on the combination of antigens. Importantly, the multi-well combinations had tighter confidence intervals compared to single well formats. This indicated lower variability in the assays. Due to greater specificity and sensitivity of the multi-well assay, this format was adopted for all further analysis and evaluation. This triage process was conducted on this smaller cohort, to enable streamlining of the assays to be conducted on the full development cohort as detailed in 4.3.2.

Table 4.1: Small-scale evaluation of peptides 11, HSP60-E2 and 443-N2 in single and multi-well format for the detection of women with *C. trachomatis* - related infertility

	CT infertile cohort (n=9)		Negative cohort (n=18)		Sensitivity [95% CI]	Specificity [95% CI]	P (Chisq)	Odds ratio [95%]
Assay (absorbance threshold)	Assay +	Assay -	Assay +	Assay -				
Peptide 11 (>0.296)	6	3	2	16	0.67[0.3-0.93]	0.89[0.65-0.99]	0.003	16[2.12-120.65]
Peptide HSP60-E2 (> 0.25)	8	1	3	15	0.89 [0.52-1]	0.83[0.59-0.96]	<0.001	40[3.56-450]
Peptide 443-N2 (>0.22)	9	0	2	16	1[0.55-1]	0.89[0.65-0.99]	<0.001	125.4[5.43-2895.88]
Single-well: [a]Peptide 11/ HSP60-E2 combo assay (>0.28)	9	0	1	17	1[0.55-1]	0.94[0.73-1]	<0.001	221.67[8.20-5989.75]
Single-well: [a]Peptide 11/HSP60-E2/443-N2 (>0.23)	7	2	3	15	0.78[0.4-0.97]	0.83[0.59-0.99]	0.002	17.5[2.36-129.51]
Multi-well: [b]Peptide 11 (>0.23) and HSP60-E2 (>0.22)	9	0	3	15	1[0.55-1]	0.78[0.52-0.94]	<0.001	61.22[2.95-1272.36]
Multi-well: [b]Peptide 11 (0.18) and 443-N2 (>0.22)	9	0	4	14	1[0.55-1]	0.78[0.52-0.94]	<0.001	61.22[2.95-1272.36]
Multi-well: [b] HSP60-E2 (>0.22) and 443-N2 (>0.22)	9	0	2	16	1[0.55-1]	0.89[0.65-0.99]	<0.001	125.4[5.43-2895.88]
Multi-well: [b]Peptide11 (>0.18) and HSP60-E2 (>0.22) and 443-N2 (>0.22)	9	0	2	16	1[0.55-1]	0.89[0.65-0.99]	<0.001	125.4[5.43-2895.88]

[a] The maximum specificity and sensitivity of combination peptide antigens in a single well at established absorbance thresholds (in parenthesis) in identifying participants with *C. trachomatis*-related infertility. The level of significance tested by chi-square test at $p<0.05$

[b]The maximum specificity and sensitivity of combination peptide antigens in multiple wells at established absorbance thresholds (in parenthesis) in identifying participants with *C. trachomatis*-related infertility. The level of significance tested by chi-square test at $p<0.05$

4.3.2 Large scale evaluation of peptides in multi-well format and selection of assay format for the QUT *Chlamydia* infertility test on the development cohort

The multi-well format was selected for larger scale assay development after the small-scale evaluation in 4.3.1 showed that individual wells allowed for higher specificity and sensitivity with smaller confidence intervals than when antigens were combined in a single-well. Peptide 11, HSP60-E2 and 443-N2 were coated on separate wells of the plate and the antibody response from women with and without chlamydia-related infertility was analyzed individually as well as in combination, in order to develop a sensitive and specific ELISA for women with chlamydial infertility. Using ROC analysis, an optimal absorbance threshold was achieved after controlling for maximum specificity in detecting *C. trachomatis* –related infertility, which is critical for assay development. The absorbance threshold for maximum specificity was determined against CT TFI cohort (n=11) and a cohort comprising of non-TFI participants and MIF negative TFI participants (n=80). The absorbance threshold that was determined in this analysis was later applied to evaluate the diagnostic performances of peptide combination multi-well assays in identifying infertile women with tubal pathology from those without (Evaluation 1). The absorbance threshold was also applied to identify women with *C. trachomatis*-related infertility in CT TFI cohort from the negative cohort (Evaluation 2). The diagnostic performance of several multi-well peptide combinations were tested on these cohorts and the combination with superior sensitivity and specificity in identifying true positives was designated as the QUT *Chlamydia* infertility test. The concordance between the assay and commercial serological assays were determined and its reproducibility was assessed through intra and inter assay variability.

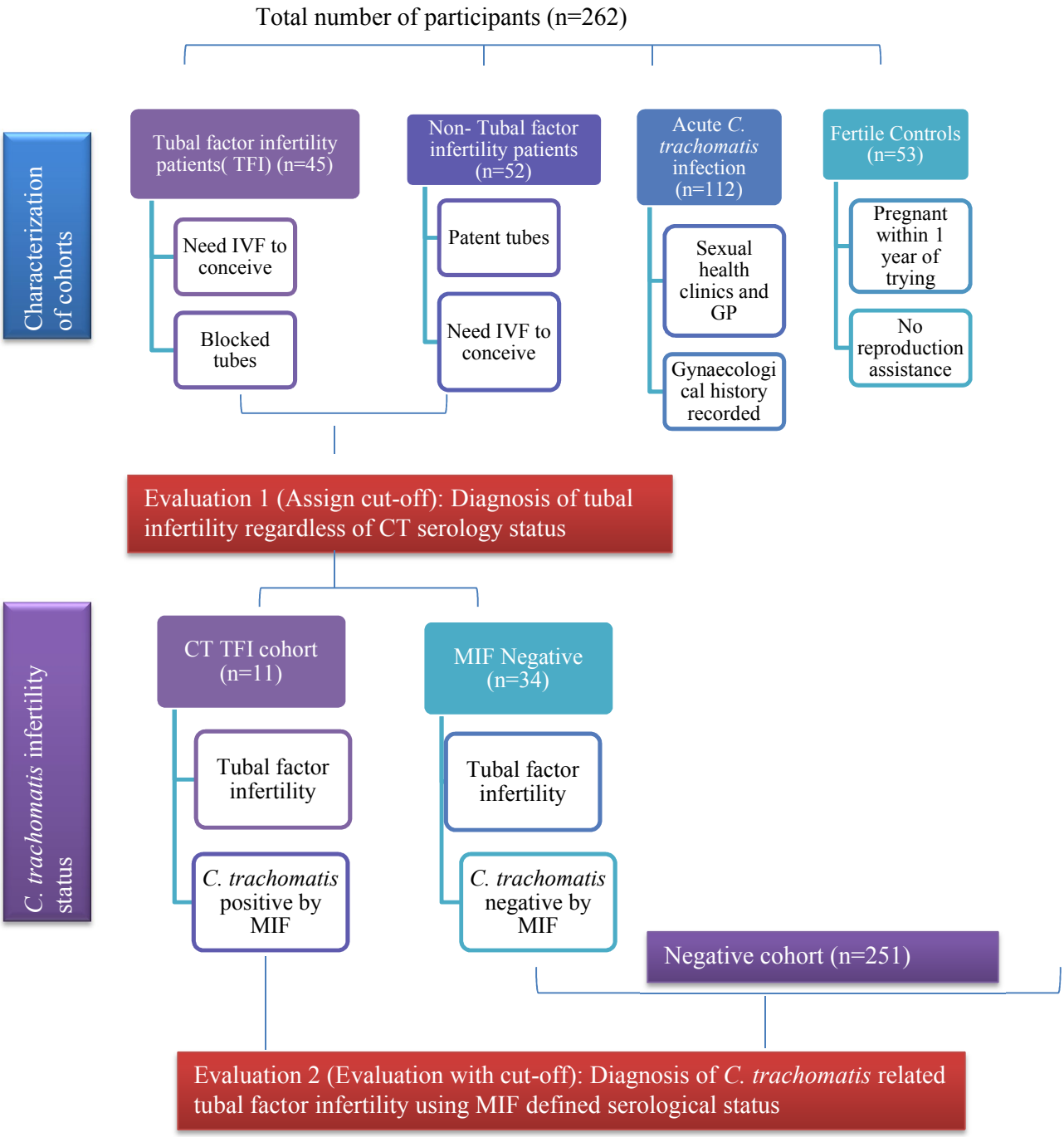

Figure 4.2: The flow chart depicts the characterization of cohorts based on the gynecological data and *C. trachomatis* infection seropositivity (MIF) of participants

Figure 4.2 depicts the serological response to *C. trachomatis* was determined by MIF, based on which the cohorts were further characterized as true positive and the negative cohort. Two main questions have been evaluated through this assay. Evaluation 1 (Assign cut-off) includes the ability of the QUT *Chlamydia* infertility test to effectively differentiate infertile participants with tubal factor infertility (TFI) from those without (excluding acute/fertile) by estimating the cut off controlled for maximum specificity. Though evaluation 2 (evaluation of cut-off), the diagnostic ability of QUT *Chlamydia* infertility test using the same cut-off to differentiate participants with CT associated TFI from participants belonging to the negative cohort (participants with acute infections, fertile healthy participants and non-TFI infertile participants and TFI participants seronegative for *C. trachomatis* infection).

Establishing the absorbance threshold for maximum specificity of the ELISA to be significantly able to differentiate women with C. trachomatis-related infertility from infertile women without TFI

The absorbance threshold controlled for maximum specificity was estimated in this assay. Individual peptides and their combination assays were tested against infertile women with and without tubal pathology (n=97). The assays were tested against women with *C. trachomatis* related infertility (women who were seropositive in MIF CT) (n=11) and negative cohort (n=80) that comprises of non-TFI infertile participants seronegative for *C. trachomatis* and non-TFI infertile women seropositive for *C. trachomatis*. When adjusting for confounders, six women were excluded due to missing information regarding BMI and smoking status. Table 4.2 lists the absorbance threshold and the corresponding sensitivity and specificity of peptide ELISA in single and multi-well format in diagnosing women with *C. trachomatis*-related TFI. All commercial serological assays such as MEDAC infertile, MEDAC MOMP and ANILabsystems could significantly differentiate women with *C. trachomatis*-related infertility from the negative cohort ($p<0.05$). MEDAC infertile had the highest sensitivity (36%) while MEDAC MOMP had the highest specificity (99%). Amongst peptide ELISAs, individual peptide could not effectively identify CT TFI cohort from negative cohort, but their combination assays could ($p=0.009$).

Double combination of peptides, peptide 11, HSP60-E2 or 443-N2 (Peptide 11 ≥ 0.686 or HSP60E2$\geq$0.559 or 443N2 $\geq$0.5715), peptide 11 with HSP60-E2 and 443-

N2 (Peptide 11 ≥0.216 and HSP60E2 ≥0.106 and 443N2 ≥ 0.4025) and peptide 11 with 443-N2 (Peptide 11≥0.216 and 443N2 ≥0.4025) yielded a sensitivity of 27% and a specificity of 95%. The odds ratio was lowered for combination assays and MEDAC infertile assay after adjusting for factors such as BMI and smoking status. This suggests that one of these factors could be a confounding variable for these assays. The absorbance threshold was applied in evaluation 1 and evaluation 2 to evaluate the performance of peptide ELISAs and identify the best ELISA format to diagnose *C. trachomatis*-related infertility in women.

Table 4.2: The diagnostic performance of individual and combination of peptides in a multi-well assay format and commercial serological assays in identifying infertile participants with *C. trachomatis* related TFI (n=11) from negative cohort (MIF-negative TFI and Non-TFI participants) (n=80)[Estimating absorbance threshold].

.

		CT TFI cohort (n=11)		**Negative cohort (n=80)**								
Assay	Absorbance Threshold(s) criteria	True positives	False negatives	False positives	True negatives	[a]Sensitivity (95% CI)	[a]Specificity (95% CI)	[b]Positive predictive value (95% CI)	[b]Negative predictive value (95% CI)	Odds Ratio (95% CI)	Chi Squared P value (unadjusted)	[c]Adjusted Odds Ratio (95% CI))
MEDAC Infertile		4	7	3	77	0.36 (0.11-0.69)	0.96 (0.89-0.99)	0.57 (0.18-0.9)	0.92 (0.84-0.97)	14.67 (2.72-79.1)	<0.001	7.9 (1.1-54)
ANIlabsystems IgG ELISA		3	8	6	74	0.27 (0.06-0.61)	0.92 (0.84-0.97)	0.33 (0.07-0.7)	0.9 (0.82-0.96)	4.62 (0.97-22.15)	0.039	4.8 (0.67-35)
MEDACMOMP	MEDAC MOMP > 1.2	1	10	1	79	0.09 (0-0.41)	0.99 (0.93-1)	0.5 (0.01-0.99)	0.89 (0.8-0.94)	7.9 (0.46-136.41)	0.096	5.1 (0.17-150)
MIF *C. trachomatis* (MIF CT)		11	0	5	75	1 (0.62-1)	0.94 (0.86-0.98)	0.69 (0.41-0.89)	1 (0.93-1)	Inf (NaN-Inf)	<0.001	7e+234 (0-Inf)
MIF *C. pneumoniae* (MIF CP)		7	4	21	59	0.64 (0.31-	0.74 (0.63-	0.25 (0.11-0.45)	0.94 (0.85-0.98)	4.92 (1.31-	0.012	4.9 (1.1-22)

						0.89)	0.83)			18.51)		
MIF *C. psittaci* (MIF CS)		4	7	1	79	0.36 (0.11-0.69)	0.99 (0.93-1)	0.8 (0.28-0.99)	0.92 (0.84-0.97)	45.14 (4.42-461.03)	<0.001	34 (2.5-470)
Peptide 11	Peptide 11 ≥0.411	1	10	4	76	0.09 (0-0.41)	0.95 (0.88-0.99)	0.2 (0.01-0.72)	0.88 (0.8-0.94)	1.9 (0.19-18.73)	0.577	0.84 (0.063-11)
HSP60- E2	HSP60E2 ≥0.522	1	10	4	76	0.09 (0-0.41)	0.95 (0.88-0.99)	0.2 (0.01-0.72)	0.88 (0.8-0.94)	1.9 (0.19-18.73)	0.577	3.3 (0.26-41)
443-N2	443n2 ≥ 0.539	1	10	4	76	0.09 (0-0.41)	0.95 (0.88-0.99)	0.2 (0.01-0.72)	0.88 (0.8-0.94)	1.9 (0.19-18.73)	0.577	0.89 (0.061-13)
Double combination of either peptides: Peptide 11or HSP60-E2 or 443-N2	Peptide 11 ≥0.686 or HSP60E2 ≥0.559 or 443N2 ≥ 0.5715	3	8	4	76	0.27 (0.06-0.61)	0.95 (0.88-0.99)	0.43 (0.1-0.82)	0.9 (0.82-0.96)	7.12 (1.35-37.65)	0.009	5.2 (0.67-39)
Triple peptide combination: Peptide11 and HSP60-E2 and 443-N2	Peptide 11 ≥ 0.216 and HSP60E2 ≥ 0.106 and 443N2 ≥0.4025	3	8	4	76	0.27 (0.06-0.61)	0.95 (0.88-0.99)	0.43 (0.1-0.82)	0.9 (0.82-0.96)	7.12 (1.35-37.65)	0.009	3.9 (0.59-25)
Double combination: Peptide 11 and 443-N2	Peptide 11 ≥0.216 and 443N2 ≥ 0.4025	3	8	4	76	0.27 (0.06-0.61)	0.95 (0.88-0.99)	0.43 (0.1-0.82)	0.9 (0.82-0.96)	7.12 (1.35-37.65)	0.009	3.9 (0.59-25)

[a]Specificity and Sensitivity: Range is 0-1, 95% confidence intervals in parenthesis; [b]Positive predictive value and negative predictive value: range 0-1, 95% confidence intervals in parenthesis; [c]Adjusted ratio after accounting for BMI and smoking

Evaluation 1 (assign cut-off): QUT Chlamydia infertility test can differentiate participants with TFI from infertile participants without TFI with greater specificity and sensitivity as compared to commercial serological assays

The peptides in multi-well format were assayed against sera from participants with TFI (n=45) and infertile participants without TFI (n=52). The participants who tested equivocal in the commercial serological assays were excluded from the analysis (n=6). Therefore, only 47 non-TFI participants and 44 TFI participants were tested against commercial serological assays. Table 4.3 showed the performance of individual peptides and combination of peptides in multi-well formats against TFI and non- TFI participants. The absorbance threshold that was determined in Table 4.2 was applied in Evaluation 1 and the corresponding specificity and sensitivity was determined.

The commercial ELISAs assayed against the cohort showed high specificity (94%-98%), however the sensitivities were low, with MEDAC MOMP being the lowest (2%) and ANILabsystems the highest (14%). The MEDAC infertile assay had a sensitivity of 11% and had higher positive predictive values (71%) in detecting participants with TFI compared to other commercial ELISAs. Although, MIF CT (MIF *C. trachomatis*) had the highest sensitivity compared to commercial assays (27%) in detecting TFI in women, the specificity was lower than other assays (89%). Furthermore, its positive predictive value (PPV) was also lower than other assays.

Amongst individual peptides assays, HSP60-E2 (cut off value=0.522; sensitivity=7%; specificity=96%) and 443-N2 (cut off value=0.539; specificity=9%; sensitivity=98%) showed better diagnostic performance as compared to peptide 11 (cut off value=0.411; sensitivity =4%; specificity=94%) in identifying TFI participants from non-TFI participants. Combination of peptide 11 (=0.686) with either HSP60-E2 (absorbance threshold =0.559) or 443-N2 (absorbance threshold =0.5715) yielded a sensitivity of 27% and a specificity of 95% in differentiating TFI participants from non-TFI infertile participants (p=0.006). The combination of all three peptides, peptide 11 (absorbance threshold =0.216), HSP60-E2 (absorbance threshold =0.106) and 443-N2 (absorbance threshold =0.4025); and combination of peptide 11 (absorbance threshold =0.216) and 443-N2 (absorbance threshold =0.4025) showed equal sensitivity and specificity in identifying TFI participants. The positive predictive value was 100% in combination assays, which shows that all the

samples that tested positive were positive. Although, double combination of any of the following peptides, peptide 11, HP60-E2 and 443-N2, yielded higher sensitivity, triple combination of all three peptides yielded higher specificity and could significantly differentiate TFI participants from Non-TFI participants ($p<0.004$). Therefore, it was considered a potential candidate for QUT *Chlamydia* infertility test.

The double and triple peptide combination assays had the highest sensitivity and specificity compared to all commercial serological assays, including MIF. The best performing commercial assay, MEDAC Infertile had only 11% sensitivity and 96% specificity, whereas triple combination assays had much higher sensitivity (16%) and specificity (100%), and could effectively differentiate infertile participants with TFI from those without TFI ($p=0.004$). The odds ratio was adjusted for factors such as birth year, smoking, BMI and history of alcohol consumption using multiple logistic regression analysis. The decrease in odds ratio after the adjustments suggest that one of these factors could be a possible confounder for the analysis and may influence the outcome of all the peptide assays.

Table 4.3: The diagnostic performance of individual and combination of peptides in a multi-well assay format and commercial serological assays in identifying infertile participants with TFI from those infertile participants without TFI [Evaluation 1 on development cohort].

	TFI Positive (n=45)		Non-TFI (n=52)								
Assay	True positives	False negatives	False positives	True negatives	[a]Sensitivity (95% ci)	[b]Specificity (95% ci)	Positive predictive value (95% CI)	Negative predictive value (95% CI)	Chi Squared P value (unadjusted)	Odds Ratio (95% CI)	[c]Adjusted odds ratio (95% CI)
MEDAC Infertile	5	39	2	45	0.11 (0.04-0.25)	0.96 (0.85-0.99)	0.71 (0.29-0.96)	0.54 (0.42-0.65)	0.203	2.88 (0.53-15.71)	1.9 (0.31-12)
ANILabsystems	6	38	3	44	0.14 (0.05-0.27)	0.94 (0.82-0.99)	0.67 (0.3-0.93)	0.54 (0.42-0.65)	0.247	2.32 (0.54-9.9)	2.8 (0.5-16)
MEDACMOMP >1.2	1	43	1	46	0.02 (0-0.12)	0.98 (0.89-1)	0.5 (0.01-0.99)	0.52 (0.41-0.62)	0.962	1.07 (0.06-17.64)	0.56 (0.027-12)
MIF *C. trachomatis*	11	33	5	42	0.25 (0.13-0.4)	0.89 (0.77-0.96)	0.69 (0.41-0.89)	0.56 (0.44-0.67)	0.072	2.8 (0.89-8.85)	3.9 (0.95-16)
MIF *C. psittaci*	4	40	1	46	0.09 (0.03-0.22)	0.98 (0.89-1)	0.8 (0.28-0.99)	0.53 (0.42-0.64)	0.145	4.6 (0.49-42.86)	3.4 (0.33-35)
MIF *C. pneumoniae*	12	32	16	31	0.27 (0.15-0.43)	0.66 (0.51-0.79)	0.43 (0.24-0.63)	0.49 (0.36-0.62)	0.484	0.73 (0.3-1.78)	0.61 (0.23-1.6)
Peptide 11 ≥0.411	2	43	3	49	0.05	0.94	0.4 (0.05-	0.53	0.701	0.7 (0.11-	0.7

					(0.01-0.15)	(0.84-0.99)	0.85)	(0.43-0.64)		4.39)	(0.093-5.3)
HSP60E2 ≥0.522	3	42	2	50	0.07 (0.01-0.18)	0.96 (0.87-1)	0.6 (0.15-0.95)	0.54 (0.44-0.65)	0.592	1.65 (0.26-10.35)	2.5 (0.36-17)
443N2 ≥0.539	4	41	1	51	0.09 (0.02-0.21)	0.98 (0.9-1)	0.8 (0.28-0.99)	0.55 (0.45-0.66)	0.145	4.6 (0.49-42.86)	5.7 (0.45-73)
Double combination of either peptides: Peptide 11≥0.686 or HSP60E2 ≥0.559 or 443N2 ≥ 0.5715	3	38	4	52	0.27 (0.06-0.61)	0.95 (0.89-0.99)	0.43 (0.1-0.82)	0.91 (0.83-0.96)	0.203	2.88 (0.53-15.71)	3.3 (0.5-21)
Triple combination: Peptide 11 ≥ 0.216 and HSP60E2 ≥ 0.106 and 443N2 ≥ 0.4025	7	38	0	52	0.16 (0.06-0.29)	1 (0.9-1)	1 (0.47-1)	0.58 (0.47-0.68)	0.004	Inf (NaN-Inf)	4.1e+07 (0-Inf)
Double combination:_ Peptide 11 ≥ 0.216 and 443N2 ≥0.4025	7	38	0	52	0.16 (0.06-0.29)	1 (0.9-1)	1 (0.47-1)	0.58 (0.47-0.68)	0.004	Inf (NaN-Inf)	4.1e+07 (0-Inf)

[a] Specificity and Sensitivity: Range is 0-1, 95% confidence intervals in parenthesis; [b] Positive predictive value and negative predictive value: range 0-1, 95% confidence intervals in parenthesis, [c] The analysis also accounts for variables such as BMI and smoking status.

Evaluation 2 (evaluation with cut-off): QUT Chlamydia infertility test can identify participants with chlamydial TFI from other cohorts

The second evaluation aimed to determine if any of the assays under development here had high specificity for women with *C. trachomatis*-related infertility and not detect fertile women, or women who have a recent or current CT infection with no reported fertility problems. Therefore through evaluation 2, the diagnostic performance of peptide ELISA in identifying participants with *C. trachomatis*-related infertility was assessed and compared with other commercial serological assays. Since, the MIF is considered the gold standard, it was used to define participants as *C. trachomatis*–related tubal infertility based on their serological status. MIF and confirmed tubal infertility was used to characterize participants into CT TFI positive cohort (n=11) and Negative cohort (comprises of infertile women with non-TFI, acute CT infections and fertile controls) (n=251) (Table 4.4). Participant sera that tested equivocal in the commercial serological assays were excluded from analysis. Therefore, 17 participants were excluded from ANILabsystems and 3 participants from MIF analysis. MEDAC infertile assay had the highest sensitivity (36%) compared to other commercial serological assays such as ANILabsystems and MEDAC MOMP. Although, its specificity was low (86%), the positive predictive (10% vs 5%) and the negative predictive value (97% vs 96%) was higher than other commercial assays. Additionally, it could effectively identify *C. trachomatis*-related TFI participants from negative cohort (p=0.04).

Multiple peptides had greater sensitivity and specificity in detecting *C. trachomatis* related infertile participants from negative cohort compared to individual peptides. Double peptide combinations (peptide 11 with either Hsp60-E2 or 443-N2 and peptide 11 and 443-N2) and triple peptide combinations (peptide 11, HSP60-E2 and 443-N2) had a sensitivity of 27%, while their specificity ranged from 93%-94%. Although the assays had low positive predictive values (18%-43%), the combination assays generally had fewer false positives as they had high negative predictive values of 97%. This suggests that out of 227 samples tested, 97% of the samples that tested negative in the assay were truly negative. This suggests that the peptide assay test were highly specific to *C. trachomatis*-related infertility.

The peptide combination assay also demonstrated greater diagnostic performance in identifying women with *C. trachomatis* –related TFI compared to the

best performing commercial assay, MEDAC infertile. Although, MEDAC infertile assay had higher sensitivity than peptide combination assays (36% vs 27%), the specificity (96%-97% vs 86%), PPV (14%-18% vs 10%) and the odds ratio (4.85-6.35 vs 3.41) were higher for the peptide combination assays (p=0.004). Additionally, Women with CT TFI were significantly more likely to be positive in the peptide combination assays than the leading commercial ELISA MEDAC infertile. Although, the diagnostic sensitivities and specificities were comparable between both double and triple peptide combination assays, the triple peptide combination assay (peptide 11, HSP60-E2 and 443-N2) showed greater odds ratio and level of significance in identifying participants with *C. trachomatis*-related infertility (p=0.004). Additionally, the triple peptide combination assays were also adept in identifying participants with TFI from those without TFI with the highest specificity (100%) compared to all other assays. Hence, this triple peptide was selected as the QUT *Chlamydia* infertility test and its performance was further validated in a secondary validation group.

Table 4.4: The diagnostic performance of individual and combination of peptides in a multi-well assay format and commercial serological assays in identifying CT TFI cohort (n=11) from negative cohort (n=251)[Evaluation 2 on development cohort].

	CT TFI cohort (n=11)		Negative cohort (n=251)							
Assay	True positives	False negatives	False positives	True negatives	[a]Sensitivity (95% CI)	Specificity (95% CI)	[b]Positive predictive value (95% CI)	Negative predictive value (95% CI)	Chi Squared P value (unadjusted)	Odds Ratio (95% CI)
MEDAC Infertile	4	7	36	215	0.36 (0.11-0.69)	0.86 (0.81-0.9)	0.1 (0.03-0.24)	0.97 (0.94-0.99)	0.047	3.41 (0.95-12.25)
ANIlabsystems	3	8	62	172	0.27 (0.06-0.61)	0.74 (0.67-0.79)	0.05 (0.01-0.13)	0.96 (0.91-0.98)	0.955	1.04 (0.27-4.05)
MEDACMOMP >1.2	1	10	24	227	0.09 (0-0.41)	0.9 (0.86-0.94)	0.04 (0-0.2)	0.96 (0.92-0.98)	0.959	0.95 (0.12-7.71)
MIF *C. trachomatis*	11	0	84	164	1 (0.62-1)	0.66 (0.6-0.72)	0.12 (0.06-0.2)	1 (0.97-1)	<0.001	Inf (NaN-Inf)
MIF *C. pneumoniae*	7	4	110	138	0.64 (0.31-0.89)	0.56 (0.49-0.62)	0.06 (0.02-0.12)	0.97 (0.93-0.99)	0.209	2.2 (0.63-7.69)
MIF *C. psittaci*	4	7	4	244	0.36 (0.11-0.69)	0.98 (0.96-1)	0.5 (0.16-0.84)	0.97 (0.94-0.99)	<0.001	34.86 (7.21-168.63)
Peptide 11 ≥0.411	1	10	27	207	0.09 (0-0.41)	0.88 (0.84-0.92)	0.04 (0-0.18)	0.95 (0.92-0.98)	0.802	0.77 (0.09-6.2)
HSP60E2 ≥ 0.522	1	10	14	220	0.09 (0-0.41)	0.94 (0.9-0.97)	0.07 (0-0.32)	0.96 (0.92-0.98)	0.72	1.47 (0.18-12.2)
443N2 ≥0.539	1	10	11	223	0.09 (0-0.41)	0.95 (0.92-0.98)	0.08 (0-0.38)	0.96 (0.92-0.98)	0.465	2.18 (0.26-18.59)

Double combination of either peptides: Peptide 11 ≥0.686 or HSP60E2 ≥0.559 or 443N2≥0.5715	3	8	14	237	0.27 (0.06-0.61)	0.94 (0.91-0.97)	0.18 (0.04-0.43)	0.97 (0.94-0.99)	0.004	6.35 (1.52-26.59)
Triple combination: Peptide 11 ≥ 0.216 and HSP60E2≥0.106 and 443N2≥0.4025	3	8	13	221	0.27 (0.06-0.61)	0.94 (0.91-0.97)	0.19 (0.04-0.46)	0.97 (0.93-0.98)	0.004	6.35 (1.52-26.59)
Double combination: Peptide 11 ≥ 0.216 and 443N2 ≥0.4025	3	8	14	220	0.27 (0.06-0.61)	0.94 (0.9-0.97)	0.18 (0.04-0.43)	0.96 (0.93-0.98)	0.006	5.9 (1.42-24.55)

[a] Specificity and Sensitivity: Range is 0-1, 95% confidence intervals in parenthesis; [b] Positive predictive value and negative predictive value: range 0-1, 95% confidence intervals in parenthesis

The diagnostic performance of variants of peptide 11 is comparable to individual peptides, but does not outperform QUT Chlamydia infertility test in identifying women with tubal pathology and C. trachomatis-related infertility

The ability to use a more soluble format of peptide 11 was an important factor for consideration as the process to dissolve the peptide 11 was labour intensive leading to concerns about possible future manufacture process. This difficulty is likely due to the high hydrophobicity of peptide 11 (58% hydrophobicity, 25% hydrophilic). Hence, several peptide variants were designed attempting to bias the selection towards the hydrophilic amino acid residues while maintaining the antigenic epitope (ADTRGILVV, SPA). The variants included DTRGI, TRGILVV, ILVVAVEAGSPA and ILLVVAVEAGSP. Table 4.5 showed that the sensitivity of peptide variants TRGILVV (sensitivity =5%; specificity =93%) and ILVVAVEAGSPA (sensitivity=7%; specificity=96%) were comparable to that of peptide 11 and HSP60-E2 in identifying infertile women with tubal pathology. Peptide variants in combination with peptide 11 and HSP60-E2 increased the sensitivity and specificity to 7% and 96%, which was higher than individual peptides; but were lower than the QUT *chlamydia* infertility test (sensitivity=16%; specificity=100%).

The peptide variants, individually and in combination with HSP60-E2 and 443-N2, were tested on CT TFI cohort and negative cohort to determine its diagnostic performance in identifying women with *C. trachomatis*-related infertility. While neither individual peptide variants nor combination assays were able to identify women with CT TFI from the negative cohort, the combination of these peptides with HSP60-E2 and 443-N2, the specificity was improved while the sensitivity was maintained at 9% (Table 4.6). The positive predictive value of these individual peptide variants and their combination (20%) were higher than peptide 11 (3%), HSP60-E2 (6%) and 443-N2 (8%) and was comparable to QUT *chlamydia* infertility test (18%). Additionally, The odds ratio of combination assays of peptide 11 variant TRGILVV and ILVVAVEGSPA were comparable to that of QUT *Chlamydia* infertility test. Thus, peptide variants of peptide 11 are likely candidates for a CT TFI diagnostic that can be further optimized for improved performance in the future.

Table 4.5 The diagnostic performance of peptide 11 variants in individual assays and in combination with HSP60 and 443-N2 in identifying women with tubal factor infertility from infertile women without tubal pathology were estimated.

	TFI positive (n=41)		Non-TFI (n=46)								
P11 variant Assay	True positives	False negatives	False positives	True negatives	[a]Sensitivity (95% CI)	[a]Specificity (95% CI)	[b]Positive predictive value (95% CI)	[b]Negative predictive value (95% CI)	Odds ratio (95% CI)	Chi Squared P value (unadjusted)	[c]Adjusted odds ratio (95% CI)
Peptide 11 ≥0.411	2	43	3	49	0.05 (0.01-0.15)	0.94 (0.84-0.99)	0.4 (0.05-0.85)	0.53 (0.43-0.64)	.7 (0.11-4.39)	0.701	0.7 (0.093-5.3)
DTRGI≥0.311	1	40	2	44	0.02 (0-0.13)	0.96 (0.85-0.99)	0.33 (0.01-0.91)	0.52 (0.41-0.63)	0.55 (0.05-6.3)	0.626	0.57 (0.036-8.9)
DTRGI. ≥0 and HSP60E2 ≥0.444 and 443N2 ≥ 0.4025	3	41	2	45	0.07 (0.01-0.19)	0.96 (0.85-0.99)	0.6 (0.15-0.95)	0.52 (0.41-0.63)	1.65 (0.26-10.35)	0.592	3 (0.42-22)
TRGILVV≥ 0.3265	2	39	3	43	0.05 (0.01-0.17)	0.93 (0.82-0.99)	0.4 (0.05-0.85)	0.52 (0.41-0.64)	0.74 (0.12-4.63)	0.742	1.2 (0.17-8.5)
TRGILVV. ≥and HSP60E2 ≥0.444 and 443N2 ≥ 0.4025	3	41	2	45	0.07 (0.01-0.19)	0.96 (0.85-0.99)	0.6 (0.15-0.95)	0.52 (0.41-0.63)	1.65 (0.26-10.35)	0.592	3 (0.42-22)
ILVVAVEAGSPA≥ 0.4825	3	38	2	44	0.07 (0.02-0.2)	0.96 (0.85-0.99)	0.6 (0.15-0.95)	0.54 (0.42-0.65)	1.74 (0.28-10.95)	0.553	2.3 (0.29-18)
ILVVAVEAGSPA. ≥ 0 and HSP60E2 ≥0.444 and 443N2 ≥ 0.4025	3	41	2	45	0.07 (0.01-0.19)	0.96 (0.85-0.99)	0.6 (0.15-0.95)	0.52 (0.41-0.63)	1.65 (0.26-10.35)	0.592	3 (0.42-22)
ILVVAVEAGSP ≥0.676	1	40	3	43	0.02 (0-0.13)	0.93 (0.82-0.99)	0.25 (0.01-0.81)	0.52 (0.41-0.63)	0.36 (0.04-3.59)	0.364	0.35 (0.032-3.8)
ILVVAVEAGSP≥0 and HSP60E2 ≥ 0.444 and 443N2 ≥0.4025	3	41	2	45	0.07 (0.01-0.19)	0.96 (0.85-0.99)	0.6 (0.15-0.95)	0.52 (0.41-0.63)	1.65 (0.26-10.35)	0.592	3 (0.42-22)

[a] Specificity and Sensitivity: Range is 0-1, 95% confidence intervals in parenthesis; [b] Positive predictive value and negative predictive value: range 0-1, 95% confidence intervals in parenthesis, [c] The analysis also accounts for variables such as BMI and smoking status.

Table 4.6: The diagnostic performance of peptide 11 variants individually and in combination with HSP60 and 443-N2 in identifying women with *C. trachomatis*-related tubal factor infertility from women non-TFI infertile women who are seronegative for *C. trachomatis* infection (for individual peptides (n=82)) and Negative cohort (for combination assays (n=244).

	CT TFI positive (n=11)		**Negative cohort (n=244)**							
Assay	True positive	False negatives	False positives	True negatives	Sensitivity (95% CI)	Specificity (95% CI)	Positive predictive value (95% CI)	Negative predictive value (95% CI)	Odds Ratio (95% CI)	Chi Squared P value (unadjusted)
Peptide 11 ≥0.411	1	10	27	207	0.09 (0-0.41)	0.88 (0.84-0.92)	0.04 (0-0.18)	0.95 (0.92-0.98)	0.77 (0.09-6.2)	0.802
DTRGI ≥0.311	0	11	4	78	0 (0-0.38)	0.95 (0.88-0.99)	0 (0-0.72)	0.88 (0.79-0.94)	0 (0-NaN)	0.454
DTRGI≥0 and HSP60E2 ≥0.444 and 443N2 ≥ 0.4025	1	10	4	240	0.09 (0-0.41)	0.98 (0.96-1)	0.2 (0.01-0.72)	0.96 (0.93-0.98)	6 (0.61-58.71)	0.081
TRGILVV≥ 0.3265	1	10	4	78	0.09 (0-0.41)	0.95 (0.88-0.99)	0.2 (0.01-0.72)	0.89 (0.8-0.94)	1.95 (0.2-19.22)	0.561
TRGILVV≥0 and HSP60E2 ≥ 0.444 and 443N2 >= 0.4025	1	10	4	240	0.09 (0-0.41)	0.98 (0.96-1)	0.2 (0.01-0.72)	0.96 (0.93-0.98)	6 (0.61-58.71)	0.081
ILVVAVEAGSPA ≥0.4825	1	10	4	78	0.09 (0-0.41)	0.95 (0.88-0.99)	0.2 (0.01-0.72)	0.89 (0.8-0.94)	1.95 (0.2-19.22)	0.561
ILVVAVEAGSPA≥0 and HSP60E2 ≥0.444 and 443N2 ≥0.4025	1	10	4	240	0.09 (0-0.41)	0.98 (0.96-1)	0.2 (0.01-0.72)	0.96 (0.93-0.98)	6 (0.61-58.71)	0.081
ILVVAVEAGSP ≥0.676	0	11	4	78	0 (0-0.38)	0.95 (0.88-0.99)	0 (0-0.72)	0.88 (0.79-0.94)	0 (0-NaN)	0.454
ILVVAVEAGSP. ≥0 and HSP60E2≥0.444 and 443N2 ≥0.4025	1	10	4	240	0.09 (0-0.41)	0.98 (0.96-1)	0.2 (0.01-0.72)	0.96 (0.93-0.98)	6 (0.61-58.71)	0.081

[a] Specificity and Sensitivity: Range is 0-1, 95% confidence intervals in parenthesis; [b] Positive predictive value and negative predictive value: range 0-1, 95% confidence intervals in parenthesis

QUT Chlamydia infertility test does not correlate with commercial serological assays in detecting C. trachomatis-related infertility in women in the development cohort

In order to determine if the same participants are being detected by the different serological tests a concordance analysis was conducted. Table 4.7 illustrated that all MOMP peptide based commercial serological assays including MEDAC Infertile assay, ANILabsystems, MEDAC MOMP showed great concordance with each other and with MIF CT ($p<0.05$). However, these assays also showed significant concordance with MIF CP (*C. pneumoniae*) and MIF CS (*C. psittaci*) ($p<0.05$). This suggests that there is a potential for cross-reactivity between other chlamydial species in commercial serological assay, which compromises the specificity of the assay. Thereby, possibly increasing the rate of false positive tests for these assays.

As for QUT *Chlamydia* infertility test, the assay showed concordance with all peptide combination assays and individual peptides such as HSP60-E2 and 443N2. Although, the assay did not show concordance with any other commercial serological assays, it exhibited high correlation with MIF CS ($p=0.032$). Since, none of the individual peptides showed correlation to MIF CS, and MIF CS had exhibited its ability to differentiate CT TFI cohort from negative cohort (Table 4.4) and also correlates with MIF CT ($p=0.002$), it could be inferred that MIF CS may recognize cross-reacting antibodies with *C. trachomatis*.

Table 4.7: Concordance between commercial serological assays and *In-house* peptide ELISA in identifying *C. trachomatis* related tubal infertility in women in the development cohort

	MEDACInfertile	ANIlabsystems	MEDACMOMP	MIF.CT	MIF.CP	MIF.CS	Peptide.11	HSP60E2	443N2	Double combo (either) Peptide.11_or_HSP60e2_or_443n2	QUT *Chlamydia* infertility test	Double combination: peptide 11 and 443N2	Peptide 11.var.DTRGI	DTRGI + HSP60E2 + 443-N2	Peptide.11.var.TRGILVV	TRGILVV + HSP60E2 +443N2	Peptide 11.var.ILVVAVEAGSPA	ILVVAVEAGSPA+HSP60E2+443N2	Peptide.11.var.ILVVAVEAGSP	ILVVAVEAGSP+_HSP60e2_+443n2
MEDACInfertile	[a]<0.001	<0.001	<0.001	<0.001	0.01	<0.001	0.065	0.094	0.494	0.077	0.327	0.395	0.531	0.768	0.35	0.768	0.481	0.768	0.531	0.768
ANIlabsystems	<0.001	<0.001	<0.001	<0.001	0.145	0.019	0.475	0.99	0.902	0.982	0.304	0.396	0.478	0.504	0.493	0.504	0.493	0.504	0.478	0.504
MEDACMOMP	<0.001	<0.001	<0.001	<0.001	0.047	0.007	0.453	0.595	0.249	0.121	0.747	0.814	0.709	0.467	0.675	0.467	0.675	0.467	0.709	0.467
MIF.CT	<0.001	<0.001	<0.001	<0.001	0.004	0.002	0.227	0.358	0.714	0.119	0.15	0.224	0.351	0.449	0.865	0.449	0.865	0.449	0.351	0.449
MIF.CP	0.01	0.145	0.047	0.004	<0.001	0.015	0.572	0.732	0.009	0.111	0.064	0.043	0.856	0.245	0.579	0.245	0.141	0.245	0.191	0.245
MIF.CS	<0.001	0.019	0.007	0.002	0.015	<0.001	0.228	0.491	0.282	0.002	0.032	0.041	0.626	0.03	0.136	0.03	0.584	0.03	0.626	0.03
Peptide.11	0.065	0.475	0.453	0.227	0.572	0.228	<0.001	0.106	0.561	0.001	0.106	0.137	<0.001	0.437	0.584	0.437	0.584	0.437	0.626	0.437
HSP60E2	0.094	0.99	0.595	0.358	0.732	0.491	0.106	<0.001	0.008	<0.001	<0.001	<0.001	0.075	<0.001	<0.001	<0.001	0.584	<0.001	0.075	<0.001
443N2	0.494	0.902	0.249	0.714	0.009	0.282	0.561	0.008	<0.001	<0.001	<0.001	<0.001	0.626	<0.001	0.136	<0.001	0.136	<0.001	0.075	<0.001

Double combo (either) Peptide.11_or_HSP 60e2_or_443n2	0.077	0.982	0.121	0.119	0.111	0.002	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.176	0.003	<0.001	0.003	0.277	0.003	0.176	0.003
Triple combination	0.327	0.304	0.747	0.15	0.064	0.032	0.106	<0.001	<0.001	<0.001	<0.001	<0.001	0.56	<0.001	0.277	<0.001	0.512	<0.001	0.176	<0.001
Double combination: peptide 11 and 443N2	0.395	0.396	0.814	0.224	0.043	0.041	0.137	<0.001	<0.001	<0.001	<0.001	<0.001	0.56	<0.001	0.277	<0.001	0.512	<0.001	0.176	<0.001
Peptide.11.var.DTRGI	0.531	0.478	0.709	0.351	0.856	0.626	<0.001	0.075	0.626	0.176	0.56	0.056	<0.001	0.626	0.075	0.626	0.626	0.626	0.665	0.626
DTRGI + HSP60E2 + 443-N2	0.768	0.504	0.467	0.449	0.245	0.03	0.437	<0.001	<0.001	0.003	<0.001	<0.001	0.626	<0.001	<0.001	<0.001	0.136	<0.001	0.075	<0.001
Peptide.11.var.TRGILVV	0.35	0.493	0.675	0.865	0.579	0.136	0.584	<0.001	0.136	<0.001	0.277	0.277	0.075	<0.001	<0.001	<0.001	<0.001	<0.001	0.626	<0.001
TRGILVV + HSP60E2 +443N2	0.768	0.504	0.467	0.449	0.245	0.03	0.437	<0.001	<0.001	0.003	<0.001	<0.001	0.626	<0.001	<0.001	<0.001	0.136	<0.001	0.075	<0.001
Peptide.11.var.ILVVAVEAGSPA	0.481	0.493	0.675	0.865	0.141	0.584	0.584	0.584	0.136	0.277	0.512	0.512	0.626	0.136	<0.001	0.136	<0.001	0.136	0.075	0.136
ILVVAVEAGSPA +HSP60E2+443N2	0.768	0.504	0.467	0.449	0.245	0.03	0.437	<0.001	<0.001	0.003	<0.001	<0.001	0.626	<0.001	<0.001	<0.001	0.136	<0.001	0.075	<0.001
Peptide.11.var.ILVVAVEAGSP	0.531	0.478	0.709	0.351	0.191	0.626	0.626	0.075	0.075	0.176	0.176	0.176	0.665	0.075	0.626	0.075	0.075	0.075	<0.001	0.075
ILVVAVEAGSP+ _HSP60e2_+443n2	0.768	0.504	0.467	0.449	0.245	0.03	0.437	<0.001	<0.001	0.003	<0.001	<0.001	0.626	<0.001	<0.001	<0.001	0.136	<0.001	0.075	<0.001

[a]Correlation and concordance between serological assay defined by level of significance ($p<0.05$).

Women predicted to have C. trachomatis-related infertility in the QUT Chlamydia infertility test are equally likely to conceive by IVF as other infertility and therefore it is valid to recommend progression to IVF

Tubal factor infertility and previous IVF success rates were evaluated as prognostic indicators of successful infertility treatment in Table 4.8. Women with Tubal factor infertility had a lower success rate for IVF in this study, as the number of live births in infertile women without tubal pathology was significantly higher than that reported from women with tubal factor infertility (p=0.032, 24 vs 13). The odds ratio also suggested that non-TFI women were twice as likely to have a live birth than women with TFI after IVF treatment. Thus, compared to previous successful live birth and pregnancy outcome in an IVF procedure (p>0.05), tubal status of women proved to be a negative prognostic indicator of IVF outcome (p=0.032; AOR (95% CI)=3.9 (1.4-11).

Table 4.9 showed that none of the *C. trachomatis* serological assays including the QUT *Chlamydia* infertility test, MEDAC infertile assay, MEDAC MOMP, MIF CT and peptide 11 variant assays could effectively differentiate participants with successful IVF outcome from those who did not achieve a successful IVF outcome (p>0.05). Thus, the inability of the QUT *Chlamydia* infertility test to differentiate participants based on IVF outcomes suggests that participants positive for *C. trachomatis*-related infertility in this assay were as likely to achieve a successful IVF outcome as those infertile *C. trachomatis* seronegative participants. The sensitivity to detect positive live births and pregnancy rates were generally low (3%-5%) in both the peptide ELISAs and commercial serological assays (MEDAC infertile and MEDAC MOMP). Contrary to other commercial serological assays, ANILabsystems was able to effectively predict the IVF outcome (p=0.04). If a participant tested positive in this assay, they were 2.5 times more likely to have a successful IVF outcome. The analysis (Figure 4.3) also showed that the number of IVF cycles taken to achieve live birth or pregnancy rates were not statistically significantly between women with *C. trachomatis*-related TFI and women with other forms of infertility. This indicates that IVF treatments are as likely to be successful for women with *C. trachomatis*-related infertility as women with other forms of infertility.

.

Table 4.8:Prediction of IVF outcome (Live birth) based on the tubal status of infertile participants, history of live births and pregnancy in previous IVF cycles.

Factor (+/-)	Live birth, factor +	Live birth, factor -	No live birth, factor +	No live birth, factor -	Sensitivity (95% CI)	Specificity (95% CI)	Positive predictive value (95% CI)	Negative predictive value (95% CI)	Odds Ratio (95% CI)	Chi Squared P value (unadjusted)	Adjusted Odds Ratio (95% CI)
Tubal status (Non-TFI (n= 52)/ TFI (n=45)	24	13	18	26	0.65 (0.47-0.8)	0.59 (0.43-0.74)	0.57 (0.41-0.72)	0.67 (0.5-0.81)	2.67 (1.08-6.58)	0.032	3.9 (1.4-11)
Previous live birth outcome (TRUE / FALSE)	7	30	6	38	0.19 (0.08-0.35)	0.86 (0.73-0.95)	0.54 (0.25-0.81)	0.56 (0.43-0.68)	1.48 (0.45-4.86)	0.519	1.6 (0.44-5.6)
Previous pregnancy outcome (TRUE / FALSE)	8	29	12	32	0.22 (0.1-0.38)	0.73 (0.57-0.85)	0.4 (0.19-0.64)	0.52 (0.39-0.65)	0.74 (0.26-2.05)	0.557	0.71 (0.24-2.2)

[a] Specificity and Sensitivity: Range is 0-1, 95% confidence intervals in parenthesis; [b] Positive predictive value and negative predictive value: range 0-1, 95% confidence intervals in parenthesis, [c] The analysis also accounts for variables such as BMI and smoking status.

Table 4.9: The sensitivity and specificity of commercial serological assays and *In-house* peptide ELISA to predict IVF outcome in infertile women (n=97) in the development cohort

	No. of women with successful live births/ pregnancies (n=44)		**No. of women with failed live births/ pregnancies (n=47)**								
Assay	True positives	False negatives	False positives	True negatives	[a]Sensitivity (95% CI)	[a]Specificity (95% CI)	[b]Positive predictive value (95% CI)	[b]Negative predictive value (95% CI)	Chi Squared P value (unadjusted)	Odds Ratio (95% CI)	[c]Adjusted odds ratio (95% CI)
MEDAC Infertile	5	39	2	45	0.11 (0.04-0.25)	0.96 (0.85-0.99)	0.71 (0.29-0.96)	0.54 (0.42-0.65)	0.528	0.57 (0.1-3.31)	1.9 (0.31-12)
ANILabsystems	6	38	3	44	0.14 (0.05-0.27)	0.94 (0.82-0.99)	0.67 (0.3-0.93)	0.54 (0.42-0.65)	0.04	4.9 (0.95-25.26)	2.8 (0.5-16)
MEDAC MOMP >1.2	1	43	1	46	0.02 (0-0.12)	0.98 (0.89-1)	0.5 (0.01-0.99)	0.52 (0.41-0.62)	0.273	Inf (NaN-Inf)	0.56 (0.027-12)
MIF *C. trachomatis*	6	31	6	38	0.16 (0.06-0.32)	0.86 (0.73-0.95)	0.5 (0.21-0.79)	0.55 (0.43-0.67)	0.745	1.23 (0.36-4.18)	0.57 (0.12-2.8)
MIF *C. pneumoniae*	15	22	10	34	0.41 (0.25-0.58)	0.77 (0.62-0.89)	0.6 (0.39-0.79)	0.61 (0.47-0.74)	0.084	2.32 (0.88-	1.9 (0.68-5.5)

										6.07)	
Peptide 11 ≥0.411	3	34	2	42	0.08 (0.02-0.22)	0.95 (0.85-0.99)	0.6 (0.15-0.95)	0.55 (0.43-0.67)	0.507	1.2 (0.16-8.96)	2 (0.24-17)
HSP60E2 ≥ 0.522	2	35	2	42	0.05 (0.01-0.18)	0.95 (0.85-0.99)	0.5 (0.07-0.93)	0.55 (0.43-0.66)	0.859	1.85 (0.29-11.73)	1.4 (0.15-12)
443N2 ≥0.539	1	36	4	40	0.03 (0-0.14)	0.91 (0.78-0.97)	0.2 (0.01-0.72)	0.53 (0.41-0.64)	0.234	1.2 (0.16-8.96)	0.29 (0.021-4.1)
Double combination (either peptides): Peptide 11≥0.686 or HSP60E2 ≥0.559 or 443N2 ≥ 0.5715	2	35	5	39	0.05 (0.01-0.18)	0.89 (0.75-0.96)	0.29 (0.04-0.71)	0.53 (0.41-0.64)	0.342	0.28 (0.03-2.6)	0.33 (0.045-2.4)
QUT *Chlamydia* infertility test	2	35	5	39	0.05 (0.01-0.18)	0.89 (0.75-0.96)	0.29 (0.04-0.71)	0.53 (0.41-0.64)	0.342	0.45 (0.08-2.45)	0.32 (0.048-2.2)
Double combination: Peptide 11 ≥ 0.216 and 443N2 ≥ 0.4025	2	35	5	39	0.05 (0.01-0.18)	0.89 (0.75-0.96)	0.29 (0.04-0.71)	0.53 (0.41-0.64)	0.342	0.45 (0.08-2.45)	0.32 (0.048-2.2)
Peptide 11 variant (DTRGI) ≥0.311	2	35	1	39	0.05 (0.01-0.18)	0.98 (0.87-1)	0.67 (0.09-0.99)	0.53 (0.41-0.64)	0.51	2.23 (0.19-25.66)	1.8 (0.13-26)
Peptide.11.variant..DTRGI. ≥0 and HSP60E2 ≥ 0.444 and 443N2 ≥ 0.4025	1	36	3	41	0.03 (0-0.14)	0.93 (0.81-0.99)	0.25 (0.01-0.81)	0.53 (0.42-0.65)	0.394	0.38 (0.04-3.81)	0.5 (0.042-6.1)
Peptide 11 variant	3	34	2	38	0.08	0.95	0.6 (0.15-	0.53 (0.41-	0.58	1.68	1.8 (0.23-13)

(TRGILVV) ≥ 0.3265					(0.02-0.22)	(0.83-0.99)	0.95)	0.65)		(0.26-10.64)	
Peptide.11.variant..TRGILVV. ≥ 0 and HSP60E2 ≥ 0.444 and 443N2 ≥0.4025	1	36	3	41	0.03 (0-0.14)	0.93 (0.81-0.99)	0.25 (0.01-0.81)	0.53 (0.42-0.65)	0.394	0.38 (0.04-3.81)	0.5 (0.042-6.1)
Peptide 11 variant (ILVVAVEAGSPA) ≥0.4825	2	35	2	38	0.05 (0.01-0.18)	0.95 (0.83-0.99)	0.5 (0.07-0.93)	0.52 (0.4-0.64)	0.936	1.09 (0.15-8.13)	0.99 (0.11-9.3)
Peptide.11.variant..ILVVAVEAGSPA. ≥ 0 and HSP60E2 ≥ 0.444 and 443N2 ≥ 0.4025	1	36	3	41	0.03 (0-0.14)	0.93 (0.81-0.99)	0.25 (0.01-0.81)	0.53 (0.42-0.65)	0.394	0.38 (0.04-3.81)	0.5 (0.042-6.1)
Peptide 11 variant (ILVVAVEAGSP) ≥ 0.676	0	37	2	38	0 (0-0.14)	0.95 (0.83-0.99)	0 (0-0.91)	0.51 (0.39-0.62)	0.168	0 (0-NaN)	5.7e-08 (0-Inf)
Peptide.11.variant..ILVVAVEAGSP. ≥ 0 and HSP60E2 ≥0.444 and 443N2 ≥ 0.4025	1	36	3	41	0.03 (0-0.14)	0.93 (0.81-0.99)	0.25 (0.01-0.81)	0.53 (0.42-0.65)	0.394	0.38 (0.04-3.81)	0.5 (0.042-6.1)

[a] Specificity and Sensitivity: Range is 0-1, 95% confidence intervals in parenthesis; [b] Positive predictive value and negative predictive value: range 0-1, 95% confidence intervals in parenthesis, [c] The analysis also accounts for variables such as BMI and smoking status.

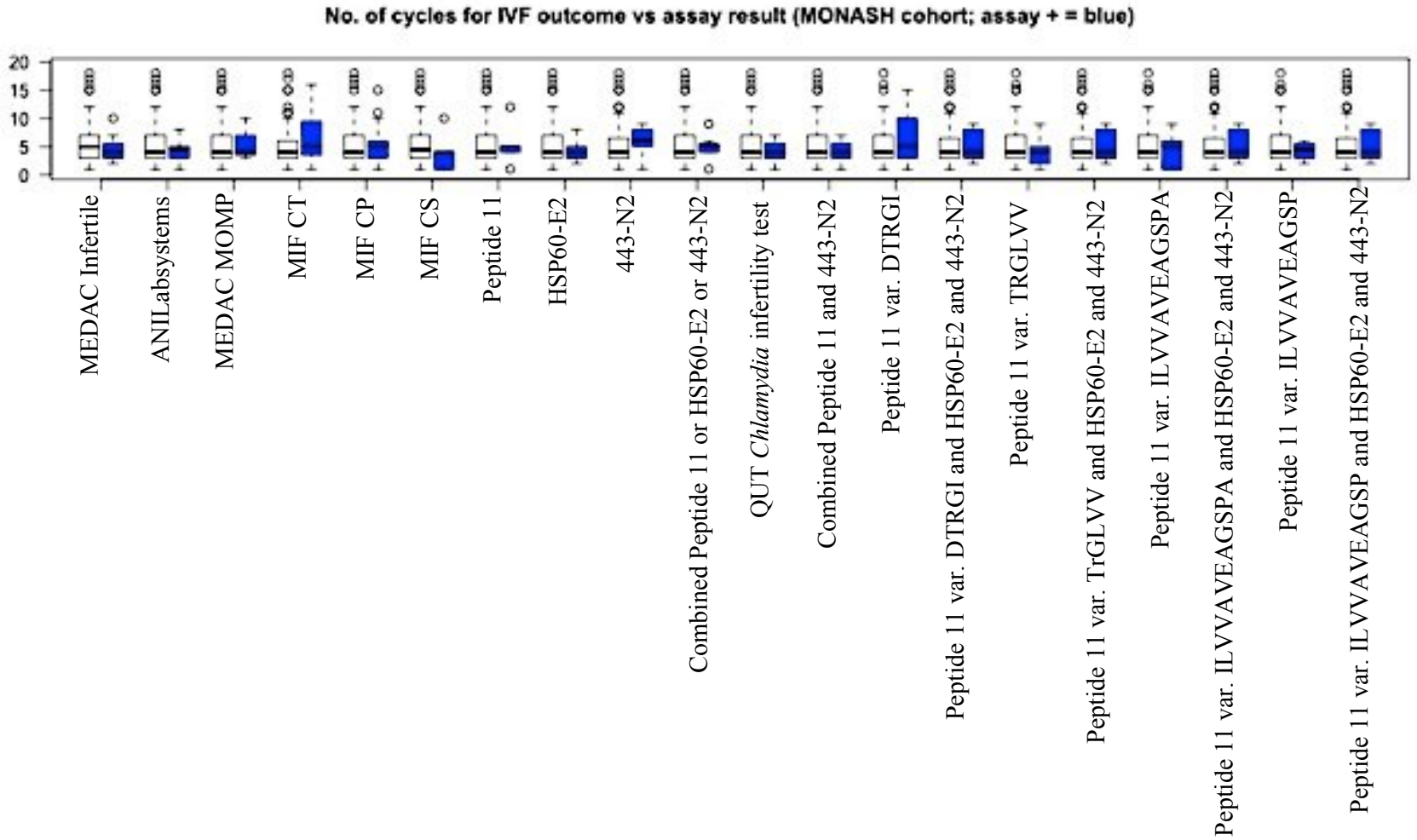

Figure 4.3: The association of number of cycles to achieve an IVF outcome in infertile women with *C. trachomatis* commercial and peptide ELISAs

Inter and intra assay variability

The inter and intra assay variability was estimated for QUT *Chlamydia* infertility test by assaying against 6 samples from each cohort; CT TFI cohort, CT negative infertile participants with no tubal pathology, participants with acute *C. trachomatis* infections, healthy fertile controls. The variability was measured across three replicates and over six days. Table 4.10 showed that the intra assay variability for peptide 11, HSP60-E2 and 443-N2 were 0.07, 0.069 and 0.074 respectively. The inter assay variability for peptide 11, HSP60-E2 and 443-N20 were 0.13, 0.139 and 0.136, respectively. Since the coefficient of variation of both inter and intra assay variability was less than 15%, the peptide ELISAs yielded reproducible results, therefore QUT *Chlamydia* infertility test that includes a combination of all three assays would be reproducible and robust.

Table 4.10: The inter and intra assay variability in 24 samples over the span of 6 days for peptide ELISAs that constitute QUT *Chlamydia* infertility test

Assay	Intra assay variability (n=24) (%CV)	Inter assay variability (%CV) (n=6)
Peptide 11	0.07	0.13
HSP60-E2	0.069	0.139
443-N2	0.074	0.136

4.3.3 Testing of QUT *Chlamydia* infertility test as a diagnostic test on infertile participants recruited from an IVF clinic (Validation cohort)

The QUT *Chlamydia* infertility test was tested on 73 infertile participants recruited from an IVF clinic (Brisbane, Australia). The diagnostic performance of the assay in identifying tubal pathology and *C. trachomatis*-related infertility was evaluated and compared against commercial serological assays.

QUT Chlamydia infertility test and commercial serological assays could not differentiate women with TFI from non-TFI infertile women in the validation cohort

The QUT *Chlamydia* infertility test was tested against another development cohort consisting of women recruited from another IVF clinic to validate its performance. The assay was tested against 19 infertile women with tubal pathology and 54 infertile women with no tubal pathology. Participant sera that tested equivocal for *C. trachomatis* infection in the commercial serological assays were excluded from the analyses. Among participants who tested equivocal, 2 participants were excluded from MEDAC Infertile and MEDAC MOMP, 3 participants from ANILabsystems and one participant from MEDACHSP60. Table 4.11 showed that while none of the commercial serological assays yielded significant differences between women with TFI and non-TFI women, MIF CT showed the highest sensitivity (26%) and positive predictive value (38%) compared to all other assays for identifying tubal pathology in women. Although, the MEDAC infertile assay and QUT *Chlamydia* infertility test showed significant difference in identifying women with *C. trachomatis*-related infertility from *C. trachomatis*-negative non-TFI women in the development cohort, the assays could not detect a single true positive in the validation cohort, but had high negative predictive value (72%). However, only peptide 11 showed a sensitivity of 11% and a specificity of 81% (not significant) in detecting *C. trachomatis*-related tubal factor infertility in women. The overall low diagnostic performance of serological assays in this cohort could be attributed to its limited sample size and that the tubal pathology in this cohort might not be a consequence of *C. trachomatis* infections.

Table 4.11: The validation of the diagnostic performance of QUT *Chlamydia* infertility test in identifying women with TFI (n=19) from infertile women not associated with TFI (n=54) in the validation cohort

	TFI positive (n=19)		**Non-TFI (n=54)**							
Assay	True positive	False negative	False positive	True negative	Sensitivity (95% ci)	Specificity (95% CI)	Positive predictive value (95% CI)	Negative predictive value (95% CI)	Odds Ratio (95% CI)	Chi Squared P value (unadjusted)
MEDAC Infertile	0	19	2	50	0 (0-0.25)	0.96 (0.87-1)	0 (0-0.91)	0.72 (0.6-0.83)	0 (0-NaN)	0.386
MEDACMOMP	1	18	3	49	0.05 (0-0.26)	0.94 (0.84-0.99)	0.25 (0.01-0.81)	0.73 (0.61-0.83)	0.91 (0.09-9.3)	0.935
MEDACHSP60	3	16	6	47	0.16 (0.03-0.4)	0.89 (0.77-0.96)	0.33 (0.07-0.7)	0.75 (0.62-0.85)	1.47 (0.33-6.57)	0.613
ANIlabsystems	3	15	6	45	0.17 (0.04-0.41)	0.88 (0.76-0.96)	0.33 (0.07-0.7)	0.75 (0.62-0.85)	1.5 (0.33-6.75)	0.595
MIF.CT	5	14	8	46	0.26 (0.09-0.51)	0.85 (0.73-0.93)	0.38 (0.14-0.68)	0.77 (0.64-0.87)	2.05 (0.58-7.29)	0.26
MIF.CP	9	10	28	26	0.47 (0.24-0.71)	0.48 (0.34-0.62)	0.24 (0.12-0.41)	0.72 (0.55-0.86)	0.84 (0.29-2.38)	0.737
MIF.CS	1	18	1	53	0.05 (0-0.26)	0.98 (0.9-1)	0.5 (0.01-0.99)	0.75 (0.63-0.84)	2.94 (0.17-49.54)	0.433

PEPTIDE11	2	17	10	44	0.11 (0.01-0.33)	0.81 (0.69-0.91)	0.17 (0.02-0.48)	0.72 (0.59-0.83)	0.52 (0.1-2.61)	0.419
HSP60E2	0	19	3	51	0 (0-0.25)	0.94 (0.85-0.99)	0 (0-0.81)	0.73 (0.61-0.83)	0 (0-NaN)	0.294
443N2	0	19	4	50	0 (0-0.25)	0.93 (0.82-0.98)	0 (0-0.72)	0.72 (0.6-0.83)	0 (0-NaN)	0.222
Double combination (either peptides)	0	19	6	48	0 (0-0.25)	0.89 (0.77-0.96)	0 (0-0.58)	0.72 (0.59-0.82)	0 (0-NaN)	0.129
QUT *Chlamydia* infertility test	0	19	6	48	0 (0-0.25)	0.89 (0.77-0.96)	0 (0-0.58)	0.72 (0.59-0.82)	0 (0-NaN)	0.129
Double combination (Peptide11 and 443N2)	0	19	6	48	0 (0-0.25)	0.89 (0.77-0.96)	0 (0-0.58)	0.72 (0.59-0.82)	0 (0-NaN)	0.129

QUT Chlamydia infertility test and commercial serological assays could not differentiate women with tubal pathology or unknown etiology from women with neither tubal pathology nor unknown etiology

The QUT *Chlamydia* infertility test was tested against women with tubal pathology or unknown etiology (n=42) against women with other forms of infertility (neither tubal nor unknown etiology) (n=31) (Table 4.12). Several participants were excluded from the study if they tested equivocal for *C. trachomatis* infection in the commercial serological assays. Therefore, only 29 participants were included in MEDAC Infertile, MEDACMOMP and ANILabsystems, while 30 participants were included in MEDACHSP60. While none of the commercial serological assays or *In-house* peptide ELISAs including QUT *Chlamydia* infertility test yielded significant difference in identifying women with either tubal pathology or unknown etiology, MEDAC cHSP60 had the highest sensitivity (17%) amongst commercial assays. 19% (8/42) of participants with tubal pathology/unknown etiology were positive in MIF, while only 2% (1/42) and 4% (2/42) of the participants were detected by MEDAC Infertile and QUT *Chlamydia* infertility assay (sensitivity =5%; specificity =87%).

Table 4.12: The validation of the diagnostic performance of QUT *Chlamydia* infertility test in identifying women with women with unknown etiology or tubal pathology (n=42) from infertile with no tubal pathology/unknown etiology (n=31) in the validation cohort

Assay	Unknown or Tubal pathology / assay +	Unknown or Tubal pathology / assay -	Pathology neither Tubal or Unknown / assay +	Pathology neither Tubal or Unknown / assay -	Sensitivity (95% CI)	Specificity (95% CI)	Positive predictive value (95% CI)	Negative predictive value (95% CI)	Odds Ratio (95% CI)	Chi Squared P value (unadjusted)
MEDACInfertile	1	41	1	28	0.02 (0-0.13)	0.97 (0.82-1)	0.5 (0.01-0.99)	0.41 (0.29-0.53)	0.68 (0.04-11.38)	0.789
MEDACMOMP	3	39	1	28	0.07 (0.01-0.19)	0.97 (0.82-1)	0.75 (0.19-0.99)	0.42 (0.3-0.54)	2.15 (0.21-21.8)	0.507
MEDACHSP60	7	35	2	28	0.17 (0.07-0.31)	0.93 (0.78-0.99)	0.78 (0.4-0.97)	0.44 (0.32-0.58)	2.8 (0.54-14.55)	0.206
ANILabsystems	6	34	3	26	0.15 (0.06-0.3)	0.9 (0.73-0.98)	0.67 (0.3-0.93)	0.43 (0.31-0.57)	1.53 (0.35-6.7)	0.571
MIF.CT	8	34	5	26	0.19 (0.09-0.34)	0.84 (0.66-0.95)	0.62 (0.32-0.86)	0.43 (0.31-0.57)	1.22 (0.36-4.18)	0.747
MIF.CP	21	21	16	15	0.5 (0.34-0.66)	0.48 (0.3-0.67)	0.57 (0.39-0.73)	0.42 (0.26-0.59)	0.94 (0.37-2.37)	0.892
MIF.CS	1	41	1	30	0.02 (0-0.13)	0.97 (0.83-1)	0.5 (0.01-0.99)	0.42 (0.31-0.55)	0.73 (0.04-12.17)	0.827
PEPTIDE11	6	36	6	25	0.14 (0.05-0.29)	0.81 (0.63-0.93)	0.5 (0.21-0.79)	0.41 (0.29-0.54)	0.69 (0.2-2.4)	0.564
HSP60E2	2	40	1	30	0.05 (0.01-0.16)	0.97 (0.83-1)	0.67 (0.09-0.99)	0.43 (0.31-0.55)	1.5 (0.13-17.33)	0.744
443N2	2	40	2	29	0.05 (0.01-0.16)	0.94 (0.79-0.99)	0.5 (0.07-0.93)	0.42 (0.3-0.55)	0.72 (0.1-5.45)	0.754
Double combination	3	39	3	28	0.07 (0.01-	0.9 (0.74-	0.5 (0.12-	0.42 (0.3-	0.72 (0.13-	0.697

(either peptides)					0.19)	0.98)	0.88)	0.54)	3.82)	
QUT *Chlamydia* infertility test	2	40	4	27	0.05 (0.01-0.16)	0.87 (0.7-0.96)	0.33 (0.04-0.78)	0.4 (0.28-0.53)	0.34 (0.06-1.97)	0.211
Double combination (Peptide11 and 443N2)	2	40	4	27	0.05 (0.01-0.16)	0.87 (0.7-0.96)	0.33 (0.04-0.78)	0.4 (0.28-0.53)	0.34 (0.06-1.97)	0.211

Concordance of QUT Chlamydia infertility test with commercial assays

The concordance between QUT *Chlamydia* infertility test and commercial serological assays in detecting tubal pathology were compared in this cohort (Table 4.13). MIF CT correlated with MOMP based serological assays, MEDAC Infertile and ANILabsystems ($p<0.001$). Although, QUT *Chlamydia* infertility showed no concordance with all commercial serological assays, it showed high concordance with MIF CS ($p<0.001$). As all the peptides in the QUT *Chlamydia* infertility assay are specific only to *C. trachomatis* (as confirmed by BLAST), the concordance to MIF CS could be due to possible cross-reactivity between *C. psittaci* and *C. trachomatis*.

Table 4.13: Concordance of QUT *Chlamydia* infertility test with commercial and In-house peptide ELISAs in the validation cohort

	MEDAC MOMP	MEDAC HSP60	ANILab	MIF.CT	MIF.CP	MIF.CS	PEPTIDE11	HSP60E2	443N2	Double combo (either)	QUT *Chlamydia* infertility test	Double combo: peptide 11 and 443N2
MEDACMOMP	<0.001	0.021	<0.001	<0.001	0.931	0.726	0.378	0.665	0.615	0.532	0.532	0.532
MEDACHSP60	0.021	<0.001	0.031	0.203	0.247	0.588	0.151	0.504	0.437	0.334	0.334	0.334
ANILab	<0.001	0.031	<0.001	<0.001	0.225	0.578	0.581	0.493	0.425	0.321	0.321	0.321
MIF.CT	<0.001	0.203	<0.001	<0.001	0.388	0.504	0.124	0.473	0.338	0.939	0.234	0.234
MIF.CP	0.931	0.247	0.225	0.388	<0.001	0.984	0.226	0.081	0.317	0.095	0.414	0.414
MIF.CS	0.726	0.588	0.578	0.504	0.984	<0.001	0.194	0.767	0.73	0.668	0.029	0.029
PEPTIDE11	0.378	0.151	0.581	0.124	0.226	0.194	<0.001	<0.001	0.062	0.001	0.001	0.001
HSP60E2	0.665	0.504	0.493	0.473	0.081	0.767	<0.001	<0.001	0.03	<0.001	0.106	0.106
443N2	0.615	0.437	0.425	0.338	0.317	0.73	0.062	0.03	<0.001	<0.001	<0.001	<0.001
Double combo. (Either peptides)	0.532	0.334	0.321	0.939	0.095	0.668	0.001	<0.001	<0.001	<0.001	<0.001	<0.001
QUT *Chlamydia* infertility test	0.532	0.334	0.321	0.234	0.414	0.029	0.001	0.106	<0.001	<0.001	<0.001	<0.001
Double combo: peptide 11 and 443N2	0.532	0.334	0.321	0.234	0.414	0.029	0.001	0.106	<0.001	<0.001	<0.001	<0.001

Women predicted to have C. trachomatis-related infertility in QUT Chlamydia infertility test is as likely to conceive with IVF as women with other forms of infertility

The specificity and sensitivity of QUT *Chlamydia* infertility test to predict IVF outcome was assessed in Table 4.14. A total of 63 infertile women who have undergone IVF were included in this study. Serum samples of participants that tested equivocal for *C trachomatis* infection in commercial were excluded in the study. Therefore only 61 participants were included in the MEDACMOMP and MEDAC Infertile analysis, while only 59 participants were included in the ANILabsystems analysis. The QUT *Chlamydia* infertility assay could not effectively predict the IVF outcome ($p>0.05$). MIF was the only serological assay that could effectively predict live birth outcome after an IVF cycle ($p=0.034$). Despite showing significant difference, MIF CT showed very low sensitivity and specificity. This shows that women who are positive for *C. trachomatis*-related infertility in QUT *Chlamydia* infertility test have equal likelihood of live birth or pregnancy via IVF as women with other forms of infertility. Since the women in this group were currently undergoing IVF, their final outcome is not yet known.

Table 4.14: The sensitivity and specificity of commercial serological assays and QUT *Chlamydia* infertility test to predict IVF outcome in infertile women (n=63) in the validation cohort

Assay	Live birth / assay +	Live birth / assay -	No live birth / assay +	No live birth / assay -	[a]Sensitivity (95% CI)	[a]Specificity (95% CI)	[b]Positive predictive value (95% CI)	[b]Negative predictive value (95% CI)	Odds Ratio (95% CI)	[c]Chi Squared P value (unadjuste)
MEDAC Infertile	0	30	2	30	0 (0-0.17)	0.94 (0.79-0.99)	0 (0-0.91)	0.5 (0.37-0.63)	0 (0-NaN)	0.16
MEDACMOMP	1	29	3	29	0.03 (0-0.17)	0.91 (0.75-0.98)	0.25 (0.01-0.81)	0.5 (0.37-0.63)	0.33 (0.03-3.4)	0.33
MEDACHSP60	2	29	5	27	0.06 (0.01-0.21)	0.84 (0.67-0.95)	0.29 (0.04-0.71)	0.48 (0.35-0.62)	0.37 (0.07-2.08)	0.24
ANILabystems	3	25	6	25	0.11 (0.02-0.28)	0.81 (0.63-0.93)	0.33 (0.07-0.7)	0.5 (0.36-0.64)	0.5 (0.11-2.22)	0.35
MIF.CT	3	28	10	22	0.1 (0.02-0.26)	0.69 (0.5-0.84)	0.23 (0.05-0.54)	0.44 (0.3-0.59)	0.24 (0.06-0.96)	0.03
MIF.CP	14	17	20	12	0.45 (0.27-0.64)	0.38 (0.21-0.56)	0.41 (0.25-0.59)	0.41 (0.24-0.61)	0.49 (0.18-1.35)	0.16
MIF.CS	1	30	1	31	0.03 (0-0.17)	0.97 (0.84-1)	0.5 (0.01-0.99)	0.51 (0.38-0.64)	1.03 (0.06-17.28)	0.98
PEPTIDE11	7	24	4	28	0.23 (0.1-0.41)	0.88 (0.71-0.96)	0.64 (0.31-0.89)	0.54 (0.39-0.68)	2.04 (0.53-7.83)	0.29
HSP60-E2	2	29	1	31	0.06 (0.01-0.21)	0.97 (0.84-1)	0.67 (0.09-0.99)	0.52 (0.38-0.65)	2.14 (0.18-24.86)	0.53
443-N2	3	28	0	32	0.1 (0.02-0.26)	1 (0.84-1)	1 (0.19-1)	0.53 (0.4-0.66)	Inf (NaN-Inf)	0.07

Double combo (either peptides)	4	27	1	31	0.13 (0.04-0.3)	0.97 (0.84-1)	0.8 (0.28-0.99)	0.53 (0.4-0.67)	4.59 (0.48-43.63)	0.15
QUT *Chlamydia* infertility test	4	27	1	31	0.13 (0.04-0.3)	0.97 (0.84-1)	0.8 (0.28-0.99)	0.53 (0.4-0.67)	4.59 (0.48-43.63)	0.15
Double combo assay (peptide 11 and 443N2)	4	27	1	31	0.13 (0.04-0.3)	0.97 (0.84-1)	0.8 (0.28-0.99)	0.53 (0.4-0.67)	4.59 (0.48-43.63)	0.15

[a] Specificity and Sensitivity: Range is 0-1, 95% confidence intervals in parenthesis; [b] Positive predictive value and negative predictive value: range 0-1, 95% confidence intervals in parenthesis, [c] The analysis also accounts for variables such as BMI and smoking status.

4.4 DISCUSSION

The QUT *Chlamydia* infertility test exhibited superior specificity and sensitivity compared to the current commercially available serological assays in diagnosing women with tubal pathology and *C. trachomatis*-related tubal infertility. The assay was developed based on the peptides that were identified in Stansfield *et al.* [411]. The peptides included Peptide 11, HSP60-E2 and 443-N2 that were derived from a wide range of antigens that were also known to be immunodominant in women with *C. trachomatis*-related tubal infertility. The initial proof of concept peptide ELISA based on peptide 11 had a specificity of 95% and a sensitivity of 47% in identifying women with *C. trachomatis*-related infertility [411]. In order to improve the sensitivity of the peptide 11 ELISA, a novel peptide ELISA was designed by combining peptide 11 with other peptides. Like peptide 11, they were specific only to *C. trachomatis* and did not show cross-reactivity with other chlamydial species (by BLAST).

A range of methods and conditions were evaluated on a small- scale assay to triage the conditions for the large-scale tests. While all peptide assays including individual and combination assay formats identified women with *C. trachomatis* related infertility ($p<0.01$), peptide combinations in multi-well format were selected for assay design due to its superior diagnostic performance (sensitivity=100%; specificity =78%-89%).

In this study, the definition of tubal infertility and inclusion (unilateral or bilateral tubal occlusion) and exclusion criteria in recruiting participants for development and validation cohort are consistent with previous studies [362, 377, 410]. The cohort definitions and experiment design applied in this study is similar to that of Bax *et al.* [377], who also evaluated the performance of two commercial ELISAs against MIF in identifying participants with tubal pathology in women with infertility (confirmed by laparoscopy). This was followed by evaluation of diagnostic performance of the commercial ELISAs in identifying *C. trachomatis*-related infertility in participants with MIF as the gold standard [377]. The study reported that MEDACMOMP ELISA had high specificity and could potentially be good alternative to MIF.

For large scale-evaluation of the diagnostic performance of peptide assays, the target groups included infertile women with tubal pathology (confirmed by laparoscopy) (evaluation 1) and women with *C. trachomatis*-related infertility (evaluation 2). Since MIF was considered the gold standard and used widely in CAT [374, 400, 404], it was used to define the infection status of the cohorts. The group included women with *C. trachomatis*- related TFI and the negative cohort included non-TFI infertile women seronegative and seropositive for *C. trachomatis* infections by MIF CT. Therefore, the assays were developed to be specific only to women with tubal infertility, and who were seropositive for *C. trachomatis*.

The absorbance threshold and the corresponding diagnostic performance were evaluated on women with CT TFI (n=11) and negative cohort (infertile seronegative for *C. trachomatis* infection and tubal pathology; n=80) (Table 4.2). The absorbance threshold that yielded highest sensitivity and specificity in both individual and peptide combination assays were applied in evaluation 1 and evaluation 2. In evaluation 1, the ability of peptide assays to identify women with tubal pathology (n=45) from infertile women without tubal pathology (n=47) was determined. The diagnostic performances of peptide assay formats (individual and combination) were compared commercial serological assays, including MIF CT. Although MIF CT had the highest sensitivity (25%) in identifying tubal infertility in women as compared to commercial and *In-house* peptide ELISAs, its specificity was the lowest (89%) (Table 4.3). This is consistent with findings of Dabekausen *et al.* [403] that showed that participants with TFI had a 9.1 times likelihood of testing positive in MIF than participants without TFI. The low sensitivity of MIF suggests that *C. trachomatis* infections may not account for all tubal pathology in women in this cohort. Therefore, overall sensitivity would be lower for all assays in this cohort. Additionally, the assay usually detects women with past CT infections regardless of infertility, thus compromising its specificity. Compared to commercial serological assays and peptide ELISAs, only double combination of peptides (peptide 11 and 443-N2) and triple combination of peptides (peptide 11, Hsp60-E2 and 443-N2) were able to significantly differentiate women with TFI from infertile women with non-TFI (p=0.004). In addition, the sensitivity (16%) and specificity (100%) of the peptide combination assays were higher than MEDAC infertile (sensitivity=11%; specificity =96%) and ANILabsystems IgG ELISA (sensitivity=14%; specificity=94%) in identifying

women with tubal pathology. BMI and smoking status have been implicated in female infertility in several studies [222-224]. Studies have shown a positive correlation of smoking and BMI, and their compounded effects on female infertility [222]. Thus, the odds ratio was adjusted for factors such as BMI and smoking status, which through step-wise logistic regression analysis were found to be associated with tubal infertility.

While the diagnostic performance of peptide ELISAs in identifying tubal pathology was evaluated in Evaluation 1, the diagnostic performance of the assays in identifying *C. trachomatis*-related infertility in women were evaluated in evaluation 2 (Table 4.4). MEDAC infertile assay and the peptide combination assays were the only serological assays that could significantly differentiate women with *C. trachomatis*-related infertility (CT TFI cohort) (n=11) from negative cohort (CT negative women without tubal infertility, women with acute infections, fertile women) (n=251) ($p<0.001$). MEDAC Infertile assay had the highest sensitivity (36%) in identifying women with *C. trachomatis*-related TFI compared to commercial serological assays. All three peptide combination assays had a sensitivity of (27%) and specificity (94%). Based on the evaluation 1 and evaluation 2, the triple combination assay (peptide 11, HSP60-E2 and 443-N2) showed the highest sensitivity (27%) and specificity (94%) and hence chosen as the QUT *Chlamydia* infertility test. Although the sensitivity of QUT *Chlamydia* infertility test was lower than MEDAC infertile assay, the positive predictive value of the assay was (18%-19%) were higher, which suggests that 19% women who tested positive in the QUT *Chlamydia* infertility test were true positive for *C. trachomatis*-related TFI as compared to 10% women who tested positive in MEDAC infertile assay. Thus, it could detect a higher proportion of participants with TFI that were missed by MEDAC Infertile assay. Additionally, the high specificity and negative predictive value (NPV) of the assay would ensure low false positive rate. The high NPV (96%-97%) obtained in all ELISAs in the study is consistent with previous reports, which report a NPV of 85%-90% for CAT [362, 410, 437]. Therefore, these characteristics make QUT *Chlamydia* infertility test a suitable diagnostic for detection of *C. trachomatis* tubal factor infertility and IVF could be recommended without any additional diagnosis for women positive in the assay.

This superior diagnostic performance of QUT *Chlamydia* infertility test as compared to commercial serological assays suggests that combined peptides from

cytosolic proteins HSP60, outer membrane protein CT443 and a periplasmic protein HtrA showed greater antibody reactivity to women with *C. trachomatis*-related TFI than traditional protein antigens such as MOMP. Multi-well formats have been evaluated in the development of peptide ELISA combining CT226, CT795 and CT694, wherein triple combination of peptides yielded higher sensitivity but the specificity was compromised when compared to double combinations [389]. Bas *et al.* [367] also showed that the combination of HSP60 and PGP3 improved the sensitivity (76%) and specificity (77%) of the assay to detect acute *C. trachomatis* infections as compared to HSP60 (sensitivity =62%; specificity =80%) and PGP3 (sensitivity=53%; specificity =80%) used alone. Genome wide identification of antigens identified HSP60 and CT443 to be preferentially recognized by TFI participants (n=33), and reported that proteins in combination yielded higher sensitivity than when used alone in identifying women with tubal infertility compared to non-TFI infertile controls (n=23) [385]. Additionally, Budrys *et al.* [387] reported that peptide fragments of CT443 had better diagnostic value in identifying tubal infertility in women than full-length CT443 proteins. This shows that epitopes on peptides are show greater antibody reactivity than epitopes in full- length proteins. This highlights the antigenicity of the linear B-cell epitopes identified through bioinformatics analysis. Linear B-cell epitopes have been previously identified in MOMP [429, 439], which identified $MOMP_{377\text{-}386}$ (TRLIDERAAH) to be immunogenic and a potential vaccine and a diagnostic candidate [429].

Several variants of peptide 11 were designed that were selected in order to improve its solubility. The diagnostic performance of peptide variants in identifying women with tubal pathology and *C. trachomatis*-related infertility were assessed. Some peptide variants were detected to have slightly improved performance than peptide 11 in detecting tubal pathology in women (Table 4.5). However, the diagnostic performance did not differ between peptide 11 and its variants when *C. trachomatis*-related infertility was considered the positive group (Table 4.6).

It was interesting to note that although MEDAC Infertile and QUT *Chlamydia* infertility test were able to identify *C. trachomatis*-related TFI in women with high sensitivity and specificity, however, there was no concordance between the two assays ($p>0.05$) (Table 4.7). All commercial serological assays had significant concordance with MIF CS and MIF CP. This suggests that the commercial serological

assays have a high rate of cross-reactivity between *C. pneumoniae* and *C. psittaci*. Land *et al.* [410] reported that with the exception of MEDAC MOMP all serological assays such as MIF, ANILabsystems showed significant cross-reactivity with *C. pneumoniae* in 315 subfertile women. *C. pneumoniae* has not been reported to contribute to tubal pathology [440], but high prevalence of serological positive people the population are thought to lead to false positive results in MIF CT [373]. Contrary to commercial serological assays, individual peptide ELISAs did not correlate with MIF CP and MIF CS, thus highlighting the specificity of the peptides to *C. trachomatis* alone. However, peptide combination assays including double combination of either peptides (p=0.002), double combination of peptide 11 and 443-N2 (p=0.041), triple combination (p=0.032) showed high correlation with MIF CS. Since the peptide combination assays were controlled for high specificity, it resulted in a higher rate of true negatives while fewer true positives were identified. The low prevalence of MIF CS indicated a high rate of true negatives, which accounts for the correlation in the diagnostic performance between both MIF CS and the peptide combination assays.

The participants in the development cohort included women who underwent treatment at IVF clinics. The factors associated with IVF outcome was evaluated in Table 4.8. Amongst other factors such as previous history of live birth and pregnancy in previous IVF treatments, tubal pathology in women significantly correlated with IVF outcome (p=0.032). Infertile women without tubal pathology were 3.9 times more likely to have a successful IVF outcome (pregnancy/ live birth) compared to women with tubal pathology. The analysis was adjusted for variables such as BMI and smoking status, and the increase in the adjusted odds ratio suggest that one of these variables may also be a confounder to IVF outcome prediction. Since women with tubal infertility are more likely to have a successful IVF outcome, the likelihood of women predicted to have *C. trachomatis*-related infertility by QUT *Chlamydia* infertility test in conceiving via IVF as women with other forms of infertility were represented in Table 4.9.

With the exception of ANILabsystems, none of the serological assays including QUT *Chlamydia* infertility test were significantly different based on the IVF outcome or the number of cycles to achieve it in infertile women (Table 4.9). This could be due to the influence of confounding factors such as BMI and smoking status. Consistent

with the findings in this study, Tasdemir *et al.* [441] evaluated 51 couples undergoing IVF and embryo transfer treatment and reported that presence of *C. trachomatis* antibodies did not alter the success rates of IVF-ET and achieved a pregnancy rate of 32.7% as compared to participants seronegative for *C. trachomatis* (17.4%). Keltz *et al.* [185] also demonstrated that per-cycle pregnancy rate did not differ significantly in CT positive and CT negative infertile participants (n=1279). The study also showed that while pregnancy rates were lower in women with *C. trachomatis* induced infertility; it did not affect the IVF outcome. Among tubal factor infertility participants, cHSP60 seropositive participants had greater pregnancy rate than cHSP60 seronegative participants after oocyte pick-up (OR = 8.9, 95% CI = 2.3 to 27.5) (n=195) [442]. Thus, it could be inferred that amongst all assisted reproductive technologies, IVF is most ideal and effective treatment strategy for women with *C. trachomatis*-related tubal pathology. Therefore, women positive in QUT *Chlamydia* infertility could proceed directly to IVF without undergoing additional investigations.

The reproducibility and robustness of QUT *Chlamydia* infertility test was validated by measuring inter and intra assay variability (Table 4.10). The coefficient of variation (%CV) was less than 15% in both assays; therefore QUT *Chlamydia* infertility test was robust and reproducible.

The diagnostic performance of the assay was validated on another cohort comprising of women retrospectively and prospectively recruited from a separate IVF clinic (n=73) (Table 4.11). Contrary to the development cohort, none of the serological assays including QUT *Chlamydia* infertility test effectively identified women with tubal pathology (n=19) ($p>0.05$). MIF CT had low sensitivity and specificity of 25% and 85% respectively. The inability of serological assays to detect tubal pathology could be due to the low prevalence of *C. trachomatis*-induced tubal pathology in the group. Group definitions may be the major factor influencing these results. Another explanation could be that this clinic may represent a distinct demographic of patients from the first, with some other aetiology being more commonly responsible for tubal fertility.

Keltz *et al.* [185] reported that women with tubal damage confirmed by laparoscopy and hysterosalpinography who are seropositive for *C. trachomatis* were less likely to conceive without IVF compared to women who were seronegative for the infection. The study also showed CAT positive women had significantly more

tubal damage on HSG (37.5% vs 10.1%; p=0.001) and laparoscopically confirmed tubal damage (85.7% vs 48.9%; p=0.002) than CAT negative participants. Therefore, HSG and laparoscopy may not detect all the tubal damage associated with *C. trachomatis* infection. Although not statistically significant, by including infertile participants with unknown etiology, the sensitivity was higher with QUT *Chlamydia* infertility test as well as other serological assays (Table 4.12). Hence, in this study women with unknown infertility were also included, so as to determine if their infertility was associated with *C. trachomatis* infections. However, MIF CT showed reduced sensitivity (19%) but higher specificity in this cohort compared to women with tubal pathology alone (not significant). This suggests that MIF CT is more likely to detect tubal pathology than any other forms of infertility. The concordance between commercial assays and QUT *Chlamydia* infertility test in identifying tubal pathology was also assessed (Table 4.13). Similar to the development cohort results, the commercial assays showed great correlation with each other and not with QUT *Chlamydia* infertility assay and other peptide ELISAs. However, contrary to the development cohort results, the commercial assays did not show cross-reactivity with other species, while QUT *Chlamydia* infertility test showed concordance with MIF CS. Since very low number of positives were detected by QUT *Chlamydia* infertility test in this group as compared to commercial assays (such as ANILabsystems and MIF CT), it is likely that of the two samples detected, one sample could have shown cross-reactivity with *C. psittaci*. However, since the prevalence of *C. trachomatis* – induced TFI has been estimated to be low in this cohort, a larger sample size would be needed to effectively independently validate the performance of QUT *Chlamydia* infertility test. The prevalence rate of *C. trachomatis*-related TFI estimated by MIF in the development cohort and validation cohort was 11.95% and 7.04% respectively, which was similar to previous literature (5.5% (n=1279)[185], 20.8% (n=1482) [361]). However, high prevalence rate has also been reported by Bax *et al.* [377] of 31.6% in 76 women with subfertility. Similar to the development cohort, the QUT *Chlamydia* infertility test could not predict the IVF outcome (Table 4.14). Since most of the women in this cohort have only begun their IVF treatment, their pregnancy outcomes are still unknown. Therefore, the analysis may not be relevant.

QUT *Chlamydia* infertility test has demonstrated a high specificity in identifying women with tubal pathology (100%) and *C. trachomatis*-related infertility

(94%). While, the assay significantly reduces the rate of false positives, making it an ideal early infertility investigative tool in fertility clinics, its sensitivity has been limited to 16% and 27% respectively. However, the low sensitivity means that a negative result in this test does not exclude the possibility of chlamydial tubal infertility. In addition, the multi peptide ELISA outperformed current commercial serological assays in identifying women with tubal pathology and chlamydial infertility. Despite the lack of validation in a separate IVF clinic, QUT *Chlamydia* infertility is highly promising and could be developed into a specific test for chlamydial infertility in women. The assay validation could be further improved by considerably increasing the sample size of target cohort, in order to evaluate and enhance its diagnostic ability.

The highly specific QUT *Chlamydia* test can be applied through a two-step diagnostic pathway that could be routinely implemented during the infertility investigation of women presenting at the fertility clinic setting. Firstly, a MIF positive result could be used to indicate if further tubal investigation is needed or possible non-detectable tubal damage is present. If other infertility aetiologies were absent in the individual, then IVF would be recommended. Secondly, a highly specific ELISA, such as the multi-peptide test developed here, could be used to identify women who (if positive) are likely to have chlamydial tubal damage that might be best recommended to progress directly to IVF treatment. Since the study demonstrated equivalent IVF success rates from both fertility clinic cohorts for women who were positive in the serological tests compared to women who were negative. Thus, IVF treatment is a valid recommendation in the context of these serological results.

Chapter 5: CXCL10, CXCL11 and IL-1β are induced in mononuclear cells from women with *C. trachomatis* related infertility

5.1 INTRODUCTION

The progression of *C. trachomatis* infection from the lower genital tract to upper genital tract and its role in female infertility has been investigated using various *in vitro* [443-445], *ex vivo* [446-450], and mouse models [451-455]. These studies indicate that a pro-inflammatory response from the local tissue is likely a major contributor to the pathological outcome in some cases, while in others it has been associated with resolution of the infection.

In vitro studies have shown that infection with *C. trachomatis* in HeLa cells induced the expression of genes that regulate innate immunity [443] and Interleukin-8 (IL-8) cytokine [444]. Buchholz *et al.* [444] further confirmed that IL-8 was produced as a result of bacterial protein synthesis at 15 hours post infection and transcription of IL-8 responses such AP-1, NFL6 and NFκB were also up regulated. Thus, the study illustrates that in *in vitro,* one of the early immune responses associated with *C. trachomatis* infection are IL-8 production and the genes associated with its transcription. Zhou *et al.* [445] further illustrated the signalling pathways regulating key pro-inflammatory immune genes that are associated with chlamydial pathology. The study showed that infection with a *C. trachomatis* pORF5 protein (secreted protein by pORF5 plasmid) in HeLa cells, induced the expression of genes such TNF-α, IL-1β and IL-8 through p38/mitogen activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK)/MAP signaling pathways. In addition to IL-8, pro-inflammatory cytokine IL-6 was reported to be upregulated in a HeLa/THP-1 co-culture model, and using a multiplexed cytometric bead array assay, the study reported a downregulation of IL-10, IL-12p70, and TNF-α [280].

Consistent with the findings in *in vitro* models, among *ex vivo* studies, infection with *C. trachomatis* serovar D on primary cells isolated from human fallopian tube showed that under hypoxic condition, IFN-γ facilitated the development of persistent infections in the upper genital tract [447]. While the study reports the role of pro-inflammatory cytokine in *C. trachomatis*-related tubal pathology, it also highlights the likely role of environmental conditions such as oxygen supply in reducing clearance of bacteria and development of pathology in the upper genital tract. Contrary to these findings, *ex-vivo* stimulation of PBMC and endometrial tissue of women with *C. trachomatis* showed an accumulation of IL-4 [449] and a polarization towards Type 2

immunity [448]. This was supported by genome-wide microarray analysis, which also revealed up-regulation of genes such as matrix metalloproteinase 10, GATA-3 (transcription factor regulating Th2 differentiation), IL-4 as compared to IL-17 and IFN-γ [448]. Several studies have elucidated the role of innate immunity in *C. trachomatis* –related pathology in the upper genital tract. Immumohistochemistry (IHC) on fallopian tubes of women with ectopic pregnancy (n=50) revealed expression of IL-6, IL-8 and its receptor CXCR1 [456]. Shaw *et al.* [267] showed an increased expression of prokineticin (PROK1) and its receptor PROKR1, a molecule that controls smooth muscle contractility and genes associated with intrauterine implantation, in fallopian tubes of women with a history of *C. trachomatis* infection and ectopic pregnancy (n=14). The study further identified higher expressions of TLR2 and TLR4 in the fallopian tubes by IHC, and cell culture models further supported these results and showed that in addition to the innate molecules, NFκB was activated on *C. trachomatis* infection [267]. Higher expression of TLR2 and TLR4 were also evident in cervical monocytes obtained from women with fertility disorders who are positive for *C. trachomatis* infection (n=57) [263]. Interestingly, cytokine analysis of laparoscopic specimens of fallopian tube and cervical secretions of infertile women (n=37) revealed that the secretion of cytokines varied between different sites [285]. Most cytokines including IL-6, IL-2 and IL-12 levels were significantly much lower in the fallopian tubes of *C. trachomatis* positive infertile women compared to infertile women negative for *C. trachomatis* infection ($p<0.05$) [285]. However, in the cervical secretions, the level of IFN-γ, IL-10 and IL-12 were significantly higher in infertile women with *C. trachomatis* infection compared to infertile women negative for the infection ($p <0.05$) [285]. The presence of IL-10 and IFN-γ in the cervical secretions of infertile participants indicates that genital mucosa produces both Th1/Th2 responses during *C. trachomatis* infection.

The *C. muridarum* mouse model can replicate most pathologies associated with *C. trachomatis* in women, including ascension of infection and subsequent oviduct scarring, hydrosalpinx and infertility; hence it is considered a good experimental model for *C. trachomatis* [457]. Khamesipour *et al.* [450] illustrated that inoculation of *C. trachomatis* MoPn biovar in the ovarian bursa of C3H/ HeN and C57BL/6 mice resulted in salpingitis in both mice which correlated with high antibody response to cHSP60. The infection adversely affected the pregnancy outcome, with significantly

lower pregnancy rates in infected mice as compared to the control ($p < 0.05$) (8% of C3H and 25% of C57BL/6). Murine models have shown that infection with *C. trachomatis* activates the development of $CD8^+$T memory cells that elicit protection against pathology on secondary infection [453]. Gondek *et al*. [458] reported that secondary *C. trachomatis* infections in upper genital pathology induced by transcervical inoculation resulted in a 100-fold level of protection by $CD4^+$T cells against the infection as compared to $CD4^+$T cell naïve control. However, *C. muriadarum* immune mice that had undergone $CD4^+$T cells depletion before secondary challenge did not exhibit any protective effect against the infection. Thus, this shows that, in addition to $CD8^+$ T cells, $CD4^+$T cell-specific cells skewed towards Th1 immunity elicit protective immunity in the upper genital tract against re-infection. However, CD8 T-cell clones derived from infected mice showed that it contributed to immunopathology through production of IL-10, TNF-α and IL-13, which revived replication of *C. muridarum* in epithelial cells and subsequent scarring of tissues [457]. Nagarajan *et al.* [446] reported that infection of mouse macrophages and fibroblasts with *C. trachomatis* MoPn resulted in the production of IFN-β and IP-10 (chemokine involved in T cell recruitment) in cells deficient in Toll like receptors, TLR2 and TLR4. The study showed that the production of the pro-inflammatory cytokines is not dependent on the TLRs, rather it adopts the MyD88 singalling pathway. Thus, it gives an insight into the innate immune markers associated with chlamydial pathology. Therefore, these studies indicate that a pro-inflammatory response from the local tissue is likely a major contributor to the pathological outcome.

Several studies have identified immune factors expressed from peripheral blood mononuclear cells from *Chlamydia* infected or infertile participants. The stimulation of PBMC from infertile women (n=133) with chlamydial 60kDa heat shock protein yielded higher production of IFN-γ, IL-10 and IL-12 cytokines, while stimulation with EB antigen induced a weaker production of IL-10 cytokine [270]. This suggests that cHP60 plays an important role regulating Th1-Th2 balance and in the immunopathogenesis of chlamydial infection in the upper genital tract. *In vitro* lymphocyte proliferation of PBMC with *C. trachomatis* 57-kDa HSP showed that the proportion of women with salpingitis (n=18) who exhibited antigen mediated lymphocyte proliferation were significantly higher than women with cervicitis (n=10),

recurrent miscarriages (n=5) and healthy fertile women (n=45) ($p<0.001$) [459] . This shows that C. trachomatis induces tubal pathology in the upper genital tract. Cytokine assay on PBMCs stimulated with cHSP60 revealed that the proportion of IFN-γ to IL-10 was significantly lower in women with PID and history of *C. trachomatis* infection (n=9; 0.006, $P<0.01$)) [273]. Thus, cHSP60 elicits a Th2 immune response in women with *C. trachomatis*- related PID. Similarly, stimulation of PBMCs from women with tubal factor infertility and a history of *C. trachomatis* infection (n=4) with chlamydial antigens CtTSp (*Chlamydia trachomatis* tail-specific protease) and CtHtrA showed higher production IL-6 and IL-10 [278]. In addition to cytokine and gene expression analysis, the influence of host gene polymorphisms were elucidated in Ohman *et al.* [249]. The study reported that infertile women (n=34) with IL-10-0182 and IFNG+874 SNPs showed an abundance of IL-10 secretion in the PBMC compared to IFN-γ on infection with *C. trachomatis*. Thus, host gene polymorphisms in IFN-γ and IL-10 genes affect the susceptibility to TFI by influencing the cytokine profile and immune response to *C. trachomatis* infection. The PBMC studies reveal that both Th1 and Th2 mediated immune responses are associated with *C. trachomatis*-related infertility in women.

Diagnosis of women with chlamydial infertility is typically conducted using surgical or sonographic investigation for fallopian tubal blockage [460]. However, even in the absence of apparent tubal occlusion women who are seropositive for *C. trachomatis* are 50% less likely to conceive other than by IVF treatment [185]. However, serology, particularly the gold standard microimmunofluorescence, is not specific enough to be used as a diagnostic to proceed directly to IVF treatment [2]. It is estimated that 5.5% of IVF patients have chlamydial infertility (due to positive *Chlamydia* serology) in the USA [185]. However, in spite of the prevalence of this condition we still only have limited understanding of the underlying disease mechanism that results in pathology.

In this study, gene expression of 88 innate and adaptive genes (excluding 5 house-keeping genes) and 10 secreted cytokines were measured from PBMC from infertile female participants who are undergoing or had recently undergone IVF treatment were isolated and cultured *ex vivo* in the presence of *C. trachomatis.* The immune response was measured in order to help understand the possible disease processes that have occurred from women with a history of *C. trachomatis* infection.

5.2 MATERIALS AND METHODS

5.2.1 Patient recruitment

Whole blood and sera was collected from fully consented voluntary participants attending an IVF hospital (Brisbane, Australia). 31 women with infertility (requiring IVF treatment with multiple etiologies) (UC Health Ethics approval 1314, QUT Human Research Ethics approval 1300000505) participated in the study. The bloods were processed and the PBMC were isolated the same day it was collected. The sera were stored at -80°C. *Chlamydia* Microimmunofluorescence (MIF) IgG (Focus Diagnostics, USA) assay (described in detail in section 3.4.4.3.) was conducted on the participant sera to determine the *C. trachomatis* infection history status.

5.2.2 *C. trachomatis* strain D and F strain culture

C. trachomatis D (ATCC VR-885) and F strain (ATCC VR-346) were cultured in McCoy cells. Confluent cells were infected with the strains and incubated at 37°C for 44 hours. Following infection, the strains were semi-purified (described in section 3.3.1.). To prepare a mixture of *C. trachomatis* D and F strains, the cultures were mixed together (equal ratio of EBs) and purified using density gradient centrifugation (29% v/v urografin Ultravist® (Bayer, USA)) (described in detail in Materials and methods 3.3.2.), and stored in sucrose phosphate buffer at -80°C.

5.2.3 PBMC proliferation and stimulation

Peripheral blood mononuclear cells (PBMC) were isolated and purified as described in the section 3.6. They were cultured in 48-well cell culture plates at a $1x10^6$ cells/well. The PBMCs were stimulated at a MOI of 5 with 4 antigens; purified *C. trachomatis* serovar F (ATCC VR-346), purified *C. trachomatis* D and F mix (50:50 elementary bodies (EBs)), phytohemaglutinin (PHA) (positive control) and media (negative control). The cells were incubated for 15 hours at 37°C. After incubation, the cells were centrifuged at 800 ×g, and the supernatant was collected. The pelleted cells were resuspended in 1mL of RNA cell protect® reagent (Qiagen, Victoria, Australia) for gene expression analysis. The plates are placed in -80°C until further use.

6.1.1. History of *C. trachomatis* infection

The presence of past *C. trachomatis* infection in women with TFI was determined using microimmunofluorescence (MIF) (Focus Diagnostics, USA) on patient sera. Those patients that were rendered positive by MIF were considered as *C. trachomatis*-positive. The method has been detailed in section 3.4.4.3.

6.1.2. Expression of innate and adaptive immune genes using RT -qPCR array

The expressions of innate and adaptive immune genes that are associated with *C. trachomatis*-related infertility in women were analysed using the Qiagen RT^2 Profiler PCR array.

5.2.4 RNA isolation from PBMC

The RNA from PBMC was extracted using the RNeasy micro kit (Qiagen, Victoria, Australia). The cell protectant agent was removed from the cells by centrifuging at 3000 ×g for 5 minutes at 4°C. The cells were resuspended in 350µL of RLT lysis buffer and the RNA was extracted as per manufacturer's instructions. The concentration and purity of RNA was determined by measuring the absorbance at 260nm and 280 nm using a NanoDrop spectrophotometer (Thermofischer, USA). The absorbance ratios of $A_{260}/_{280}$ >2 and $A_{230}/_{260}$ >1.2 were indicative of pure RNA. An on-column Dnase (Qiagen, Australia) digestion for 10 minutes at room temperature was conducted to remove genomic DNA. The RNA was eluted into nuclease-free water and stored at -80°C until use.

5.2.5 cDNA synthesis

The total RNA was reverse-transcribed to cDNA using the Qiagen First Strand synthesis (Qiagen, Victoria, Australia). The kit includes a genomic DNA elimination buffer, random hexamers and oligo dT primers for reverse transcription. The RNA was added to the reverse transcription mix, and incubated 42°C for 15 minutes followed by 95°C for 5 minutes. 6µL of the resulting mixture (1:10 dilution) was used directly in the RT-PCR profiler array.

5.2.6 Expression of genes using RT-PCR profiler array

The expression of innate and adaptive immune genes were determined using Qiagen RT^2 Profiler PCR arrays which contains preset primers for 88 genes. The expression levels of all genes were normalized to 5 reference genes (ACTB, B2M,

GAPDH, HPRT1, RPLP0) as per manufacturer's instructions. The RT-PCR profiler array involves a combination of real-time PCR performance and ability of microarrays to detect multiple gene expressions. The kit includes a RT^2 SYBR Green mastermix and the 384-well plates are embedded with several controls such as Genomic DNA control, Reverse transcription and positive PCR controls. The arrays were run on the Applied Biosystems ABI7900HT PCR systems. The reaction was initiated at 95°C for 10 minutes, followed by 40 cycles of 15s at 95°C and 1 minute at 60°C. The fold change between *C. trachomatis*-positive infertile women and *C. trachomatis*-negative infertile women were determined using $2^{-\Delta\Delta Ct}$ method [461].

5.2.7 Cytokine ELISAs

The level of IFN-γ, IL-12, IL-1B, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17 and TNF-α were measured from the supernatants using cytokine ELISA kits (Elisakit.com, Melbourne, Australia) as per manufacturer's instructions.

5.3 RESULTS

5.3.1 Classification of women into cohorts based on *C. trachomatis* serology and gynecological history

Participants were classified as *C. trachomatis*-positive infertile women using *Chlamydia* MIF IgG assay (four out of 31 were MIF positive and considered positive for *C. trachomatis*-related infertility). All participants were women undergoing or recently had IVF treatment for infertility (Table 5.1). The type of infertility was diagnosed using laparoscopy and the participants were characterized as positive for tubal infertility if they had tubal occlusion, damage or salpingitis. The participants who were seronegative to *C. trachomatis* infection were characterized as *C. trachomatis*-negative (n=27). The innate and adaptive immune responses associated with chlamydial infertility was analysed using cytokine ELISAs and qRT-PCR analysis. The immune responses induced by *C. trachomatis* D and F strain treatments within the same group were compared to the cells stimulated by media (negative control).

The gynecological history and demographic status of participants are outlined in Table 5.1. The average age of women in the *C. trachomatis*-positive infertile group was 39.5 years while the average age of women in the *C. trachomatis*-negative group was 36.8 years. In *C. trachomatis*-positive infertile group (n=4), 75% had tubal infertility accompanied by other gynecological disorders such salpingitis (n=1) PID (n=1) and tubal adhesion (n=2). The IVF outcome based on number of successful or failed live births or pregnancies after IVF treatment were reported for both *C. trachomatis*-positive infertile women and *C. trachomatis*-negative infertile women (Table 5.1). Among *C. trachomatis*-positive participants who underwent IVF, 75% were nulliparous in the past and 50% attained a successful IVF outcome.

In the *C. trachomatis*-negative infertile group (n=27), 18.5% of the women had tubal infertility (n=5), accompanied by gynecological disorders and surgeries such as PID (n=1), salpinigitis (n=1), ovary removal (n=2), tubal obstruction (n=2), endometriosis (n=2) and polycystic ovarian syndrome (n=1). Amongst non-tubal *C. trachomatis*-negative infertile women, 33.3% (n=9) had for unknown etiology, 18.5% (n=5) had endometriosis (n=2). 22.2% (n=6) of the participants had polycystic ovarian syndrome and only one person had ectopic pregnancy and non-tubal ovary

removal. 77% of C. trachomatis-negative infertile women were nulliparous and about 62% achieved a successful IVF outcome.

Table 5.1: Demographic and gynecological history of study participants

C. trachomatis serology status by MIF	Mean Age (years)	Type of infertility *ectopic (n=) *tubal(n=)	IVF outcome (live birth/pregnancy) *success(n=) *failure (n=)	Gravidas past *nulliparous(n=) *multiparous (n=)	Gravidas at the time of collection (n=)
C. trachomatis-positive infertile women (n=4)	39.5	Tubal (n=3) [PID (n=1); salpingitis (n=1); tubal adhesion (n=2)] Unknown etiology (n=1)	Success (n=2) Failure (n=2)	Nulliparous (n=3) Multiparous (n=1)	0
C. trachomatis-negative infertile women (n=27)	36.8	Tubal (n=5)[ovary removal (n=2); PID (n=1); salpingitis (n=1); tubal obstruct (n=2); PCOS (n=1), Endometriosis (n=2)] Ectopic pregnancy (n=1) Polycystic ovarian syndrome (PCOS) (n=6) Endometriosis (n=5) [PID=(n=2)] Unknown etiology (n=9)[PCOS (n=2)] Non-tubal ovary removal (n=1)	Success (n=17) Failure (n=10)	Nulliparous (n=21) Multiparous (n=6)	n=7

5.3.2 The level of IL-1β was significantly higher in women with *C. trachomatis*-related infertility than in women with other forms of infertility

The level of secreted cytokines produced by stimulated PBMCs isolated from C. trachomatis- positive infertile and C. trachomatis-negative infertile women was analysed in Figure 5.1. In response to *C. trachomatis* D and F EBs, high levels of IL-8 (617.5pg/mL versus 602.5pg/mL) and TNF-α (111 pg/mL versus 71.73pg/mL) were secreted in PBMCs isolated from *C. trachomatis*-positive infertile women and *C. trachomatis*-negative infertile women. However, only IL-1β (96.42pg/mL versus 41.42pg/mL) production in the PBMCs of *C. trachomatis*-positive infertile women was significantly higher than the levels in the PBMCs of *C. trachomatis*-negative women ($p<0.05$). Within the *C. trachomatis*-positive infertile women, the levels of IL-8 (617.5pg/mL and 515pg/mL), TNF-α (111pg/mL and 7.557pg/mL) and IL-1β (96.42 pg/mL vs 7.76pg/mL) were significantly higher in the PBMC stimulated with *C. trachomatis* D and F strains compared to the negative control (Media; unstimulated PBMC) (Figure 5.2). Therefore, the secreted cytokines observed in the PBMC of *C. trachomatis*-positive infertile provides insight into the immune markers associated with *C. trachomatis*-related infertility.

5.3.3 CXCL10, CXCL11 and HLA-A were significantly up-regulated in women with *C. trachomatis*-related infertility than in women with other forms of infertility

Expression of 88 innate and adaptive immune genes in the stimulated PBMCs of *C. trachomatis*-positive infertile and *C. trachomatis*-negative infertile women were analysed using a RT-PCR array. The expression of each gene was normalized to reference genes and the fold change in gene expression between *C. trachomatis* infertile women and women with infertility for other reasons tested ($2^{-\Delta\Delta Ct}$). Three genes showed increased expression levels (not significant; $p > 0.05$) in *C. trachomatis*-positive infertile women (n=4) compared to *C. trachomatis*-negative infertile women (n=27) (Table 5.2). The chemokines CXCL10 (5.48-fold) and CXCL11 (2.31-fold), and human leukocyte antigen HLA-A (2.22-fold) showed the most notable differences between the two participant groups (these were not significantly different, possibly due to the small sample size of women with *C. trachomatis*-related infertility).

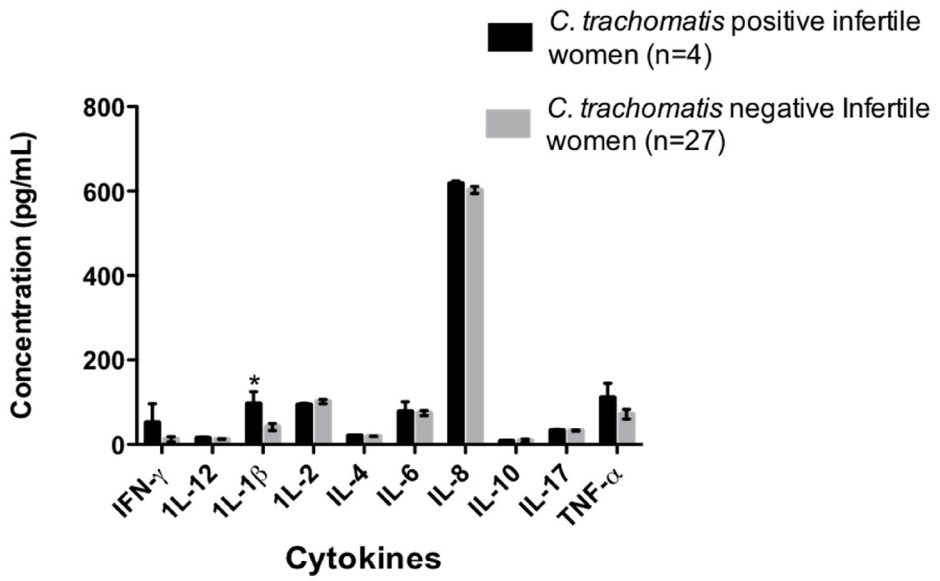

Figure 5.1: The graph shows the concentration of cytokines detected in the stimulated PBMCs (*C. trachomatis* D and F strains) of *C. trachomatis*- positive infertile women and *C. trachomatis*-negative infertile women .

C. trachomatis-positive infertile women (n=4) and *C. trachomatis*-negative infertile women (n=27) were compared. * indicates $p<0.05$.

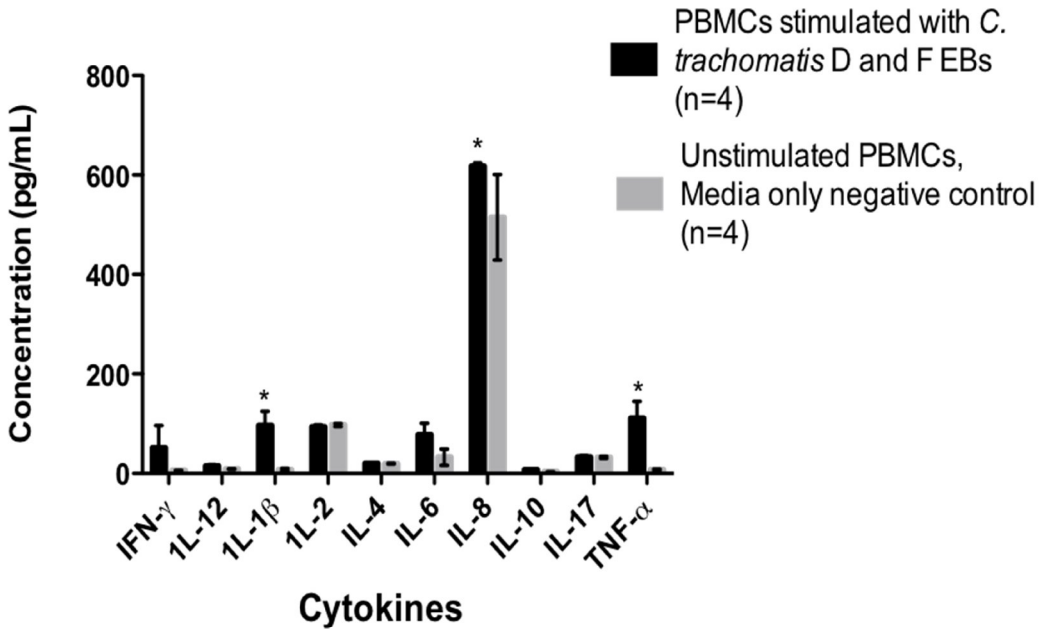

Figure 5.2: The graph shows the concentration of cytokines detected in the stimulated PBMCs (*C. trachomatis* D and F strains) and unstimulated PBMCs (Media; negative control) of *C. trachomatis*- positive infertile women.

The PBMCs stimulated with *C. trachomatis* D and F EBs (n=4) was compared with the unstimulated PBMCs (media only) (n=4). * indicates $p<0.05$

Table 5.2: Gene expression ($2^{-\Delta\Delta Ct}$) for PBMCs from *C. trachomatis*-positive infertile women compared to *C. trachomatis*-negative infertile women

Gene symbol	Gene function [462]	Fold change (*C. trachomatis*-positive infertile vs *C. trachomatis*-negative infertile)*	P value
CXCL10	CXC motif ligand 10 or interferon gamma inducible protein 10 (IP10)- stimulation of T cells, natural killer cells and monocytes	5.480	0.0557889
CXCL11	Induced by IFN-γ, a chemotactic for T cells	2.319	0.0717008
HLA-A	Human leucocyte antigen presents peptides from endoplasmic reticulum lumen	2.224	0.0796689
MX1	Gene encodes guanosine triphosphate metabolizing protein that is induced by type I and type II interferons.	2.044	0.15578
TNF	Tumour necrosis factor regulates cell proliferation, differentiation, apoptosis, lipid coagulation and metabolism.	1.689	0.374361
HLA-E	Human leucocyte antigen belongs to class I heavy chain that binds to a subset of peptides derived from leader peptides of other class I peptides	1.317	0.590575
C3	Complement component 3	1.190	0.471629
NFKBIA	Nuclear factor of Kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.105	0.867405

	inhibits NFK-β/REL complexes that are involved in inflammatory responses		
IRF3	Interferon regulatory transcription factor (IRF)	0.185	0.97611
IL1B	Interleukin 1 induces cytokine inflammatory response and regulates a variety of cellular activities, including cell proliferation, differentiation, and apoptosis	1.08	0.980821
CCL5	Chemokine, chemoattractant for blood monocytes, eosinophils and memory T helper cells	1.055	0.806007
STAT3	Mediates the expression of a variety of genes in response to cell stimuli, cell growth and apoptosis	1.010	0.979167
IL8	Chemoattractant, and a potent angiogenic factor.	0.995	0.657476
FASLG	Member of the tumor necrosis factor superfamily FAS/FASLG signalling pathway is essential for immune system regulation, including activation-induced cell death (AICD) of T cells and cytotoxic T lymphocyte induced cell death.	0.988	0.961924
DDX58	DEAD box proteins, putative RNA helicases	0.962	0.816789
STAT1	Signal transducer and activator of transcription (STAT1) is vital for cell viability in response to different pathogens and cell stimuli	0.926	0.96934
MYD88	Cytosolic adapter protein; central role in the innate and adaptive immune response.	0.913	0.870936
IFNAR1	Receptor for interferons alpha and beta	0.912	0.829671
CD14	Surface antigen expressed on monocytes/macrophages; mediates the innate immune response to bacterial lipopolysaccharide.	0.875	0.886719

IL1A	Interleukin 1 cytokine family; pleiotropic cytokine involved in various inflammatory processes, immune responses, and hematopoiesis	0.854	0.974281
STAT4	Signal transducer and activator of transcription; essential in regulating the differentiation of T helper cells and for mediating responses to IL12 in lymphocytes	0.831	0.906692
CSF2	Colony stimulating factor 2; production, differentiation and function of granulocytes and macrophages	0.818	0.972042
IL23A	Subunit of the cytokine interleukin 23 (IL23). Activate the transcription activator STAT4, and stimulate the production of interferon-gamma (IFNγ).	0.782	0.746775
NFKB1	Transcription regulator activated by intra- and extra-cellular stimuli such as cytokines, ultraviolet irradiation, oxidant-free radicals, and bacterial or viral products.	0.744	0.537021
ICAM1	Intercellular adhesion molecule 1 cell surface glycoprotein; typically expressed on endothelial cells and cells of the immune system.	0.710	0.988701
CD40	Member of the TNF-receptor superfamily; receptor on antigen-presenting cells. memory B cell development, T cell-dependent immunoglobulin class switching, and germinal centre formation.	0.689	0.708097
CXCR3	G protein-coupled receptor with selectivity for three chemokines, termed CXCL9/Mig (monokine induced by interferon-g), CXCL10/IP10 (interferonγ-inducible 10 kDa protein) and CXCL11/I-TAC (interferon-inducible T cell a-chemoattractant).	0.629	0.524409
IL6	Cytokine that functions in inflammation and the maturation of B cells	0.622	0.862861

TBX21	T-box genes encode transcription factors that regulate developmental processes.	0.591	0.556515
CASP1	Cysteine-aspartic acid protease (caspase) family; apoptosis.	0.580	0.708687
CD80	Membrane receptor; induces T-cell proliferation and cytokine production.	0.578	0.923101
IRF7	IRF7 encodes interferon regulatory factor 7 (IRF); role in the transcriptional activation of virus-inducible cellular genes.	0.546	0.500677
STAT6	Exert IL4 mediated biological responses that induce the expression of BCL2L1/BCL-xL, which is responsible for the anti-apoptotic activity of IL4	0.532	0.01862
IFNG	Type II interferon family	0.519	0.741645
IFNGR1	This gene (IFNGR1) encodes the ligand-binding chain (alpha) of the gamma interferon receptor.	0.515	0.253977
MAPK1	MAP kinases, also known as extracellular signal-regulated kinases (ERKs); involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development.	0.448	0.928017
ITGAM	This gene encodes the integrin alpha M chain.	0.441	0.797051
IFNA1	Produced by macrophages and has antiviral activity	0.4068	0.621741
CD8A	The CD8 antigen is a cell surface glycoprotein found on cytotoxic T lymphocytes. Mediates efficient immune cell-cell interactions	0.403	0.874176
IFNB1	Production of Interferon β	0.394	0.606598

TRAF6	TNF receptor associated factor (TRAF) protein family; mediate signal transduction	0.392	0.8323
NOD1	NOD (nucleotide-binding oligomerization domain) family; cytosolic protein,	0.374	0.857525
CXCR5	This gene encodes a multi-pass membrane protein that belongs to the CXC chemokine receptor family; binds to B-lymphocyte chemoattractant (BLC).	0.359	0.678219
CD4	Membrane glycoprotein of T lymphocytes; interacts with major histocompatibility complex class II antigens.	0.340	0.700329
CD86	Expressed by antigen-presenting cells; is the ligand for two proteins at the cell surface of T cells, CD28 antigen and cytotoxic T-lymphocyte-associated protein 4	0.323	0.549186
MAPK8	MAP kinases; involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development.	0.310	0.591922
CCL2	Chemokine displays chemotactic activity for monocytes and basophils	0.294	0.601704
IL10	Cytokine pleiotropic effects in immunoregulation and inflammation.	0.292	0.539064
TICAM1	Adaptor protein containing a Toll/interleukin-1 receptor (TIR) homology domain, mediates signal-transduction components and protein-protein interactions between the Toll-like receptors (TLRs).	0.271	0.651261
JAK2	Janus kinase 2 involved in a cytokine receptor signalling pathways; is required for responses to gamma interferon.	0.257	0.291592
LYZ	Lysozyme, targets bacterial cell wall peptidoglycan	0.257	0.72527
TLR3	Toll-like receptor (TLR) family; pathogen recognition and activation of innate	0.2541	0.722248

	immunity.		
IL2	Proliferation of T and B lymphocytes	0.225	0.712847
CCR6	Beta chemokine receptor family; preferentially expressed by immature dendritic cells and memory T cells.	0.211	0.573793
TLR7	Toll-like receptor (TLR) family which plays a fundamental role in pathogen recognition and activation of innate immunity	0.204	0.416802
IRAK1	Interleukin-1 receptor-associated kinase 1; partially responsible for IL1-induced upregulation of the transcription factor NF-kappa B.	0.198	0.637532
CD40LG	Surface of T cells; regulates B cell function by engaging CD40	0.191	0.866972
SLC11A1	Solute carrier family 11 (proton-coupled divalent metal ion transporters) family divalent transition metal transporter	0.183	0.574461
FOXP3	Member of forkhead/winged-helix family of transcriptional regulator	0.162	0.614845
NLRP3	Upstream activator of NF-kappaB signaling, and it plays a role in the regulation of inflammation, the immune response, and apoptosis.	0.159	0.604511
TLR2	Member of the Toll-like receptor (TLR) family; fundamental role in pathogen recognition and activation of innate immunity	0.143	0.172989
CCR5	Beta chemokine receptor family,	0.142	0.39416
IL18	Proinflammatory cytokine; augments natural killer cell activity and stimulates interferon gamma production in T-helper type I cells.	0.138	0.639216

IL1R1	Cytokine receptor interleukin-1 alpha, interleukin-1 beta, and interleukin-1 receptor antagonist	0.117	0.579764
TLR1	Plays a fundamental role in pathogen recognition and activation of innate immunity	0.098	0.544638
TLR4	Lipopolysaccharide (LPS) found in most Gram-negative bacteria induces signal transduction events	0.098	0.532769
LY96	Protein associates with toll-like receptor 4 on the cell surface and confers responsiveness to lipopolysaccyaride (LPS)	0.089	0.675998
NOD2	Nod1/Apaf-1 family; primarily expressed in the peripheral blood leukocytes; role in the immune response to intracellular bacterial lipopolysaccharides (LPS)	0.088	0.653912
TLR8	Predominantly expressed in lung and peripheral blood leukocytes	0.086	0.569824
TLR6	Receptor functionally interacts with toll-like receptor 2 to mediate cellular response to bacterial lipoproteins	0.081	0.552111
RAG1	Involved in activation of immunoglobulin V-D-J recombination and recognition of the DNA substrate	0.081	0.658209
IL5	Interleukin 5 cytokine acts as a growth and differentiation factor for both B cells and eosinophils	0.080	0.626102
GATA3	GATA family of transcription factors; endothelial cell biology.	0.080	0.663112
IL17A	Proinflammatory cytokine produced by activated T cells; regulates the activities of mitogen-activated protein kinases and NF-kappaB	0.078	0.655239

TLR5	Receptor mobilizes the nuclear factor NF-κB; inflammatory gene regulation	0.076	0.632839
CXCL13	Chemokine; promotes the migration of B lymphocytes	0.075	0.642325
IL4	Interleukin 4; pleiotropic cytokine produced by activated T cells	0.073	0.648235
MBL2	Soluble mannose-binding lectin found in serum; innate immune	0.073	0.651894
APCS	Glycoprotein;	0.072	0.637162
TLR9	Toll-like receptor (TLR) family; role in pathogen recognition and activation of innate immunity	0.072	0.636229
CRP	Host defence related functions; recognize foreign pathogens	0.071	0.651894
IL22	Interleukin-22 (IL-22); host defence at mucosal surfaces [463]	0.045	0.646686
IL13	Immunoregulatory cytokine; B-cell maturation	0.041	0.646695
MPO	Myeloperoxidase (MPO).	0.037	0.611395
CCR4	G-protein-coupled receptor family; receptor for the CC chemokine	0.036	0.688041
TYK2	Tyrosine kinase Janus kinases (JAKs) protein families; interferon signalling pathways.	0.034	0.671997
CCR8	Beta chemokine receptor family; G protein-coupled receptors; important for migration of various cell types into the inflammatory sites.	0.015	
RORC	DNA-binding transcription factor; inhibits the expression of Fas ligand and IL2.	0.081	0.640822

*ΔCT was obtained by normalizing the level of expression of gene of interest to the expression level of housekeeping genes (HKG) (ΔCT= CTGOI - CTAVG HKG). Fold change in gene expression (2-ΔΔCT) was determined by dividing the normalized expression of gene of interest of the experimental sample by the normalized expression of the same gene of interest in the control sample; where $\Delta\Delta C_T = \Delta C_T$ (*C. trachomatis*-positive infertile sample)- ΔC_T (*C. trachomatis*-negative infertile sample

5.4 DISCUSSION

The cytokines secreted in response to CT stimulation and expression of immune genes in peripheral blood mononuclear cells were tested to provide information into the immune pathway modulated by *C. trachomatis* in women with chlamydia-related infertility. Within 15 hours of co-culture with *C. trachomatis*, three immune genes, HLA-A (2.224-fold), CXCL10 (5.480- fold) and CXCL11 (2.31-fold) were expressed at greater than 2 fold higher levels in *C. trachomatis*-positive infertile women compared to *C. trachomatis*-negative infertile women (not significant). Amongst secreted cytokines, IL-1β was significantly up-regulated in women with *C. trachomatis*-related infertility compared to infertile women seronegative for *C. trachomatis* ($p<0.05$). The PBMCs were stimulated with a mix of two well characterized and common *C. trachomatis* strains, rather than a single strain, although these may not have been the same as the strain that originally infected the participants to cause the infertility. Serovar F is one of the most prevalent *C. trachomatis* strains in Australia [124]. Serovar D was reportedly found to be predominant in women with PCR confirmed *C. trachomatis* infection and elicited the highest mean IgG response (Mean IgG titre of 200) compared to other serovars such as such as serovas B, C and I [293]. Genotyping of *C. trachomatis* from endocervical specimens of infertile Mexican women (n=152) who were positive for *C. trachomatis* infection (n=24), revealed that serovar F was the most prevalent serovar (54.2%), as compared to other serovars (serovars K, E, LGV, D, H, Ia) [464]. These two strains will not cover all of the relevant diversity of *C. trachomatis*, but were used to provide more diversity in the simulating antigen mix than a single strain.

In this study, the expression of HLA-class I molecule, ecncoded by the HLA-A gene was upregulated in women with *C. trachomatis*-related infertility (not significant). Infection by *C. trachomatis* is likely to trigger a range of immune responses as a result of antigen binding human leukocyte antigens (HLA) molecules. Although several studies have established strong association between HLA class II molecules and their genotypes in women with chlamydial infertility [172, 465], HLA class I molecules and alleles have also been reported to in increasing the susceptibility to tubal pathology and infertility caused by *C. trachomatis* infections

[466, 467]. Kimani *et al.* [466] reported that HLA-A31 allele was a significant risk factor for *C. trachomatis* PID (OR (95%CI): 3.9(1.04-14.5); p=0.04; n=23) in a longitudinal study of urban female sex worker in Kenya (n=23 PID cases). Additionally, HLA-A2 was reported to elicit cytotoxic T lymphocyte responses to *C. trachomatis* MOMP in peripheral blood of patients infected by *C. trachomatis* [467]. Therefore, the upregulation of HLA-A genes in women with *C. trachomatis*-related infertility indicate that functional $CD8^+$ T cytotoxic lymphocytes may play a role in chlamydial pathology.

The study also showed an increased expression of two chemokines, CXCL10 (IP-10) and CXCL11 (IFN-inducible T cell α-chemoattractant or i-TAC) in PBMCs fromwomen with *C. trachomatis*-related infertility compared to PBMCs from infertile women seronegative for *C. trachomatis*. Chemokines are small pro-inflammatory molecules that induce migration of leucocytes [468]. The role of CXCL10 in protective immunity against chlamydial infections has been implicated in several studies [469-475]. The cytokine response to *C. trachomatis* in endocervical epithelium showed that there was a reduction of CXCL10 in polarized polA2EN epithelial cells [470]. CXCL10 recruits CXCR3 and CCR5 positive leukocytes such as T cells and natural killer cells to the site of infection and regulates IFN-γ-mediated resolution of *C. trachomatis* [470]. The reduced levels of CXCL10 indicate that endocervial epithelial cells could subvert the chemokine, which prevents the migration of IFN-γ producing T cells to the site of infection, thus leading to pathology [470]. The *C. muridarum* model has also been used to investigate the role of CXCL10 in protective immunity against *C. trachomatis* infections [469]. The study showed that the levels of CXCL10 was highest 21 days post infection and declined after 42 days which corresponded with resolution of the disease. Additionally, previous studies using mouse models have shown that CXCL10 is predominantly expressed in upper genital tract infection and mediate protection through Th1 or pro-inflammatory immunity [469, 473, 476]. Similar to CXCL10, CXCL11 is also induced by IFN-γ, which recruit CCR3, and is exclusively associated with Th1 responses [477].Wan *et al.* [478] showed that CXCL11 gene expression levels were higher in the secretory phase of endocervical epithelial cells on infection with *C. trachomatis*, thus indicating the potential role of hormones in modulating immune system. Thus, the upregulation of both CXCL10 and CXL11 in

the mononuclear cells of women with chlamydial infertility are indicative of Th1 mediated immune-responses against *C. trachomatis* infection.

Whilst a trend towards an increase in mRNA levels were observed for IL-1β and TNF-α (not significant), ELISAs detection of the secreted protein showed that secreted IL-1β levels were significantly higher in PBMC isolated from women with *C. trachomatis*-related infertility as compared to PBMCs from *C. trachomatis* seronegative infertile women ($p<0.05$). Consistent with the findings of Hvid *et al.* [266], this study also shows that IL-1β is the pro-inflammatory cytokine induced by *C. trachomatis* infection, and an important contributor to pathology during *C. trachomatis* infections. Here the IL-1β was detected from primary *ex vivo* culture of PBMC rather than reproductive tract epithelia (as in Hvid *et al.*, [266]), suggesting that both the innate epithelial response and innate mononuclear response from some women in response to *C. trachomatis* infection is pro-inflammatory and involves IL-1β. TNF-α, a pro-inflammatory multifunctional cytokine that triggers a variety of immune responses on infection, also regulates production of several cytokines by activating various components of cellular signal transduction that has detrimental effect on the genital tract [479, 480].

Murine models have previously established the role of $CD8^+$T cells mediated upper genital tract pathology via production on TNF-α by examining the oviduct pathology in TNF-α deficient mice [481] and OT-1 transgenic mice ($CD8^+$T respond only to Ova 257-264 peptide, and not *C. trachomatis*- specific antigens) [482] transgenic mice. In cervical mononuclear cells isolated from infertile women (n=70) stimulated with cHSP60 and cHSP10, an increase in the production of TNF-α suggests that it maybe involved in the immunopathological condition associated with infertility [303]. Both IL-1β and TNF-α are potent inducers of IL-8 [479], hence high levels of the cytokine were observed in the supernatants of women with *C. trachomatis*-related infertility.

The serology and gynaecology status of the participants recruited from IVF clinics showed that patients who were positive for chlamydial serology were predominantly positive for tubal pathology (75%) as compared to those who were negative for chlamydial serology (18.5%). *C. trachomatis* specific IgG antibodies in sera are indicative of chlamydial infertility as it was reported to be higher in women

with tubal factor infertility (41.4%; n=33) [165]. Analysis of follicular liquid of 253 IVF patients revealed that cHSP60 antibodies were predominant in infertile women with tubal occlusion (69.5%), and it was also predominant in 74.1% of women whose embryo implantation failed during IVF [184]. Sharara *et al.* [483] showed that the presence of elevated serum *C. trachomatis* IgG in infertile women undergoing gamete intra-fallopian transfer cycles significantly lowered the implantation rate (7.1% in *C. trachomatis*-positive group vs 16.5% in negative *C. trachomatis*-negative group) and increased the early pregnancy loss (33.3% in *C. trachomatis*-positive vs 14.6% in *C. trachomatis*-negative group). In this study, 75% of women with *C. trachomatis* –related infertility were nulliparous compared to 62% of infertile seronegative for *C. trachomatis*. This is consistent with the findings of Coppus *et al.* [484] that showed that positive *C. trachomatis* IgG serology in women with tubal pathology (n=1882) was associated with 33% lower conception rate. Thus, CXCL10, CXCL11, HLA-A and IL-1β maybe associated with the pathology in the upper genital tract that lead to severe tubal damage and lower pregnancy rate in women with *C. trachomatis*-related infertility.

The limitations of our study include a small sample size that might account for the lack of significance between the groups for many factors. By restricting the stimulation to 15 hours, we were able to effectively identify early mononuclear cellular responses that are associated with *C. trachomatis*-related infertility in women. The observed differences in responses here for women with *C. trachomatis*-related infertility compared to women with other causes of infertility supports the model that some women launch a more pronounced pro-inflammatory response to the infection and thus develop more serious pathology and disease sequelae and these responses may reflect the original response to the infection in the local tissue.

Chapter 6: Sero- epidemiological assessment of *C. trachomatis* infection and infertility in Samoan women

6.1 INTRODUCTION

Chlamydia (C.) trachomatis is one of the most common bacterial sexually transmitted infections in the world [102]. Due to the asymptomatic nature of the disease, the infection is frequently undiagnosed, resulting in the development of serious sequelae such as pelvic inflammatory disease, ectopic pregnancy and tubal infertility in women [485, 486]. Clinical infertility or subfertility is defined as the inability to become pregnant after 12 months of unprotected intercourse [374]. The lack of adequate diagnosis and timely treatment of the disease has allowed this infection to reach endemic levels in developing countries [487].

The prevalence rate (estimated by PCR) ranges from 1.5% to 5% in the developed affluent countries [488, 489] while it is significantly higher, ranging from 3.7% to 15% in the developing world [490, 491]. In the United States, between the years 1999 and 2000, a cross-sectional population assessment of *C. trachomatis* infection in 20,836 people aged 14-39 years estimated that the overall prevalence as 1.6% [492]. However, in the years 2007-2008, an increase in the prevalence rate to 2.2% was reported in 2667 women aged between 14- 39 years [492]. A similar prevalence rate of 2.0% was recorded among pregnant women aged 15-24 years recruited from prenatal clinics in the United States between the years 2004-2009 [493]. The prevalence rate in Europe was similar to that reported in United States. For instance, a prevalence rate of 2.2% was reported in in German adolescents (n=1136 girls) using a nucleic acid amplification test (strand displacement amplification)[494]. A prevalence rate similar to Germany was reported in Ireland, where the prevalence of *C. trachomatis* infection in women recruited from antenatal clinics was 3.7% using polymerase chain reaction (PCR) (n=945)[495]. Conversely, a high prevalence rate of 7.3% (estimated by PCR) was reported in a cross-sectional population based study of adolescent girls in Norway, and the study further reported that girls were twice as more likely to be infected as boys (95% CI 5.3-9.7 vs 2.3-6) [496]. Similarly, a cross-sectional population based sero-surveillance study in Netherlands showed that seroprevalence of *C. trachomatis* infection in women aged 15-39 years was 10% [497]. Davies *et al.* [111] reported that in 307 sex workers recruited from Genito- Urinary Medicine (London), the rate of PID in women with recent chlamydial infection was 27.4 per 100 person-years as compared to the 11.2 in

women with previous chlamydial infection as estimated by direct immunofluorescence.

In developing countries such as Nigeria, the overall prevalence rate of asymptomatic *C. trachomatis* infection estimated by PCR in 132 infertile women attending Gynaecological clinic was 20.5% as opposed to 10.4% in the UK and other developed countries [125, 126]. Out of 125 patients attending infertility and STD clinics in Nigeria, the prevalence rate was 8.7% in the fertility clinic (estimated by *Chlamydia* Rapid test, an immunoassay technique that detects *C. trachomatis* from swab or urine) and 10.7% in an STD clinic [128]. Using PCR, a similar prevalence rate to Nigeria of 18.6% also reported amongst infertile women (n=264) in India [498]. A higher prevalence rate of 52.8% (n=106) was reported in infertile women attending fertility clinics in the Manaus-Amazon (Brazil) using PCR [348]. The higher incidence of *C. trachomatis* infection has been reported in developing countries could be accounted by lack of regular screening strategies or untimely treatment of the infection, resulting in recurrent re-infection and transmission of the infection. Table 6.1 lists the prevalence of *C. trachomatis* infections in developed and developing countries.

Table 6.1:The prevalence of *C. trachomatis* infections in developed and developing countries

Country	Study setting (number of women)	Prevalence	Testing method	Reference
USA	Population-based assessment (n=2667)	2.2%	PCR	[492]
USA	Prenatal clinics (n=166)	2%	PCR	[493]
Germany	Adolescent girls (n=1136)	2.2%	PCR	[494]
Ireland	Antenatal clinics (n=945)	3.7%	PCR	[495]
Norway	Adolescent girls (n=800)	7.3%	PCR	[496]
Netherlands	Population sero-surveillance study	10%	CAT	[497]
India	Gynaecology outpatient clinic (n=593)	23%	PCR	[487]
Italy	STD outpatient clinic (n=43)	18.6%	PCR	[489]
Kenya	Antenatal clinic (n=300)	6%	*Chlamydia* Rapid Test	[490]
Egypt	IVF clinics	40%	PCR and CAT	[499]
Nigeria	Population based prospective study (n=286)	29.4%	CAT	[500]
Haiti	Women's health clinic (n=303)	11.9%	PCR	[501]
Spain	Cross-sectional population study (n=277)	4%	PCR	[502]
Suva	Antenatal clinics (n=440)	50%	CAT	[503]
Iran	Obstetrics and Gynaecology clinics (n=255)	2.4%	PCR	[504]
Samoa	Antenatal clinics (n=299)	26.8%	PCR	[505]

Sullivan *et al.* [506] previously estimated the prevalence rate of *C. trachomatis* infection in Samoa to be 30.9%, based on pregnant women (mean age =26 years) attending antenatal clinics between the years 1999 and 2000 (n = 427). The prevalence of sexually transmitted infections in pregnant women from antenatal clinics was also studied by Cliffe *et al.* [505] between the years 2004 and 2005 who reported that amongst all STIs such as Gonorrhoea and Syphilis, chlamydial prevalence (n=299) was the highest in women of all ages (26.8%) who were recruited from antenatal clinics. However, this group may not represent the general or at risk members of the population and consequently, an accurate estimate of the prevalence remains unknown. The high incidence of *C. trachomatis* infection may imply a high probability of increased risk of sequelae such as infertility in Samoan women.

Serological assays have been used to detect anti-chlamydial antibodies during the infertility investigation in women as a method to predict women with tubal factor or chlamydial infertility [359, 377]. There are several serological tests that could be used to detect *C. trachomatis* infection. The Microimmunofluorescence (MIF) test developed by Wang and Grayston [366] showed a high sensitivity and it is still considered the serologic "gold standard". However, it is labour intensive and highly subjective [2]. This has accounted for poor interpretation of MIF results that has led to conclusions about its poor specificity. Enzyme linked immunosorbent assay (ELISA), overcomes the problems posed by MIF with high specificity to *C. trachomatis* as it is based on recombinant chlamydial antigens [371]. Since *Chlamydia* IgG antibodies in serum are associated with tubal pathology, ELISAs have been introduced as non-invasive screening for implementation during the initial fertility investigation in some countries [365, 400]. For instance, Muvunyi *et al.* [359] demonstrated that three commercial ELISA kits, ANILabsystems IgG, Vircell IgG and ANILabsystems IgA had a high specificity of 84%, 86%, 95% respectively and a positive predictive values of 73%, 76% and 81% respectively, in detecting tubal pathology (n=312). Thomas *et al.* [400] estimated that in participants with infertility (n=57), an IgG titer greater than 1 in 32 was effective identifying those with tubal damage.

While IgG titres are indicative of past infection, positive chlamydial IgA titres indicate active ongoing infection [357]. Serum IgA antibodies have a half-life of

about 5-7 days; hence they are useful markers of active chlamydial infections [507]. A number of commercial ELISAs are available to detect *C. trachomatis* infections. They are often based on variable domains of MOMP synthetic peptide specific to *C. trachomatis* [371]. MOMP makes up to 60% of the outer membrane protein content and contains species- specific and serotype- specific epitopes [390, 393, 410, 437]. Several studies have implicated strong serological responses to cHsp60 (Heat Shock Protein60) and cHsp10 in women with subfertility [304, 508-510]. Therefore, several commercial kits utilise cHSP60 protein or peptide as the antigen to detect persistent infection or past infection. In a meta-analysis of published evaluations of various assays, Broeze and co-workers identified that micro immune-fluorescence (MIF) (various companies) was the most sensitive, but relatively low in specificity. In the same study, the MEDAC and ANIlabsystems ELISAsystems enzyme linked immunosorbent assays (ELISA) appeared to be the most specific, although less sensitive than MIF [365] to diagnose women with uni or bi-lateral tubal damage detected by surgical or sonographic technologies. These commercial tests are based on whole *Chlamydia* (MIF), or antigens (typically the major outer membrane protein (MOMP) and/or cHSP60). Numerous studies have reported a correlation with *Chlamydia* antibody testing (CAT) against MOMP and/or cHSP60 with diagnosed tubal infertility [365]. However, a proportion of women with infertility and who are serologically positive by CAT are absent of detectable tubal blockage or adhesions, but still require IVF to conceive [365].

Nucleic acid amplification tests are highly accurate techniques that are widely used in *C. trachomatis* diagnosis [318]. They detect chlamydial nucleic acids, transcripts from genital specimens, and non-invasive samples such urine specimens and self -obtained vaginal swabs, and are highly sensitive [318]. The evaluation of the sensitivity and specificity of NAAT techniques such as strand displacement amplification (SDA), PCR and Abbott ligase reaction (LCR) to *C. trachomatis* showed that the three techniques performed similarly with regards to sensitivity and specificity [326]. However, when compared to the performance of enzymeimmunoassay, the detection rate for PCR was 62% higher than that of ELISA [327].Although it cannot be used to detect past infections, its superior sensitivity and specificity allows efficient diagnosis of active *C. trachomatis* infection [511]. In fertility studies, several reports on women with infertility have shown that PCR

prevalence rate of *C. trachomatis* may be lower than seroprevalence rate which indicates that markers of past chlamydial infections are important in determining chlamydial infertility as infertile women may not longer have the infection [512-514].

In developing countries, very little is known about *C. trachomatis* and infertility. In Rwanda, 67.8% of sub-fertile women attending a fertility clinic were found to have tubal pathology by hysterosalpinography, and of these 38 (20.5%) were also found to have *C. trachomatis* serum antibodies using the ANILabsystems IgG ELISA for *C. trachomatis* infertility [365]. In India, 55% of women with secondary infertility had *C. trachomatis* IgG antibodies, and of these 63.6% had confirmed tubal occlusion [406]. In Ghana, women reporting infertility were more likely to have *C. trachomatis* IgG antibodies (odds ratio 2.1) compared to fertile women in a case-control study design that included 439 women (39% vs. 19%) [514]. Additionally in Alexandria, Egypt, higher titers of anti-*Chlamydia* IgG antibodies were significantly more likely in women with TFI and ectopic pregnancy compared to fertile or healthy pregnant controls [515]. These studies suggest that in fertility clinic studies within developing countries, *C. trachomatis* infertility is more common than that reported in developed countries.

In the current study, a sero-epidemiological analysis on a Samoan population of women was conducted using existing commercial serological kits to evaluate both, the prevalence of, and the epidemiological risk factors associated with infertility caused by *C. trachomatis*.

6.2 MATERIALS AND METHODS

6.2.1 Epidemiological and serological assignment of groups

The following figure (Fig 6.1) summarises the assignment and characterization of Samoan women using clinical history and serological techniques. It includes the number of samples analysed in the study and the tests conducted on the samples on the sexually active women in Samoa. The inclusion criteria were that participants were (1) sexually active (2) do not use contraceptives (3) have stayed a minimum of one year in the village, and (4) they should not have undergone any gynaecological surgeries that would render them infertile or unable to reproduce.

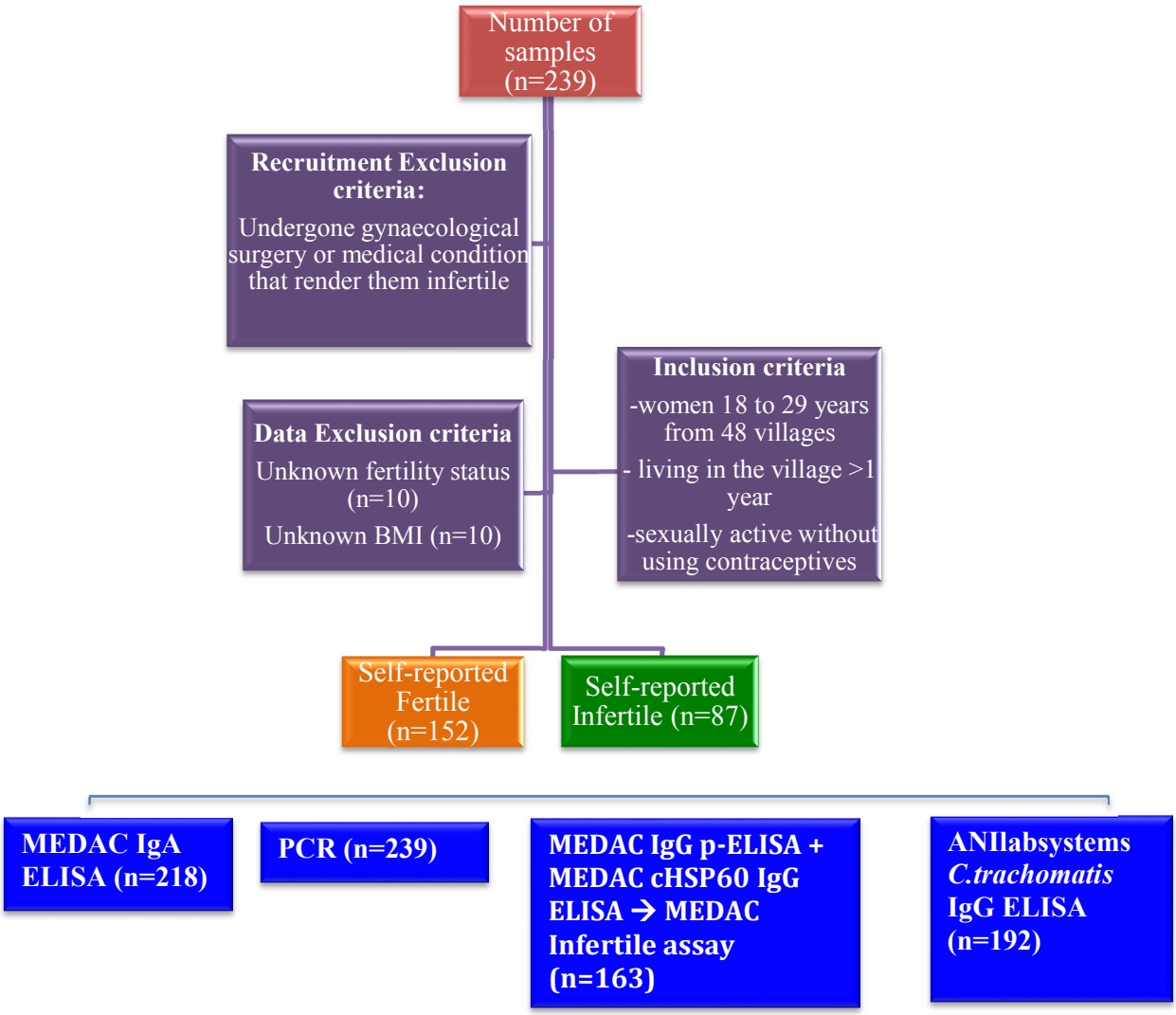

Figure 6.1: Schematic representation of the cross-sectional study design and analysis including the epidemiological assignment of cohorts and the serological assays.

The cross-sectional study included 239 female participants between the ages 18-29. Based on the patient data sheet, patients with incomplete data or unknown status were excluded from data analysis and the participants that satisfied the inclusion criteria were categorized as self-reported fertile (n=152) and self-reported infertile (n=87).

6.2.2. Cross-sectional participant recruitment in Samoan population

Study design and sample size estimation

The study design and sampling for this study has already been published in Walsh *et al.* [516]. This was a cross-sectional study set on the island of Samoa, which comprises of two main islands, Savaii and Upolu, and six smaller outer islands. Samoa is divided into four regions, the Apia Urban Area (63 villages), Upolu (111 villages), North-West Upolu (54 villages), and Savai'i (102 villages). Based on the Samoa demographic and health survey of 2009 [517], 47% of women were sexually active, of which 30% were assumed to meet the inclusion criteria and could participate in the study. The estimated *C. trachomatis* prevalence was 30% [505], and in order to obtain 95% confidence interval from 26.8 to 33.2%, 800 participants needed to be recruited through cluster sampling method. The cluster sampling method included grouping the villages into 16 clusters and the samples were collected randomly using a sampling interval number. This yielded a total of 48 villages, with each cluster encompassing between 1 and six villages. This cluster design would ensure that a minimum of 125 participants be recruited, with 50 participants recruited from each cluster [516]. Due to the subsequent damage caused by the cyclone that hit in 2011, seven villages were excluded from the study. For the 41 selected villages, the Ministry of Women, Community and Social Development provided the contact details of Sui o le Malo or Sui tama'ita'i o le Nu'u (representative of the village women's committee) for each village. 249 sexually active women from the ages of 18-29 years were recruited from 41 villages. The approval was obtained from National University of Samoa Research Ethics Committee, the Oceania University of Medicine, The Samoan Ministry of Health and the Lower South Regional Ethics Committee in New Zealand (LRS/10/11/059). The approval for the study was also obtained from Queensland University of Technology Human Research Ethics Committee (approval number 1100000276).

Collection of demographic and epidemiological information

Participants provided informed written consent, and completed an interview-led questionnaire addressing their reproductive history, sexual, lifestyle and medical (general health, current levels of smoking, drinking, exercise, BMI) and demographic information (age, marital status, educational level, own and partner's occupation). Table 8.4.in the appendix lists the demographic and epidemiological information of

the participants. The questionnaire responses were used to assign women to 'infertile' (or otherwise 'fertile') groups based on reporting at least 12 months of unprotected intercourse without conceiving a pregnancy. Based on the questionnaire, women were classified as self-reported infertile if they reported that they have been trying to get pregnant, and they have been doing so for more than a year . Women who have indicated no problems in getting pregnant and have taken less than 1 year to do so were categorized as fertile. The recruitment was conducted in schools, churches and community halls in larger villages, and local Women's committee members house was used in smaller villages. A nurse was appointed to record all the responses of the participants and the participants who tested positive for *C. trachomatis* were given 1000 mg of azithromycin. Their partners or recent sexual contact were also provided with 1000mg of azithromycin.

6.2.3. Diagnosis of *Chlamydia trachomatis* infection using NAAT

After completing their questionnaire, the first catch urine specimen and blood (processed to obtain sera) samples were collected from these patients. All questionnaires and urine specimens were de-identified labelled with the participant's unique study code, and the urine specimens were analysed locally with BD ProbeTech *Chlamydia* Amplified DNA assay according to manufacture's recommendations (Becton, Dickson, Franklin Lake, New Jersey, USA) as previously reported [516]. Urine specimens were transported to the laboratory at the end of each village visit. Any sample showing inhibition in the test was repeated twice and reported as inhibited if the tests continued to fail.

6.2.4. Serological diagnosis of *C. trachomatis* infection

The participant sera were tested for *C. trachomatis* antibodies using three commercial tests; *Chlamydia trachomatis*-IgG ELISA-plus MEDAC (peptides from MOMP protein) and cHSP60-IgG ELISA MEDAC (cHSP60 protein). The kits were used according to the manufacturer's instructions. According to the MEDAC manufacturer-provided instructions, samples positive in both MEDAC IgG *C. trachomatis* pELISA and cHSP60 IgG ELISA indicate a high risk of developing *C. trachomatis* induced tubal factor infertility (MEDAC Infertile). ANILabsystems *C. trachomatis* IgG ELISA based on synthetic peptides from *C. trachomatis* -specific variable domain of MOMP (major outer membrane protein), identified women with *C. trachomatis* PCR diagnosed infection and infertility with a sensitivity and specificity of 84% and 91% respectively (n=303) [365]. *C. trachomatis* IgA pELISA MEDAC which is also based on the immunodominant region of MOMP peptide measures the level of IgA antibodies which is an indication of current infection. Mouton *et al.* showed that MEDAC IgA pELISA had a sensitivity of 53.3% and specificity of 90.2% in identifying women with tubal factor pathology (n=15)[437]. According to the manufacturer's instructions, samples positive for MEDAC IgG p-ELISA and *C. trachomatis* IgA pELISA MEDAC are definite positive for *C. trachomatis* infection. However, if they were positive only in MEDAC IgA assay, then the participant has a possibility of early stages of infection.

6.2.5. Statistical analysis

Serological assays were conducted on all serological specimens collected during this study and analysed against epidemiological and PCR data. All statistics were calculated in R statistical environment (3.0.3) using the 'EpiR (0.9-57) and 'metafor' package (1.9-2) for calculation and presentation of odds ratios (forest plots) and embedded routines for generalized linear models for logistic regression [413]. Odds ratios (OR) were calculated with restricted maximum likelihood estimates of error.

6.3. RESULTS

6.3.1. Epidemiological and demographic factors associated with infertility

Table 6.2 lists the epidemiological and demographic factors that are associated with infertility. The factors that significantly associated to infertility were age, alcohol (frequency of drinking beer and spirits) and relationship status ($p<0.05$). The BMI of 10 women (5 infertile) were not reported. Hence, the BMI of only 82 women were included. Younger women between the 18-24 years, who were single, with no children and did not consume alcohol, were more likely to be infertile than fertile. The likelihood of developing infertility was four times higher in women who were between the ages of 18-24 years as compared to women who between the ages of 25-29 years (OR=4.22, 95% CI=2.27-7.84), and in women who were single as compared to married/de-facto/separated/widowed (OR=4.00, 95% CI=2.2342-7.1613). Although not significant, women who were unemployed were five times more likely to develop infertility as compared to women who were employed (OR=5.32, 95% CI= 2.3-12.4).

Table 6.2:The effect of epidemiological and demographic factors on infertility using chi-square tests

Infertility vs Fertility	Number of infertile women	Chi square value	P value	OR (95% CI)
Age				
18-24 years	70	22.453	<0.01	4.22 (2.2786-7.8431)
25-29 years	17			
BMI				
Underweight	0			-
Normal weight	14	3.216	0.36	-
Over weight	37			1.3974 (0.686-2.8458)
Obese	31			1.567 (0.75-3.26)
Problems becoming pregnant				
No	1	0.155	0.694	-
Yes	1			1.76 (0.107-28.7)
Age of first sex				
19years or older	55			0.8679 (0.5-1.50)
18 years or lesser	32	0.255	0.614	
Number of sex partners				
1 sex partner	54			1.77 (1.03-3.03)
2 sex partners	20	4.629	0.099	
3 or more sex partners	13			
Paid work				
No	66	3.502	0.061	5.32 (2.3-12.4)
Yes	21			

6.3.2. A high proportion of women reporting infertility by an epidemiological questionnaire were serologically predicted to have chlamydial infertility

There were 239 participants in the study, and 152 of these were identified as being fertile. The remaining 87 women were defined as infertile. The commercial assays that are specifically developed to detect women with *C. trachomatis* infertility (MEDAC Infertile, ANILabsystems ELISA) or women with *C. trachomatis* infection history (MEDAC MOMP, MEDAC cHSP60), or current infection (MEDAC IgA) were used to investigate the participant's serological responses.

Figure 6.2. illustrates the correlation between the diagnostic tests; PCR, MEDAC infertile, MEDAC MOMP, MEDAC IgA and ANILabsystems ELISA. A total of 9 participants were positive in all five assays including PCR. 4 participants tested positive only in ANILabsystems IgG ELISA, while 19 participants were uniquely recognized by MEDAC cHSP60 IgG ELISA. PCR, MEDAC IgA ELISA and MEDAC MOMP ELISA were able to identify only 5, 2 and 1 participant respectively. 11 participants showed co-positivity between all three IgG serological assays, while 16 participants showed positivity in all three IgG serological and PCR.

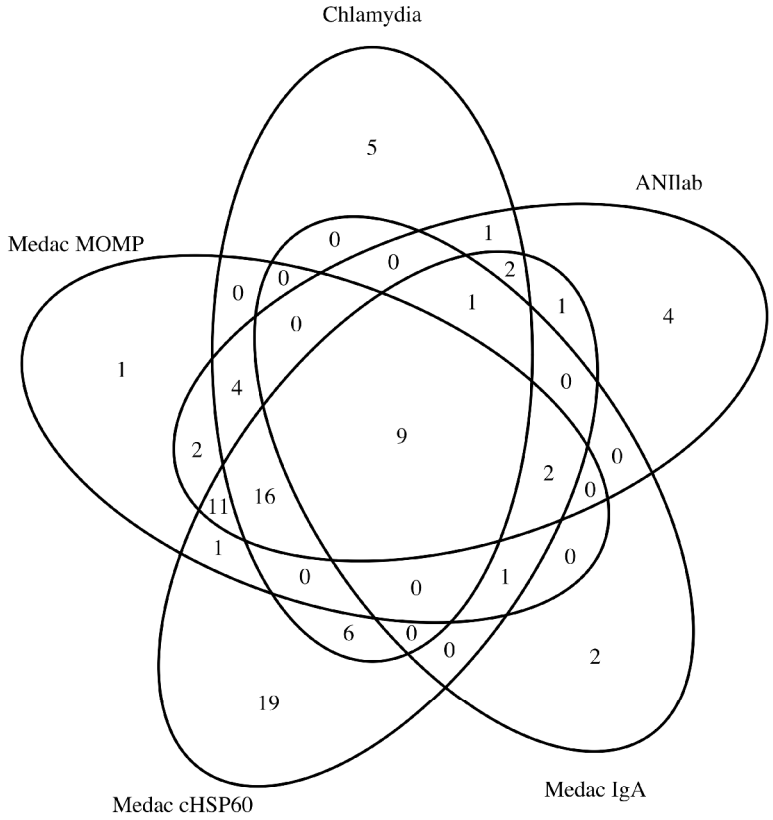

Figure 6.2: Venn diagram representing concordance of positive results for PCR status, MEDAC Infertile and ANILabsystems ELISA, MEDAC cHSP60 and MEDAC IgA serology

112 participant results, excluding 127 participants who were unequivocal or beyond the detection range of the assay, were analysed for positive results in either/both or all ANILabsystems ELISA, MEDAC Infertile, MEDAC MOMP, MEDAC cHSP60, MEDAC IgA or current infection (PCR) tests. 9 of the 112 women testing ANILabsystems ELISA, MEDAC MOMP, MEDAC cHSP60, MEDAC IgA and MEDAC Infertile positive demonstrated current infection.

Table 6.3 summarizes the ability of commercial serological assays to differentiate self-reported infertility in women from the fertile women in the population. Participant sera that tested equivocal or whose absorbance values lay beyond the upper and lower limit range of positive and negative controls were excluded from analysis. Therefore, out of 239 participants in the study, MEDAC infertile assay comprised of 163 participants, while 205 participants were included in

MEDAC MOMP. 182 participants, 218 participants and 192 participants were included in MEDAC cHSP60 IgG ELISA, MEDAC IgA ELISA and ANILabsystems IgG ELISA respectively. With 38 infertile participants in MEDAC MOMP assay (61.2%; OR (95%CI): 1.94 (1.09, 3.46), the assay showed significant association with infertility (p=0.024) and were almost twice as more likely to be infertile than fertile. Although not significant, participants who tested positive in MEDAC infertile assay and ANILabsystems were 1.7 and 1.75 times more likely to be infertile than fertile, respectively.

Among 85 participants positive by PCR (Figure 6.2.B), 34 women (were seropositive in MEDAC Infertile and was significantly associated with the PCR status ($p<0.01$; OR=4.36, 95% CI= 2.19-8.68). In addition to MEDAC Infertile, all serological assays including MEDAC MOMP, MEDAC IgA and ANILabsystems IgG could significantly differentiate participants with acute *C. trachomatis* infections from those without ($p<0.01$). Amongst the serological assays, ANILabsystems IgG ELISA had the highest odds ratio, with PCR positive participants testing positive in ANILabsystems 6 times more than women who are PCR negative (OR=6.68, 95% CI=3.46-12.93).

Table 6.3: Correlation of *Chlamydia* antibodies detected by commercial serological assays with fertility status

	Infertile		Fertile			
Assay (+/-)	Assay +	Assay -	Assay +	Assay -	Odds Ratio (95% CI)	P value
MEDAC Infertile (+/-)	26	36	30	71	1.71 [0.88, 3.31]	0.11
MEDAC MOMP (+/-)	38	37	45	85	1.94 [1.09, 3.46]	0.024
MEDAC cHSP60 (+/-)	40	30	64	48	1.00 [0.55, 1.83]	1
MEDAC IgA (+/-)	11	69	12	126	1.67 [0.7, 3.99]	0.242
ANILab (+/-)	40	32	50	70	1.75 [0.97, 3.16]	0.062
MEDAC C. pneumoniae (+/-)	77	4	107	28	5.04 [1.70, 14.95]	0.002

Table 6.4:Correlation of *Chlamydia* antibodies detecting by commercial serological assays with current infection status as determined by PCR

	Chlamydia +		Chlamydia -			
Assay (+/-)	Assay +	Assay -	Assay +	Assay -	Odds Ratio (95% CI)	P value
MEDAC Infertile (+/-)	34	28	22	79	4.36 [2.19. 8.68]	<0.001
MEDAC MOMP (+/-)	46	27	37	95	4.37 [2.38, 8.04]	<0.001
MEDAC cHSP60 (+/-)	52	19	52	59	3.11 [1.63, 5.91]	<0.001
MEDAC IgA (+/-)	14	66	9	129	3.04 [1.25, 7.39]	0.011
ANILab (+/-)	53	18	37	84	6.68 [3.46, 12.93]	<0.001
MEDAC C. pneumoniae (+/-)	71	8	113	24	1.88 [0.8, 4.43]	0.141

6.3.1. *Chlamydia pneumoniae* serology correlates with infertility in this population

The prevalence of antibodies to *C. pneumoniae* in this population was high at 85.1% (184/216 participants). However, it was surprising that antibodies to *C. pneumoniae* were found to be significantly more likely to be detected in women with infertility (p=0.002; OR 5.04, 95% CI: 1.7–14.95) (Fig 6.3A). This was not a false positive detection of antibodies against *C. trachomatis* in the *C. pneumoniae* assay because a current infection (*C. trachomatis* PCR positive result) and a positive result in *C. pneumoniae* serological assay did not correlate (of the 216 positive, only 71 (35.6%) with the PCR positive result (Pearson's chi-square test, p=0.141).

6.4. DISCUSSION

A cross-sectional sero-epidemiological study of sexually active Samoan women between the ages of 18-29 years was undertaken to determine the overall prevalence of *C. trachomatis* infection and the risk of developing chlamydial infertility. In addition to estimating the risk of chlamydial infertility using serology, the epidemiological and demographic factors associated with chlamydial infertility were determined.

The high prevalence rate (35.6%) of *C. trachomatis* infection, as diagnosed by PCR, in the Samoan population indicates that the infection may be endemic. High prevalence of *C. trachomatis* in Samoa has been reported previously. In 2005, Sullivan *et al.* [506] reported a prevalence rate of 30.8% in 452 pregnant women attending antenatal clinics in Samoa using PCR. Similarly in 2008, Cliffe *et al.* [505], reported prevalence rate of 26.1% for 1618 pregnant women recruited from antenatal clinics. However, because these studies only evaluated women who attended antenatal clinics for the first time, the prevalence could be expected to be much higher in the population of sexually active women in Samoa. In other developing countries such as Kenya and Cameroon, the prevalence rate was estimated based on sexually active urban women recruited in antenatal clinics and student volunteers respectively. A cross-sectional study was conducted in Kenya, where vaginal swabs from sexually active women aged 18-45 years were tested by PCR and the prevalence was estimated to be 6% (95%CI: 3.31%-8.69%) [490]. Using Direct fluorescence assay (DFA) and PCR on cervical and urethral swabs, the prevalence rate was estimated to be 3.96% (95% CI: 2.61%-5.93%) in Cameroon [491]. In Bangladesh, the prevalence of *C. trachomatis* was estimated based on 110 sexually active women and 40 sex workers between the ages of 15-35 years. The prevalence was determined from endocervical swab specimens from the participants through PCR and/or immunochromatography and was estimated to be 27% in local female populations and 58% in female sex workers [519]. This suggests that the prevalence of *C. trachomatis* infections in Samoa is relatively higher than some other developing countries, thus drawing attention to the immediate need to implement effective diagnosis and control of the disease.

The prevalence of approximately 50% *C. trachomatis* serum antibody positive results (MEDAC Infertile 52.5%, MEDAC MOMP 55.3%, MEDAC cHSP60

42.02%, ANILabsystems ELISA 50.9%) for infertile women was high in this population compared to that reported in developed countries. It is not unexpected that the infertility assays did not correlate significantly with the infertile group, as there are multiple reasons for infertility. In addition, these assays were developed in Western countries, where the prevalence of *C. trachomatis* infections was much lower than Samoa. Most studies where a significant association is found is when the tubal factor infertility is the specified outcome. Consistent with the findings in this study, Agholor *et al.* [134] reported that the prevalence of serum antibodies to *C. trachomatis* in women with ectopic pregnancy in Nigeria was 48% (98 ectopic pregnant women) compared to 16.3% in women with normal intrauterine pregnancy. Although the study did report a significant difference in the prevalence of chlamydial antibodies between women with ectopic pregnancy and fertile controls, the absence of *C. trachomatis* antibodies in 52% of women with ectopic pregnancy highlights that *Chlamydia* infection may play a less than significant role in the aetiology of the ectopic pregnancy in this population. In this population there is a limited knowledge of other fertility factors, as this was a village based survey study in the absence of any gynecological investigations that would normally be conducted in a fertility clinic. This lack of gynecological data is a limitation of the study. It is important to note that this study is also limited by the number of villages that were included as sites to recruit participants, however, the age range and sexual behavior used for inclusion criteria are highly relevant for women likely to be seeking pregnancy. The important observation is that by any of the assays it appears that approximately half of the women who are identified as infertile in this population have evidence of *C. trachomatis* infertility. This is higher than most studies report; even those conducted in fertility clinics, but are consistent with a fertility clinic study in India that found a similar prevalence [406]. This is likely a reflection of the absence of routine screening and treatment for *C. trachomatis* infections in this population. This may support that the widespread implementation of screening programs in several developed countries is effectively reducing the sequelae such as infertility.

Whilst a higher proportion of infertile participants were positive compared to fertile participants in most serological assays, only MEDAC MOMP showed significant association to infertility (43.6%; $P<0.05$) (Table 6.3.). Mouton *et al.* [437] showed that *C. trachomatis* specific IgG was reported in 41-57% of women with

tubal factor infertility (n=85), and MEDAC MOMP detected women with infertility with a sensitivity of 55.3% and a specificity of 75.5%. Similar to the results obtained in this study, MEDAC MOMP detected *C. trachomatis* specific IgG in 39.1% (n=23) of women with tubal pathology as compared to 10.4% of infertile women with ovulation dysfunction and idiopathic infertility (n=48) [105]. Consistent with the findings in Jeremiah *et al.* [520], the absorbance values that correspond to anti-chlamydial antibodies in MEDAC MOMP showed a significant difference between women with chlamydial infertility from those without. Additionally, they were able to distinguish women with current *C. trachomatis* infection than those without. Whilst not recommended by the manufacturer for the test used here, many studies use cHSP60 antibodies as a test for chlamydial infertility. In this population, 40 of the 70 infertile women were positive in this test; however, this was not significantly higher than in the infertile compared with the fertile group. Nevertheless, the high prevalence of cHSP60 antibodies in this population suggests that chronic infections or sequelae are high in this population. A possible limitation is that the serological results may be influenced by a higher number of repeat infections given the high prevalence of infection in this population. However, the prevalence of antibodies to *C. trachomatis* was reported to be lesser in women with repeat infections than in women with primary infections. This was elucidated in van den Broek *et al*. [357], which reported that the prevalence of IgG was 78% in primary infection and 23% in recurrent infection. Similarly, the prevalence of IgA was 52% in women with primary infection and 37% in women with recurrent infection.

All IgG serological assays, including MEDAC MOMP, MEDAC cHSP60, ANILabsystem ELISA and MEDAC IgA, showed significant association with PCR status ($p<0.01$) (Table 6.4). The IgA antibody response to *C. trachomatis* infection and its correlation with PCR positivity (active infection) in the Samoan population confirms that *C. trachomatis* infection elicits humoral response. This is consistent with the findings of van der Broek [357], where in 94% of participants who were positive for *C. trachomatis* infection by PCR were also seropositive in MEDAC MOMP. In this study, amongst those women who were positive for PCR confirmed *C. trachomatis* infection, 8.03% (9/112) were also seropositive in MEDAC MOMP, ANILabsystems IgG ELISA, MEDAC IgA ELISA and MEDAC cHSP60 IgG ELISA. Using PCR alone, *C. trachomatis* infections was detected in 47.1% (41/87)

of infertile participants, while 33.3% (16/48) of infertile participants with PCR confirmed *C. trachomatis* infection also showed seropositivity in IgG serological assays [357]. The study also highlighted that the presence of IgA antibodies to *C. trachomatis* in the vaginal mucosa of women (PCR negative for *C. trachomatis* infection), might indicate an ongoing *Chlamydia* infection. This also indicated a chronic low-grade immune response in the upper genital tract [357]. Therefore, humoral response is detectable in women following chlamydial infection. Consistent with the results obtained in the study, Abdella *et al.* [499] estimated that the prevalence of chlamydial infertility in Egyptian women as 40% (n=50) by both PCR and ELISA. Using PCR alone, current infection was detected in 6% women, and *C. trachomatis* specific IgG antibodies were detected in 38.9% of women with primary infertility (n=36) and 28.6% of women with secondary infertility (n=14). Marashi *et al.* [347] reported that in Iran, the *C. trachomatis* prevalence estimated from endocervical swabs using indirect immunofluorescence was 22.6% for infertile women (n=150) and 4.5% for fertile healthy controls (n=200). PCR analysis of endocervical swabs on the same cohorts estimated the prevalence rate to be 32% in infertile women and 8.7% in fertile healthy controls [347]. Therefore, while PCR can be used to determine with current infections, serological assays are instrumental in identifying women with past *C. trachomatis* infections.

The demographic and epidemiological factors associated with infertility included age, alcohol and relationship status. In this study, younger women between ages of 18-24 years were at a higher risk of developing infertility than women between the ages of 25-29 years (OR: 4.22; 95% CI = 2.27-7.84) (Table 6.2). Contrary to the findings in this study, majority of the studies conducted on women with *C. trachomatis*-related infertility or ectopic pregnancy showed that the mean age was above 28 years [134, 348, 520, 521]. Mueller *et al.* [522] estimated that a higher proportion of women between the ages of 25-29 years had a history of inflammatory disease as compared to women less than 25 years and greater than 30 years (38.8%; n=129). A similar result was determined by Iris *et al.*, [523] in which the mean age of infertile women with primary infertility was determined as 31.2± 3.8 years. Savolainen *et al.* [524] reported that over a period of 16 years (1999-2006) the mean age of patients with fertility complications was 25.1 years.

The increased prevalence observed could be explained by various factors. The reports of Samoa demographic and health survey, 2009 by Ministry of Health (Apia, Samoa) [517] showed that 55% of younger women between the ages of 15-29 years do not use contraceptives due to the lack of knowledge of method or source of the method and 59% of women are opposed to the use of contraceptives. Due to low condom usage rates in Samoa, there is an increasing incidence in adolescent pregnancy rates [517]. Additionally, only 11% of married women and 12% of never married women between the ages of 15-29 self-reported being likely to refuse having sexual intercourse with a man who has STI, the rest were supportive of having sexual intercourse with their partner knowing that they have an STI [517].

IgA antibodies in sera is an indication of current infection [165, 357], and consistent with the literature, the serum IgA was significantly associated with PCR status (p=0.011). Additionally, 17.5% participants who were serum IgA positive, were three times more likely to test positive for *C. trachomatis* infection in PCR as compared to participants who were serum IgA negative (OR=3.04, 95% CI= 1.24-7.39). van der Broek [357] reported that 33% (n=24) of women seropositive for serum IgA were also positive in PCR testing, and serum IgA had a sensitivity of 32% and a specificity of 95% in detecting women with active infection. In this study MEDAC IgA antibodies were prevalent in 13.75% of women with infertility and were unable to differentiate women with infertility from those without. Consistent with this study, Muvunyi *et al.* [359] reported a higher prevalence of 14% (n=14) in women with acute infections than women with subfertility (7.8%, n=303) a specificity and sensitivity of 93% (95%CI= 91-95) and 14% (95%CI: 3.00-44.00) respectively. However, contrary to results obtained in this study, *C. trachomatis* specific IgA levels were reported to be higher in women with TFI and persistent chlamydial infection (83.3%), and were further able to discriminate between participants with TFI from those with spontaneous miscarriage [165]. Komoda *et al.* [528], showed that in 52 women clinically diagnosed with cervicitis/ and or PID, very low levels of *C. trachomatis* specific serum IgA was observed using a synthetic peptide- based ELISA test. However, it is present at early stages of infection and the IgA positivity rate decreased from 82.7% at the first testing to zero after 200 days [528]. Hence, it is important to conduct additional tests such as PCR to confirm *C. trachomatis* infection. Serum IgG levels generally show greater association with

PCR status as evident in several studies [357, 365]. The low IgA levels were also reflected in the earliest studies conducted in Samoa by Ushijima *et al.* [529], where antibody testing using microplate Fluorescent antibody method reported that amongst 192 adolescents recruited from health centres, 39% were *C. trachomatis* IgG positive, while only 10% were IgA positive.

C. *pneumoniae* is a common respiratory infection associated with community acquired pneumonia and has been associated with systemic dissemination and chronic diseases such as atherosclerosis and Alzheimer's disease [530]. Serology to *C. pneumoniae* is highly prevalent in many populations with 50–70% prevalence reported [531]. More recently, 57% seropositivity was reported in Australia in women attending sexual health or fertility clinics, suggesting the previously reported 50–70% range is relevant [373]. However, there were no reports of the prevalence of *C. pneumoniae* serology in Samoa, therefore, this test was included in the serological assays conducted on this population. In this study, *C. pneumoniae* was significantly associated with infertility (p=0.002) but it did not correlate with the *C. trachomatis* PCR result (p=0.141). Serology to *C. pneumoniae* has been previously significantly correlated with various diseases [530], but not infertility. One possible explanation for high sero-prevalence to *C. pneumoniae* in infertile population is that the infertile women here have tissue lesions or adhesions in the fallopian tube that may form a reservoir for the pathogen (similar to the lesions in the other chronic diseases, although this has never been reported) and therefore these participants more frequently have a positive serological response to the pathogen. Additionally, the antigens associated with pathogenesis may be highly conserved between *C. trachomatis* and *C. pneumoniae*. Another concern for the high prevalence of *C. trachomatis* in the Samoan population was that the commercial serological assays were possibly showing cross-reactivity with ocular *C. trachomatis* strains. Ocular *C. trachomatis* strains have been reported to be endemic in the Pacific Islands [532]. However, a study by Lees *et al*. [532] reported that among 200 participants who attended the clinic in 2011 and 2012, and 150 participants who attended in 2013, none of them were positive for trachoma. The study was conducted from 2009 to 2013 and investigated eye diseases in patients attending Ophthalmology outreach clinics in Pacific islands, Fiju, Kiribati, Papua New Guinea, Samoa, Solomon Islands, Tonga, Vanuatu. Thus, the prevalence of trachoma is extremely low and it is

unlikely that it would interfere with the serological diagnosis of genital *C. trachomatis* infections.

In summary, the high prevalence of *C. trachomatis* infections in Samoa could be leading to increased preventable infertility in this population. Women need to be educated about safe-sex practices and made aware of the symptoms and diagnosis of *C. trachomatis* infections. Public health strategies such as implementation of routine testing and treatment for the infection could reduce the disease burden from infertility and could also reduce the other morbidities (PID and ectopic pregnancy) within this population.

Chapter 7: General Discussion and Conclusions

C. trachomatis infections are one of the major causes of tubal pathology in women, and tubal pathology accounts for up to 35% of all causes of infertility [416]. Since infertility treatments are financially as well as psychologically costly, it poses a significant burden on health care systems and individuals. Laparoscopy and hysterosalpinography, which cost up to AUD $4500, are currently used for diagnosing *C. trachomatis*-related tubal factor infertility. These are costly and have limited availability in developing countries [423]. Additionally, the invasive nature of the technique increases the risk of surgical complication [421]. These shortcomings have the potential to be overcome by a sensitive and specific serological diagnosis of *C. trachomatis* tubal infertility, a technique that is non-invasive, easy, cost-effective [362, 403, 404]. However, none of the current serological diagnostic assays have been adopted for routine implementation during infertility investigation in fertility clinics owing to their lack of specificity, possibly due to cross-reactivity between other chlamydial species and lack of sensitivity [533].

Through this study, the QUT *Chlamydia* infertility test was developed that had the highest specificity of 94% and a sensitivity of 27% in identifying women with *C. trachomatis*-related TFI when compared to current commercial serological assays. The QUT *Chlamydia* infertility test comprised of three peptides; peptide 11, HSP60-E2 and 443-N2 that were derived from antigens that are immuno-dominant in women with *C. trachomatis* induced TFI. Novel antigens for diagnosis of *C. trachomatis* associated infertility in women have been identified previously, most commonly, through proteomic analysis via two- dimensional electrophoresis and mass spectrometry [353, 426, 427], and proteome array using glutathione fusion proteins [386, 387]. However in this study, the peptides used in the development of the assay were identified through *in silico* analysis based on their antigenicity and specificity to *C. trachomatis* (BLAST E value for *C. trachomatis* in HSP60-E2 and CT443 peptides are 0.0003 and 0.005 respectively) [411]. Frikha-Gargouri *et al.* [534] identified an OmcB antigen through *in silico* analysis, and although it showed 72.7% concordance with MIF and a specificity of 94.3% in detecting *C. trachomatis* antibodies in participants positive in MIF, its sensitivity was very low at 23.9% (n=24).

Although, The QUT chlamydia infertility test had the highest specificity (100%) in identifying women with tubal pathology, its sensitivity was lower than MIF (16% vs 27%) (Table 4.3). However, MIF had the lowest specificity (89%) in detecting women with TFI (n=45). Bax *et al.* [377] also reported that compared to MEDAC MOMP, MIF had the highest sensitivity (63.6% vs 36.4%) but a lower specificity (81% vs 85.7%) in detecting tubal pathology (n=76). Consistent with the findings in the study, the sensitivity, specificity, PPV and NPV of commercial MIF (MIF FOCUS and MIF ANILabsystems) and ELISA (ELISA ANILabsystes and ELISA Vircell) in identifying sub-fertile women with tubal damage compared to women without (n=104) were in the ranges of 15%-27%, 88%-93%, 53%-60% and 62%-64% respectively [359]. Therefore, compared to the commercial assays in this study, and those assays in previously published literature, the QUT *Chlamydia* infertility test had a much higher specificity (100%) and PPV (100%). Additionally, this assay could significantly differentiate women with tubal damage from women without tubal damage (P=0.004) unlike the commercial assays used in this study. Thus, the QUT *Chlamydia* infertility test could be a potential screening test for identifying tubal pathology in infertile women.

The QUT *Chlamydia* infertility assay also demonstrated the highest specificity (94%), sensitivity (27%) and PPV (19%) compared to other commercial serological assays in detecting women with *C. trachomatis*-related tubal factor infertility (n=11) from the negative cohort (n=251) (seronegative *C. trachomatis* women with no TFI, women with acute *C. trachomatis* infections and fertile women). Although the commercial ELISA, MEDAC infertile assay (combination of MEDAC MOMP and MEDAC cHSP60) had a higher sensitivity and could significantly differentiate CT TFI cohort from negative cohort, like the QUT *Chlamydia* test, its PPV (10%) was lower than the peptide assay. Consistent with the literature, the MEDAC infertile assay was the best performing assay (sensitivity=36%; specificity =86%) amongst all commercial assays [535]. Jones *et al.* [375] also reported that MEDAC MOMP had the highest specificity in diagnosing chlamydial infertility compared to five commercial ELISAs, including ANILabystems.

The high diagnostic performance of the QUT *Chlamydia* infertility test could be attributed to the use of non-traditional antigens in this assay, unlike commercial assay that are generally based on MOMP and HSP60. The combination of peptides

from the cytosolic protein HSP60 (HSP60-E2), a periplasmic protein HtrA (peptide 11) and the outer membrane protein CT443 (443-N2) potentially increased the range of epitopes that would improve the antibody response in women with *C. trachomatis*-related TFI. Several novel antigens specific to *C. trachomatis*-related TFI have been identified previously, however none of these antigens have been developed for a clinical setting. Whilst Rodgers *et al.* [385] identified antigens that were uniquely recognized by participants with *C. trachomatis*-related infertility and combination of antigens that could improve the sensitivity (67%) and specificity (100%) of detecting women with TFI (combination of four antigens HSP60, CT376, CT381 and CT798 and combination of two antigens CT443 and CT381), it was a predictive model rather than a functional diagnostic. Budrys *et al.* [387] further evaluated the ability of these antigens to discriminate between different disease groups and reported that the combination of six antigens HSP60, CT376, CT557 and CT443 could differentiate women with TFI (n=24) from fertile women (n=25) (sensitivity =63%; specificity=100%). However, unlike QUT *Chlamydia* infertility test, it was unable to discriminate between women with TFI and women with acute infections.

The QUT *Chlamydia* infertility assay did not show complete concordance with any of the commercial serological tests used in this assay to identify *C. trachomatis*-related TFI in women ($P>0.05$). However, the commercial assays correlated with each other as they share a common antigen (MOMP), and even with MEDAC infertile correlated with MIF *C. pneumoniae* and MIF *C. psittaci.* This indicates that the commercial assays may have a high rate of cross-reactivity between *C. pneumoniae* and *C. psittaci.* Although, individual peptide assays did not correlate with MIF *C. psittaci*, QUT *Chlamydia* infertility test did. This could be due to the high specificity of the assay, which ensures effective identification of true negatives and elimination of false positives. Since, the prevalence of *C. psittaci* was very low in the population, the number of true negatives may coincide with that of the peptide assay. Thus, a concordance was observed between QUT *Chlamydia* infertility test and MIF CS. Freidank *et al.* [536] reported a high number of *C. pneumoniae* antibodies in women with tubal infertility (53% in women with one occluded tube (n=30) and 75% in bilateral tubal occlusion (n=64)). However, it did not correlate to cHSP60 from *C. trachomatis* [384] and was reported not to be associated with tubal pathology but are likely to cause false positive results in MIF [373].

The assay was validated on a separate group that only included infertile women who were recruited from a different IVF clinic (n=74). Contrary to the development cohort, none of the serological assays including QUT *Chlamydia* infertility test showed a statistically significant difference in identifying women with TFI from women with other forms of infertility ($p>0.05$) in the validation cohort. However, the inclusion of women with unexplained infertility resulted in an increase in sensitivity and specificity of all serological assays. Interestingly, MEDAC MOMP (sensitivity =7%, 97%), MEDAC cHSP60 (sensitivity= 17%, specificity =93%) and ANILabsystems (sensitivity =15%, specificity =90%) showed greater sensitivity and specificity than MEDAC infertile (sensitivity= 2%, specificity 97%) and QUT *Chlamydia* infertility test (sensitivity= 5%, specificity =87%) in identifying women with unknown infertility or pathology from infertile women without tubal pathology and known infertility. However, the differences were not significant ($P>0.05$). The poor performance of the serological assays on this group could have been due to the small sample size. The low overall prevalence of *C. trachomatis* in the validation cohort could also be due to the geographical location of the IVF clinic from where these participants were recruited. Since the participants for assay development and validation were recruited from separate IVF clinics situated at different geographical locations, the prevalence of *C. trachomatis* will differ between both groups.

Although, *C. trachomatis* seropositivity is associated with decreased pregnancy rates in infertile women, the literature suggests that it does not influence the IVF outcome. Tasdemir *et al.* [441] and Claman *et al.* [442] reported that after an IVF cycle, TFI women who were seropositive for *C. trachomatis* had a higher pregnancy rate than infertile women who were seronegative for *C. trachomatis*. Keltz *et al.* [185] also demonstrated that participants who were seropositive for *C. trachomatis* were more likely to conceive with IVF treatment than other non-IVF treatments. Consistent with these findings, QUT *Chlamydia* infertility test also could not predict IVF outcome (pregnancy and live birth rates) in both test and validation cohorts. Therefore, women who are positive for chlamydial infertility as diagnosed by the QUT *Chlamydia* infertility test are as likely to conceive via IVF as those women with other forms of infertility. This is important for a diagnostic assay, as the clinician could recommend these women to proceed to IVF without additional treatments or diagnosis. The limitations of this study are that due to the small sample

size in the validation cohort, the assay performance could not be validated effectively. Since, the prevalence of chlamydial infertility was higher in the diagnostic cohort (11 %) compared to the validation cohort (7%), an increased sample size would also allow higher number of participants with tubal pathology associated with *C. trachomatis*,. Future studies should include the evaluation of QUT *Chlamydia* infertility test on larger numbers of infertile participants

Thus, QUT *Chlamydia* infertility test is highly specific for tubal pathology and can effectively differentiate women with *C. trachomatis* induced tubal infertility from non-TFI infertile women seronegative for *C. trachomatis*, women with acute *C. trachomatis* infections and healthy fertile women. The assay is cost effective, robust and easy to use. Additionally, women positive in the assay could be recommended treatment by IVF directly. These characteristics make QUT *Chlamydia* test an ideal diagnostic tool for early infertility investigation. The accessibility and ease of use would also be potentially useful in low-resource setting or in countries with high prevalence of *C. trachomatis* infections.

While several techniques have been developed for the diagnosis of *C. trachomatis*-related infertility in women, the underlying mechanism leading to tubal factor infertility is yet to be understood. Cytokine analyses of peripheral blood mononuclear cells (PBMC) from infertile women have shown the involvement of both cytotoxic pathological response (Th1) [270] or a humoral antibody mediated response (Th2)[273]. In this study the expression of 88 innate and adaptive immune genes and the levels of 10 secreted cytokine from PBMC s were compared between infertile women seronegative for *C. trachomatis* (n=27) and infertile women seropositive for *C. trachomatis* (n=4). After 15 hours of stimulation with *C. trachomatis* EBs, the genes that were predominant in PBMC of women with *C. trachomatis*-related infertility were CXCL10, CXCL11 and HLA-A, while the secreted cytokine was IL-1β. Although, HLA class II molecules have been reported to be associated with cHSP60 and enhance Th2-type immune responses in infertile women (n=53) [172], HLA-A2 was reported to elicit cytotoxic T lymphocyte responses to *C. trachomatis* MOMP in the peripheral blood of *C. trachomatis* infected patients [467]. In a longitudinal study of urban female sex workers in Kenya (n=23), HLA-A31 was reported to be a significant risk factor *C. trachomatis*-related PID [466]. Proinflammatory chemokines, CXCL10 and CXCL 11 that induce

migration of leucocytes, were highly expressed in stimulated PBMCs from women with *C. trachomatis*-related infertility. Both chemokines play an important role in regulating the Th1 type immune pathway, as CXCL10 recruits CXCR3 and CCR5 positive leukocytes such as T cells and natural killer cells to the site of infection [470] and upregulation of CXCL11 (IFN-inducible T cell α-chemoattractant or i-TAC) leads to further infiltration of IFN-γ secreting T cells resulting in a positive feedback loop [539]. Additionally, several studies have reported that CXCL10 is predominantly expressed during upper genital tract infection by *C. trachomatis* [469, 473, 476]. Therefore, it could be inferred that Th1 type immune pathway is one of the early mononuclear immune responses associated with *C. trachomatis* pathology.

Amongst the secreted cytokines, IL-1β, IL-8 and TNF-α were predominantly secreted in PBMCs from women with *C. trachomatis*-related infertility, however only IL-1β showed significantly higher levels compared to infertile women seronegative for *C. trachomatis* PBMCs. The synergistic effects of secreted cytokines IL-1β, IL-8 and TNF-α have been elucidated in several studies [173, 479, 480]. Though *ex vivo* fallopian tube studies, Hvid *et al.* [173] showed that addition of IL-1RA receptor antagonists, blocked IL-1β and IL-8 production preventing the pathology from chlamydial infection. This confirms that IL-1β along with IL-8 were likely major factors for tubal pathology. Additionally, IL-1 production triggers and primes superoxide production which lead to destruction of ciliated cells and subsequent scarring of the fallopian tubes [173]. *C. trachomatis* infection in epithelial cells is associated with high levels of IL-8 and TNF-α, triggering a pro-inflammatory response could either resolve the infection or lead to tissue fibrosis and scarring [269]. The study identified significant up-regulation and production of pro-inflammatory cytokines and chemokines, including a trending increase in IL-8 and TNF-α in women with *C. trachomatis*-related TFI. This suggests that *C. trachomatis* infection triggers pro-inflammatory cytokines that augment cellular inflammatory response resulting in tissue damage and subsequent tubal pathology [540]. The IgG and IgA antibodies that were identified in women with chlamydial infertility indicate that humoral immunity plays an active role in infection clearance. The B-lymphocytes serve as antigen-presenting cells to T-lymphocytes, and the resolution of infection is facilitated by Th1 activation [541]. Since key Th1 (IFN-γ, IL-12) and Th2 (IL-10, IL-4) were not upregulated in stimulated PBMCs from women with

chlamydial infertility, the exact role of Th1 and Th2 in chlamydial pathology cannot be elucidated. However, the study has identified markers that contribute to a better understanding of mechanisms that lead to *C. trachomatis*-related tubal factor infertility in women.

This study also evaluated the sero-epidemiological prevalence of *C. trachomatis*-related infertility in Samoa, a developing country with high prevalence of *C. trachomatis* infections in the population. Using PCR, Sullivan *et al.* [506] and Cliff *et al.* [505] previously reported high prevalence rates of 30.9% and 26.8% in women attending antenatal clinics. Given that these prevalence rates have been estimated on a small cohort of women attending antenatal clinics, the prevalence in the population has the potential to be much higher. The study tested the sera of 239 sexually active women between the ages of 18-29 from 41 villages for *C. trachomatis* infections. Walsh *et al.* [516] estimated the prevalence of *C. trachomatis* in the population as 35.6% using PCR.

MEDAC infertile, characterized by a combination of MEDAC MOMP and MEDAC cHSP60 was used to identify women with *C. trachomatis*-related infertility in the population. Additionally, the assay had the best diagnostic performance compared to other commercial serological assays in identifying women with tubal pathology and women with *C. trachomatis*-related infertility (as reported during the evaluation of QUT *Chlamydia* infertility assay). There was a high proportion of women with self-reported infertility (36.4%) based on their epidemiological questionnaire. In support of the high prevalence of self-reported infertility in the population, it was reported that women in low resource settings suffer from high rates of secondary infertility [395]. The WHO estimated the prevalence of secondary infertility caused by sexually transmitted infections to be higher in developing countries such as Sub-Saharan Africa (119/1000 population), Latin America/ Caribbean (71/1000) compared to developed countries such North America/ Western Europe (19/1000) [542].

The concordance between PCR (detects active *C. trachomatis* infection) and serological assays (MEDAC infertile, MEDAC MOMP, MEDAC cHSP60, MEDAC IgA and ANILabsystems) was evaluated and only 8% of samples showed concordance with all serological assays and PCR. Although the serological assays reported a high prevalence rate of *C. trachomatis* infections of 50% (MEDAC

Infertile 52.5%, MEDAC MOMP 55.3%, MEDAC cHSP60 42.02%, ANIlabsystems ELISA 50.9%) in infertile women in the Samoan populations, only MEDAC MOMP could significantly differentiate self-reported infertile women from fertile women (Table 6.3).

This study found that younger women between the ages of 18-24 years were at a higher risk of developing infertility than women between the ages of 25-29 years. Kennedy *et al.* [544] noted that based of DHS reports (Demographic health and survey reports, 2009), the median age for first marriages in women in Samoa were 23.6 years, and while 2.7% of the women between the ages of 15-19 were currently married, 9.4% had commenced childbearing. Thus, high infertility rates reported amongst younger women in this population may be associated with early childbearing.

IgA antibodies in sera is an indication of current infection [165, 357], and in this study, the IgA levels in serum significantly associated with women with PCR positive *C. trachomatis* infection ($p<0.001$). However, contrary to results obtained in this study, *C. trachomatis* specific IgA levels have been reported by others to be higher in women with TFI and persistent chlamydial infection (83.3%), and were further able to discriminate between participants with TFI (n=33) from those with spontaneous miscarriage (n=54)[165]. Van den Broek *et al.* [357] reported that a higher proportion of women from the STI clinic (n=116) who were PCR positive had higher serum IgG (98%) than serum IgA (38%), and further concluded that serum IgG was a better predictor of current/past infection. Interestingly, the study also reported that mucosal IgA showed a high specificity for tubal pathology, comparable to that of serum IgG (Kappa IgA 0.41, $P<0.001$) (n=87). The low prevalence of IgA antibodies and a high prevalence of IgG antibodies in the population may reflect a high rate of repeat infections or past infections.

Antibodies to *C. pneumoniae* are highly prevalent in many populations with 50–70% prevalence reported [531]. Gijsen *et al.* [373] reported that 87% of *C. pneumoniae* were found in 240 subfertile women, which suggests that the prevalence rate of *C. pneumoniae* in women with infertility. Interestingly, it was more prevalent in women without TFI and positive for *C. trachomatis* by MIF (84%) compared to without TFI and seronegative for *C. trachomatis* infections (69%) [373]. Additionally, the *C. pneumoniae* antibodies did not differ significantly between

women with tubal pathology and without tubal pathology [440]. In the Samoan population, antibodies to *C. pneumoniae* showed significant correlation with infertility, but it did correlate with acute *C. trachomatis* as determined by PCR. This reflects on the high prevalence of *C. pneumoniae* in this population, and possible cross-reactivity of chlamydial antibodies exhibited by commercial serological assays.

In this population we have limited knowledge of the other fertility factors, as this was a village based survey study in the absence of any gynecological investigations that would be conducted in a fertility clinic. This lack of gynecological data is a limitation of the study. The important observation is that by any of the assays it appears that approximately half of the women who are identified as infertile in this population have evidence of *C. trachomatis*-related infertility. This is higher than most studies report, even those conducted in fertility clinics, but is consistent with a fertility clinic study in India that found a similar prevalence of 55% in 96 women with secondary infertility [406]. This is likely a reflection of the absence of routine screening and treatment for *C. trachomatis* infections in this population. This may support that the widespread implementation of screening programs in several developed countries is effectively reducing the sequelae such as infertility.

7.1 CONCLUSION

Through this study, the QUT *Chlamydia* infertility test was designed, which is a peptide based diagnostic assay that showed superior sensitivity and specificity compared to all commercial serological assays in identifying women with *C. trachomatis*-related infertility. It is non-invasive, user-friendly, cost-effective and we found that women with predicted chlamydial infertility by QUT infertility test were as likely to conceive via IVF as women with other forms of infertility; thus, making it an ideal diagnostic for early infertility investigation that could recommend IVF without additional tests. The QUT *Chlamydia* infertility test would be an ideal diagnostic tool, particularly in routine testing for low resource settings like Samoa, where the prevalence of *C. trachomatis*-related infertility is very high. This study also estimated the prevalence of chlamydial infertility in a developing country, Samoa, which has a high prevalence of *C. trachomatis* infections. The study also showed that one of the earliest immune response in women with *C. trachomatis*-related infertility include a pro-inflammatory response to infection, which may be associated with tubal pathology an subsequent infertility.

7.2 FUTURE DIRECTIONS

The QUT *Chlamydia* infertility has higher sensitivity and specificity in identifying women with *C. trachomatis*-related infertility compared to other commercial assays. Its performance could be further validated in longitudinal studies that would assess the ability of QUT *Chlamydia* infertility test to predict the risk of women developing tubal pathology and subsequent infertility. QUT *Chlamydia* infertility test could also be applied on the Samoa cohort to determine the risk of infertility in the population and compare its diagnostic performance with the commercial assays used in the study. The assay needs to be further tested for reproducibility and repeatability, an eventually developed for clinical purposes by improving its fitness of purpose by improving its performance costs, sample throughput, shelf-life, turn-around times for test results and quality control and assurance.

The immunological aspect of the study identified the initial markers associated with *C. trachomatis* induced infertility. Further transcriptomic and translational studies on a larger cohort of samples could reveal a better understanding of the pro-inflammatory cytokines in influencing the Th1/Th2 balance that induce pathology. Understanding the immune mechanisms associated with pathogenesis and protective immunity could be instrumental in the development of vaccines against *C. trachomatis* infection. The high prevalence of chlamydial infertility in the women of Samoa stresses the need for routine testing and increasing the awareness regarding STIs and their consequences. Since the mean likelihood of *C. trachomatis* - related infertility was higher in younger women, screening should be implemented for women starting from the age of 15 years. Steps must be taken to ensure that these women are checked for infertility and recommended treatment immediately.

Chapter 8: Appendix

Table 8.1: Demographic and gynaecological characteristics of participants from the infertile cohort recruited from an IVF clinic (development cohort)

Participant ID	Average age (years)	Gynaecological history	Average BMI	Parity	Duration of infertility	Smoking status	Alcohol	Average no. IVF cycles for successful outcome
Tubal factor infertility (n=45)	36.88	History of fibroids (n=12) History of endometriosis (n=14) History of hydrosalpinx (n=9) Polycystic ovaries (n=8) Unilateral tubal blockage (n=16) Bilateral tubal blockage (n=29) History of PID (n=4) Ectopic pregnancy (n=11)	23.75	No pregnancy (n=14) Miscarriage (n=19)	<1year (n=5) 1-2 years (n=10) 2-4 years (n=14) >4 years (n=14)	Past (n=17) Never (n=27) Current (n=1)	Never (n=27) Yes (n=18)	3.55
Non-TFI associated infertility (n=52)	38.26	History of fibroids (n=10) History of endometriosis (n=12) Endometritis (n=3) Polycystic ovaries (n=9) Ectopic pregnancy (n=2)	25.01	No pregnancy (n=10) Miscarriage (n=18)	<1year (n=4) 1-2 years (n=10) 2-4 years (n=22) >4 years (n=14)	Past (n=12) Never (n=37) Current (n=1)	Never (n=31) Yes (n=21)	4.34

Table 8.2: Correlation between demographic and lifestyle factors to tubal infertility (development cohort)

Infertility	Age [Correlation coefficient (p value)]	BMI [correlation coefficient (p value)]	Smoking [correlation coefficient (p value)]	Alcohol consumption [correlation coefficient (p value)]
Tubal pathology	0.136 (0.093)	0.052 (0.306)	0.179 (0.04)*	0.004 (0.485)

*Smoking showed significant correlation with tubal pathology (Spearman correlation coefficient)

Table 8.2: Demographic and gynaecological characteristics of participants from the infertile cohort recruited from IVF clinic (Brisbane) (validation cohort)

Participant ID	Average age (years)	Gynecological history	Average BMI	Parity	No. of sex partners	Smoking status	Pregnancy outcome after IVF	Live birth oucome after IVF
Tubal factor infertility (n=19)	37.33	Tubal adhesion (n=4) Tubal obstruction (n=7) Ovarian cystecty (n=5) PCOS (n=4) Endometritis (n=5) Fibroids (n=1) Salpingitis (n= 6) Ectopic pregnancy (n= 3) PID (n=3)	23.2	No pregnancy (n=10)	<10 (n=2) >10(n=6) <5 (n=11)	Past (n=1) Never (n=18)	No (n=12) Yes (n=7)	No (n=13) Yes (n=4)
Non-TFI associated infertility (n=54)	37.10	History of fibroids (n=1) Endometritis (n=13) Polycystic ovaries (n=14) Ectopic pregnancy (n=1) PID (n=4) Unknown etiology (n=23)	22.76	No pregnancy (n=40)	<10 (n=20) >10(n=12) <5 (n=20)	Past (n=12) Never (n=40) Current (n=2)	Yes (n=19) No (n=35)	Yes (n=28) No (n=26)

Table 8.3: **Demographic and epidemiological information of participants from Samoa**

Demographic/ epidemiological variables	**Number of participants (N=239)**
Employed	
No	196
Yes	43
Occupation	
None	196
Clerical/ office work	12
Hospitality	8
Retail/ sales	6
Health care worker	1
Government worker	2
Police/ military	1
Farmer	3
Street sales	1
Teacher	3
Other	6
Education qualifications	
University degree	4
Tertiary level diploma	11
Tertiary level certificate	30
High school	129
None	65
Fertility problem diagnosed	
Blocked fallopian tubes	1
Unexplained infertility	3
Other	1
No diagnosis	234
Relationship status	
Married	102
Living with partner	43
Separated	19
Single	75

Age of first sex	
19 or older	156
18 or less	83
Number of sex partners in life	
1 sex partner	127
2 sex partners	72
3 or more sex partners	40
Past pregnancy	
No	88
Yes	151
Not intending to stop pregnancy	
Yes, not preventing pregnancy now	13
Yes, in the future	42
No	8
Missing	176
Duration of preventing pregnancy	
Less than 1 year	2
1-2 years	10
Over 2 years	5
Missing	222
Seek medical advice	
Upto 6 months	25
1 year	14
2 years	15
3 years	1
Over 3 years	23
Missing	165
Number of children	
1 child	94
2 children	70
3 children	75
Time taken for first pregnancy	
Less than 12 months	74

12-24 months	41
Greater than 24 months	34
None	94
Trying for a child after last pregnancy	
No	152
Yes	87
Time taken to get pregnant after last pregnancy	
Less than a year	12
1-2 years	10
Over 2 years	26
Don't know	12
Missing	179
Regard family complete	
No	61
Yes	33
Unsure	57
Missing	88
Problems becoming pregnant	
No	96
Yes	55
Missing	88
Age	
18-24 years	145
25-29 years	94
BMI	
Underweight	1
Normal weight	45
Over weight	87
Obese	96
Missing	10
Smoking status	
Current smoker	44
Ex-smoker	7

Never smoked	188
Number of cigarettes per day	
1	1
2	7
3	6
4	3
5	9
6	4
8	1
9	1
10	7
15	1
Missing	199
Frequency of drinking wine	
Never	229
Less than once a week	9
Once or twice a week	1
Frequency of drinking beer	
Never	210
Less than once a week	23
Once or twice a week	4
More than twice a week	2
Frequency of drinking spirits	
Never	219
Less than once a week	17
Once or twice a week	3
Health problems	
None	230
Tubal surgery	3
Pelvic surgery	1
Other pelvic infection	5

Chapter 9: Bibliography

1. Chapron, C.M., F. Pierre, S. Lacroix, D. Querleu, J. Lansac, and J.B. Dubuisson, *Major vascular injuries during gynecologic laparoscopy.* J Am Coll Surg, 1997. **185**(5): p. 461-5.
2. Baud, D., L. Regan, and G. Greub, *Comparison of five commercial serological tests for the detection of anti-Chlamydia trachomatis antibodies.* Eur J Clin Microbiol Infect Dis, 2010. **29**(6): p. 669-75.
3. Data, W.L.C.-i.-P., *Global incidence and prevalence of selected curable sexually transmitted infections – 2008*. 2012, World Health Organization.
4. CDC. http://www.cdc.gov/std/stats/sti-estimates-fact-sheet-feb-2013.pdf. 2013.
5. Haggerty, C.L., S.L. Gottlieb, B.D. Taylor, N. Low, F. Xu, and R.B. Ness, *Risk of sequelae after Chlamydia trachomatis genital infection in women.* J Infect Dis, 2010. **201 Suppl 2**: p. S134-55.
6. Wyrick, P.B., *Intracellular survival by Chlamydia.* Cell Microbiol, 2000. **2**(4): p. 275-82.
7. Land, J.A. and J. Evers, *Chlamydia infection and subfertility.* Best practice & research. Clinical obstetrics & gynaecology, 2002. **16**(6): p. 901.
8. Horn, M., A. Collingro, S. Schmitz-Esser, C.L. Beier, U. Purkhold, B. Fartmann, P. Brandt, G.J. Nyakatura, M. Droege, D. Frishman, T. Rattei, H.W. Mewes, and M. Wagner, *Illuminating the evolutionary history of chlamydiae.* Science, 2004. **304**: p. 728-730.
9. Hackstadt, T., M.A. Scidmore, and D.D. Rockey, *Lipid metabolism in Chlamydia trachomatis-infected cells: directed trafficking of Golgi-derived sphingolipids to the chlamydial inclusion.* Proceedings of the National Academy of Sciences, 1995. **92**(11): p. 4877-4881.
10. Fraiz, J. and R.B. Jones, *Chlamydial infections.* Annual review of medicine, 1988. **39**: p. 357-70.
11. Hatch, T.P., *Disulfide cross-linked envelope proteins: the functional equivalent of peptidoglycan in chlamydiae?* Journal of Bacteriology, 1996. **178**(1): p. 1-5.
12. Ghuysen, J.-M. and C. Goffin, *Lack of Cell Wall Peptidoglycan versus Penicillin Sensitivity: New Insights into the Chlamydial Anomaly.* Antimicrobial Agents and Chemotherapy, 1999. **43**(10): p. 2339-2344.
13. Liechti, G.W., E. Kuru, E. Hall, A. Kalinda, Y.V. Brun, M. VanNieuwenhze, and A.T. Maurelli, *A new metabolic cell-wall labelling method reveals peptidoglycan in Chlamydia trachomatis.* Nature, 2014. **506**(7489): p. 507-10.
14. Batteiger, B.E., W.J.t. Newhall, P. Terho, C.E. Wilde, 3rd, and R.B. Jones, *Antigenic analysis of the major outer membrane protein of Chlamydia trachomatis with murine monoclonal antibodies.* Infect Immun, 1986. **53**(3): p. 530-3.
15. Morre, S.A., J.M. Ossewaarde, J. Lan, G.J. van Doornum, J.M. Walboomers, D.M. MacLaren, C.J. Meijer, and A.J. van den Brule, *Serotyping and*

genotyping of genital Chlamydia trachomatis isolates reveal variants of serovars Ba, G, and J as confirmed by omp1 nucleotide sequence analysis. J Clin Microbiol, 1998. **36**(2): p. 345-51.
16. Mylonas, I., *Female genital Chlamydia trachomatis infection: where are we heading?* Archives of Gynecology and Obstetrics: p. 1-15.
17. Beagley, K., W.M. Huston, P.M. Hansbro, and P. Timms, *Chlamydial Infection of Immune Cells: Altered Function and Implications for Disease.* 2009. **29**(4): p. 275-305.
18. Wyrick, P.B., *Chlamydia trachomatis Persistence In Vitro: An Overview.* Journal of Infectious Diseases, 2010. **201**(Supplement 2): p. S88-S95.
19. Nguyen, B.D., D. Cunningham, X. Liang, X. Chen, E.J. Toone, C.R. Raetz, P. Zhou, and R.H. Valdivia, *Lipooligosaccharide is required for the generation of infectious elementary bodies in Chlamydia trachomatis.* Proc Natl Acad Sci U S A, 2011. **108**(25): p. 10284-9.
20. AbdelRahman, Y.M. and R.J. Belland, *The chlamydial developmental cycle.* FEMS Microbiology Reviews, 2005. **29**(5): p. 949-959.
21. Brickman, T.J., C.E. Barry, and T. Hackstadt, *Molecular cloning and expression of hctB encoding a strain-variant chlamydial histone-like protein with DNA-binding activity.* Journal of Bacteriology, 1993. **175**(14): p. 4274-4281.
22. Hatch, T.P., E. Al-Hossainy, and J.A. Silverman, *Adenine nucleotide and lysine transport in Chlamydia psittaci.* J Bacteriol, 1982. **150**(2): p. 662-70.
23. Hatch, T.P., M. Miceli, and J.E. Sublett, *Synthesis of disulfide-bonded outer membrane proteins during the developmental cycle of Chlamydia psittaci and Chlamydia trachomatis.* J Bacteriol, 1986. **165**(2): p. 379-85.
24. Hatch, T.P., M. Miceli, and J.A. Silverman, *Synthesis of protein in host-free reticulate bodies of Chlamydia psittaci and Chlamydia trachomatis.* J Bacteriol, 1985. **162**(3): p. 938-42.
25. Omsland, A., B.S. Sixt, M. Horn, and T. Hackstadt, *Chlamydial metabolism revisited: interspecies metabolic variability and developmental stage-specific physiologic activities.* FEMS Microbiol Rev, 2014. **38**(4): p. 779-801.
26. Skipp, P., J. Robinson, C.D. O'Connor, and I.N. Clarke, *Shotgun proteomic analysis of Chlamydia trachomatis.* Proteomics, 2005. **5**(6): p. 1558-73.
27. Saka, H.A., J.W. Thompson, Y.S. Chen, Y. Kumar, L.G. Dubois, M.A. Moseley, and R.H. Valdivia, *Quantitative proteomics reveals metabolic and pathogenic properties of Chlamydia trachomatis developmental forms.* Mol Microbiol, 2011. **82**(5): p. 1185-203.
28. Omsland, A., J. Sager, V. Nair, D.E. Sturdevant, and T. Hackstadt, *Developmental stage-specific metabolic and transcriptional activity of Chlamydia trachomatis in an axenic medium.* Proc Natl Acad Sci U S A, 2012. **109**(48): p. 19781-5.
29. Wolf, K., H.J. Betts, B. Chellas-Géry, S. Hower, C.N. Linton, and K.A. Fields, *Treatment of Chlamydia trachomatis with a small molecule inhibitor of the Yersinia type III secretion system disrupts progression of the chlamydial developmental cycle.* Molecular Microbiology, 2006. **61**(6): p. 1543-1555.
30. Hammerschlag, M.R., *Chlamydia trachomatis and Chlamydia pneumoniae infections in children and adolescents.* Pediatr Rev, 2004. **25**(2): p. 43-51.
31. Ajonuma, L.C., K.L. Fok, L.S. Ho, P.K. Chan, P.H. Chow, L.L. Tsang, C.H. Wong, J. Chen, S. Li, D.K. Rowlands, Y.W. Chung, and H.C. Chan, *CFTR is*

required for cellular entry and internalization of Chlamydia trachomatis. Cell biology international, 2010. **34**(6): p. 593-600.
32. Su, H., N.G. Watkins, Y.X. Zhang, and H.D. Caldwell, *Chlamydia trachomatis-host cell interactions: role of the chlamydial major outer membrane protein as an adhesin.* Infect Immun, 1990. **58**(4): p. 1017-25.
33. Hodinka, R.L., C.H. Davis, J. Choong, and P.B. Wyrick, *Ultrastructural study of endocytosis of Chlamydia trachomatis by McCoy cells.* Infection and Immunity, 1988. **56**(6): p. 1456-1463.
34. Majeed, M. and E. Kihlström, *Mobilization of F-actin and clathrin during redistribution of Chlamydia trachomatis to an intracellular site in eucaryotic cells.* Infection and Immunity, 1991. **59**(12): p. 4465-4472.
35. Boleti, H., A. Benmerah, D.M. Ojcius, N. Cerf-Bensussan, and A. Dautry-Varsat, *Chlamydia infection of epithelial cells expressing dynamin and Eps15 mutants: clathrin-independent entry into cells and dynamin-dependent productive growth.* Journal of Cell Science, 1999. **112**(10): p. 1487-1496.
36. Carabeo, R.A., S.S. Grieshaber, E. Fischer, and T. Hackstadt, *Chlamydia trachomatis Induces Remodeling of the Actin Cytoskeleton during Attachment and Entry into HeLa Cells.* Infection and Immunity, 2002. **70**(7): p. 3793-3803.
37. Reynolds, D.J. and J.H. Pearce, *Endocytic mechanisms utilized by chlamydiae and their influence on induction of productive infection.* Infection and Immunity, 1991. **59**(9): p. 3033-3039.
38. Norkin, L.C., Wolfrom, S.A. and Stuart, E.S. *Association of Caveolin with Chlamydia trachomatis Inclusions at Early and Late Stages of Infection.* Experimental Cell Research, 2001. **266**(2): p. 229-238.
39. Stuart, E.S., Webley, W.C. and Norkin, L.C. *Lipid rafts, caveolae, caveolin-1, and entry by Chlamydiae into host cells.* Experimental Cell Research, 2003. **287**(1): p. 67-78.
40. Hybiske, K. and R.S. Stephens, *Mechanisms of Chlamydia trachomatis entry into nonphagocytic cells.* Infect Immun, 2007. **75**(8): p. 3925-34.
41. Clifton, D.R., K.A. Fields, S.S. Grieshaber, C.A. Dooley, E.R. Fischer, D.J. Mead, R.A. Carabeo, and T. Hackstadt, *A chlamydial type III translocated protein is tyrosine-phosphorylated at the site of entry and associated with recruitment of actin.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(27): p. 10166-10171.
42. Backert, S. and M. Selbach, *Tyrosine-phosphorylated bacterial effector proteins: the enemies within.* Trends in Microbiology, 2005. **13**(10): p. 476-484.
43. Lane, B.J., C. Mutchler, S. Al Khodor, S.S. Grieshaber, and R.A. Carabeo, *Chlamydial entry involves TARP binding of guanine nucleotide exchange factors.* PLoS Pathog, 2008. **4**(3): p. e1000014.
44. Wyrick, P.B., C.H. Davis, S.T. Knight, J. Choong, J.E. Raulston, Schramm, and Nara, *An In Vitro Human Epithelial Cell Culture System for Studying the Pathogenesis of Chlamydia trachomatis.* Sexually Transmitted Diseases, 1993. **20**(5): p. 248-256.
45. Maslow, A.S., C.H. Davis, J. Choong, and P.B. Wyrick, *Estrogen enhances attachment of Chlamydia trachomatis to human endometrial epithelial cells in vitro.* American journal of obstetrics and gynecology, 1988. **159**(4): p. 1006-14.

46. Hall, J.V., M. Schell, S. Dessus-Babus, C.G. Moore, J.D. Whittimore, M. Sal, B.D. Dill, and P.B. Wyrick, *The multifaceted role of oestrogen in enhancing Chlamydia trachomatis infection in polarized human endometrial epithelial cells.* Cell Microbiol, 2011. **13**(8): p. 1183-99.
47. Lutter, E.I., C. Martens, and T. Hackstadt, *Evolution and Conservation of Predicted Inclusion Membrane Proteins in Chlamydiae.* Comparative and Functional Genomics, 2012. **2012**: p. 13.
48. Carabeo, R.A., S.S. Grieshaber, E. Fischer, and T. Hackstadt, *Chlamydia trachomatis induces remodeling of the actin cytoskeleton during attachment and entry into HeLa cells.* Infect Immun, 2002. **70**(7): p. 3793-803.
49. Rzomp, K.A., A.R. Moorhead, and M.A. Scidmore, *The GTPase Rab4 Interacts with Chlamydia trachomatis Inclusion Membrane Protein CT229.* Infection and Immunity, 2006. **74**(9): p. 5362-5373.
50. Rzomp, K.A., L.D. Scholtes, B.J. Briggs, G.R. Whittaker, and M.A. Scidmore, *Rab GTPases Are Recruited to Chlamydial Inclusions in Both a Species-Dependent and Species-Independent Manner.* Infection and Immunity, 2003. **71**(10): p. 5855-5870.
51. Capmany, A. and M.T. Damiani, *Chlamydia trachomatis Intercepts Golgi-Derived Sphingolipids through a Rab14-Mediated Transport Required for Bacterial Development and Replication.* PLoS ONE, 2010. **5**(11): p. e14084.
52. Marci A, S., *Recent advances in Chlamydia subversion of host cytoskeletal and membrane trafficking pathways.* Microbes and Infection, 2011. **13**(6): p. 527-535.
53. Moorhead, A.M., J.-Y. Jung, A. Smirnov, S. Kaufer, and M.A. Scidmore, *Multiple Host Proteins That Function in Phosphatidylinositol-4-Phosphate Metabolism Are Recruited to the Chlamydial Inclusion.* Infection and Immunity, 2010. **78**(5): p. 1990-2007.
54. Vicinanza, M., G. D'Angelo, A. Di Campli, and M.A. De Matteis, *Function and dysfunction of the PI system in membrane trafficking.* EMBO J, 2008. **27**(19): p. 2457-2470.
55. Wylie, J.L., G.M. Hatch, and G. McClarty, *Host cell phospholipids are trafficked to and then modified by Chlamydia trachomatis.* Journal of Bacteriology, 1997. **179**(23): p. 7233-42.
56. Moore, E.R., E.R. Fischer, D.J. Mead, and T. Hackstadt, *The Chlamydial Inclusion Preferentially Intercepts Basolaterally Directed Sphingomyelin-Containing Exocytic Vacuoles.* Traffic, 2008. **9**(12): p. 2130-2140.
57. Hackstadt, T., E.R. Fischer, M.A. Scidmore, D.D. Rockey, and R.A. Heinzen, *Origins and functions of the chlamydial inclusion.* Trends in Microbiology, 1997. **5**(7): p. 288-293.
58. Mital, J. and T. Hackstadt, *Role for the Src Family Kinase Fyn in Sphingolipid Acquisition by Chlamydiae.* Infection and Immunity, 2011. **79**(11): p. 4559-4568.
59. Heuer, D., A.R. Lipinski, N. Machuy, A. Karlas, A. Wehrens, F. Siedler, V. Brinkmann, and T.F. Meyer, *Chlamydia causes fragmentation of the Golgi compartment to ensure reproduction.* Nature, 2009. **457**(7230): p. 731-735.
60. Cocchiaro, J.L., Y. Kumar, E.R. Fischer, T. Hackstadt, and R.H. Valdivia, *Cytoplasmic lipid droplets are translocated into the lumen of the Chlamydia trachomatis parasitophorous vacuole.* Proceedings of the National Academy of Sciences, 2008.

61. Soupene, E., D. Wang, and F.A. Kuypers, *Remodeling of host phosphatidylcholine by Chlamydia acyltransferase is regulated by acyl-CoA binding protein ACBD6 associated with lipid droplets.* Microbiologyopen, 2015.
62. Kumar, Y., J. Cocchiaro, and R.H. Valdivia, *The Obligate Intracellular Pathogen Chlamydia trachomatis Targets Host Lipid Droplets.* Current Biology, 2006. **16**(16): p. 1646-1651.
63. Bannantine, Griffiths, Viratyosin, Brown, and Rockey, *A secondary structure motif predictive of protein localization to the chlamydial inclusion membrane.* Cellular Microbiology, 2000. **2**(1): p. 35-47.
64. Rockey, D.D., R.A. Heinzen, and T. Hackstadt, *Cloning and characterization of a Chlamydia psittaci gene coding for a protein localized in the inclusion membrane of infected cells.* Molecular Microbiology, 1995. **15**(4): p. 617-626.
65. Bannantine, J.P., W.E. Stamm, R.J. Suchland, and D.D. Rockey, *Chlamydia trachomatis IncA Is Localized to the Inclusion Membrane and Is Recognized by Antisera from Infected Humans and Primates.* Infection and Immunity, 1998. **66**(12): p. 6017-6021.
66. Li, Z., C. Chen, D. Chen, Y. Wu, Y. Zhong, and G. Zhong, *Characterization of Fifty Putative Inclusion Membrane Proteins Encoded in the Chlamydia trachomatis Genome.* Infection and Immunity, 2008. **76**(6): p. 2746-2757.
67. Rockey, D.D., D. Grosenbach, D.E. Hruby, M.G. Peacock, R.A. Heinzen, and T. Hackstadt, *Chlamydia psittaci IncA is phosphorylated by the host cell and is exposed on the cytoplasmic face of the developing inclusion.* Molecular Microbiology, 1997. **24**(1): p. 217-228.
68. Hackstadt, T., M.A. Scidmore-Carlson, E.I. Shaw, and E.R. Fischer, *The Chlamydia trachomatis IncA protein is required for homotypic vesicle fusion.* Cellular Microbiology, 1999. **1**(2): p. 119-130.
69. Delevoye, C., M. Nilges, P. Dehoux, F. Paumet, S. Perrinet, A. Dautry-Varsat, and A. Subtil, *SNARE Protein Mimicry by an Intracellular Bacterium.* PLoS Pathog, 2008. **4**(3): p. e1000022.
70. Scidmore, M.A. and T. Hackstadt, *Mammalian 14-3-3β associates with the Chlamydia trachomatis inclusion membrane via its interaction with IncG.* Molecular Microbiology, 2001. **39**(6): p. 1638-1650.
71. Mital, J., N.J. Miller, E.R. Fischer, and T. Hackstadt, *Specific chlamydial inclusion membrane proteins associate with active Src family kinases in microdomains that interact with the host microtubule network.* Cellular Microbiology, 2010. **12**(9): p. 1235-1249.
72. Brumell, J.H. and M.A. Scidmore, *Manipulation of Rab GTPase Function by Intracellular Bacterial Pathogens.* Microbiology and Molecular Biology Reviews, 2007. **71**(4): p. 636-652.
73. Derré, I., R. Swiss, and H. Agaisse, *The Lipid Transfer Protein CERT Interacts with the Chlamydianclusion Protein IncD and Participates to ER ChlamydiaInclusion Membrane Contact Sites.* PLoS Pathog, 2011. **7**(6): p. e1002092.
74. Dehoux, P., R. Flores, C. Dauga, G. Zhong, and A. Subtil, *Multi-genome identification and characterization of chlamydiae-specific type III secretion substrates: the Inc proteins.* BMC Genomics, 2011. **12**(1): p. 109.
75. Brinkworth, A.J., D.S. Malcolm, A.T. Pedrosa, K. Roguska, S. Shahbazian, J.E. Graham, R.D. Hayward, and R.A. Carabeo, *Chlamydia trachomatis Slc1*

is a type III secretion chaperone that enhances the translocation of its invasion effector substrate TARP. Molecular Microbiology, 2011. **82**(1): p. 131-144.
76. Christian, J.G., J. Heymann, S.A. Paschen, J. Vier, L. Schauenburg, J. Rupp, T.F. Meyer, G. Häcker, and D. Heuer, *Targeting of a Chlamydial Protease Impedes Intracellular Bacterial Growth.* PLoS Pathog, 2011. **7**(9): p. e1002283.
77. Wu, X., L. Lei, S. Gong, D. Chen, R. Flores, and G. Zhong, *The chlamydial periplasmic stress response serine protease cHtrA is secreted into host cell cytosol.* BMC Microbiology, 2011. **11**(1): p. 87.
78. Wolf, K., H. Betts, B. Chellas-Gery, S. Hower, C. Linton, and K. Fields, *Treatment of Chlamydia trachomatis with a small molecule inhibitor of the Yersinia type III secretion system disrupts progression of the chlamydial developmental cycle.* Mol Microbiol, 2006. **61**(6): p. 1543 - 1555.
79. Chen, Y.S., R.J. Bastidas, H.A. Saka, V.K. Carpenter, K.L. Richards, G.V. Plano, and R.H. Valdivia, *The Chlamydia trachomatis type III secretion chaperone Slc1 engages multiple early effectors, including TepP, a tyrosine-phosphorylated protein required for the recruitment of CrkI-II to nascent inclusions and innate immune signaling.* PLoS Pathog, 2014. **10**(2): p. e1003954.
80. Dille, S., K. Herbst, L. Volceanov, T. Nolke, O. Kretz, and G. Hacker, *Golgi fragmentation and sphingomyelin transport to Chlamydia trachomatis during penicillin-induced persistence do not depend on the cytosolic presence of the chlamydial protease CPAF.* PLoS One, 2014. **9**(7): p. e103220.
81. Hybiske, K. and R.S. Stephens, *Mechanisms of host cell exit by the intracellular bacterium Chlamydia.* Proc Natl Acad Sci U S A, 2007. **104**(27): p. 11430-5.
82. Shaw, E.I., C.A. Dooley, E.R. Fischer, M.A. Scidmore, K.A. Fields, and T. Hackstadt, *Three temporal classes of gene expression during the Chlamydia trachomatis developmental cycle.* Molecular Microbiology, 2000. **37**(4): p. 913-925.
83. Klint, M., M. Thollesson, E. Bongcam-Rudloff, S. Birkelund, A. Nilsson, and B. Herrmann, *Mosaic structure of intragenic repetitive elements in histone H1-like protein Hc2 varies within serovars of Chlamydia trachomatis.* BMC Microbiology, 2010. **10**(1): p. 81.
84. Belland, R.J., G. Zhong, D.D. Crane, D. Hogan, D. Sturdevant, J. Sharma, W.L. Beatty, and H.D. Caldwell, *Genomic transcriptional profiling of the developmental cycle of Chlamydia trachomatis.* Proceedings of the National Academy of Sciences, 2003. **100**(14): p. 8478-8483.
85. Chin, E., K. Kirker, M. Zuck, G. James, and K. Hybiske, *Actin recruitment to the Chlamydia inclusion is spatiotemporally regulated by a mechanism that requires host and bacterial factors.* PLoS One, 2012. **7**(10): p. e46949.
86. Volceanov, L., K. Herbst, M. Biniossek, O. Schilling, D. Haller, T. Nolke, P. Subbarayal, T. Rudel, B. Zieger, and G. Hacker, *Septins arrange F-actin-containing fibers on the Chlamydia trachomatis inclusion and are required for normal release of the inclusion by extrusion.* MBio, 2014. **5**(5): p. e01802-14.
87. Christian, J.G., J. Heymann, S.A. Paschen, J. Vier, L. Schauenburg, J. Rupp, T.F. Meyer, G. Hacker, and D. Heuer, *Targeting of a chlamydial protease*

impedes intracellular bacterial growth. PLoS Pathog, 2011. **7**(9): p. e1002283.
88. Matsumoto, A. and G.P. Manire, *Electron Microscopic Observations on the Effects of Penicillin on the Morphology of Chlamydia psittaci.* Journal of Bacteriology, 1970. **101**(1): p. 278-285.
89. Skilton, R.J., L.T. Cutcliffen, D. Barlow, Y. Wang, O. Salim, P.R. Lambden, and I.N. Clarke, *Penicillin induced persistence in Chlamydia trachomatis: high quality time lapse video analysis of the developmental cycle.* PLoS One, 2009. **4**(11): p. e7723.
90. Huston, W., C. Theodoropoulos, S. Mathews, and P. Timms, *Chlamydia trachomatis responds to heat shock, penicillin induced persistence, and IFN-gamma persistence by altering levels of the extracytoplasmic stress response protease HtrA.* BMC Microbiol, 2008. **8**: p. 190.
91. Ong, V.A., J.W. Marsh, A. Lawrence, J.A. Allan, P. Timms, and W.M. Huston, *The protease inhibitor JO146 demonstrates a critical role for CtHtrA for Chlamydia trachomatis reversion from penicillin persistence.* Front Cell Infect Microbiol, 2013. **3**: p. 100.
92. Roshick, C., H. Wood, H.D. Caldwell, and G. McClarty, *Comparison of gamma interferon-mediated antichlamydial defense mechanisms in human and mouse cells.* Infect Immun, 2006. **74**(1): p. 225-38.
93. Beatty, W.L., T.A. Belanger, A.A. Desai, R.P. Morrison, and G.I. Byrne, *Tryptophan depletion as a mechanism of gamma interferon-mediated chlamydial persistence.* Infect Immun, 1994. **62**(9): p. 3705-11.
94. Leonhardt, R.M., S.J. Lee, P.B. Kavathas, and P. Cresswell, *Severe tryptophan starvation blocks onset of conventional persistence and reduces reactivation of Chlamydia trachomatis.* Infect Immun, 2007. **75**(11): p. 5105-17.
95. Belland, R.J., D.E. Nelson, D. Virok, D.D. Crane, D. Hogan, D. Sturdevant, W.L. Beatty, and H.D. Caldwell, *Transcriptome analysis of chlamydial growth during IFN-gamma-mediated persistence and reactivation.* Proc Natl Acad Sci U S A, 2003. **100**(26): p. 15971-6.
96. Akers, J.C. and M. Tan, *Molecular mechanism of tryptophan-dependent transcriptional regulation in Chlamydia trachomatis.* J Bacteriol, 2006. **188**(12): p. 4236-43.
97. Rodel, J., C. Grosse, H. Yu, K. Wolf, G.P. Otto, E. Liebler-Tenorio, V. Forsbach-Birk, and E. Straube, *Persistent Chlamydia trachomatis infection of HeLa cells mediates apoptosis resistance through a Chlamydia protease-like activity factor-independent mechanism and induces high mobility group box 1 release.* Infect Immun, 2012. **80**(1): p. 195-205.
98. Thompson, C.C. and R.A. Carabeo, *An optimal method of iron starvation of the obligate intracellular pathogen, Chlamydia trachomatis.* Front Microbiol, 2011. **2**: p. 20.
99. Dill, B.D. and J.E. Raulston, *Examination of an inducible expression system for limiting iron availability during Chlamydia trachomatis infection.* Microbes Infect, 2007. **9**(8): p. 947-53.
100. LaRue, R.W., B.D. Dill, D.K. Giles, J.D. Whittimore, and J.E. Raulston, *Chlamydial Hsp60-2 is iron responsive in Chlamydia trachomatis serovar E-infected human endometrial epithelial cells in vitro.* Infect Immun, 2007. **75**(5): p. 2374-80.

101. Rekart, M.L., M. Gilbert, R. Meza, P.H. Kim, M. Chang, D.M. Money, and R.C. Brunham, *Chlamydia public health programs and the epidemiology of pelvic inflammatory disease and ectopic pregnancy.* Journal of Infectious Diseases, 2012.
102. Paavonen, J. and W. Eggert-Kruse, *Chlamydia trachomatis: impact on human reproduction.* Human reproduction update, 1999. **5**(5): p. 433-447.
103. Price, M.J., A.E. Ades, N.J. Welton, J. Macleod, K. Turner, I. Simms, and P.J. Horner, *How much tubal factor infertility is caused by Chlamydia? Estimates based on serological evidence corrected for sensitivity and specificity.* Sex Transm Dis, 2012. **39**(8): p. 608-13.
104. Wiesenfeld, H.C., S.L. Hillier, L.A. Meyn, A.J. Amortegui, and R.L. Sweet, *Subclinical Pelvic Inflammatory Disease and Infertility.* Obstetrics & Gynecology, 2012. **120**(1): p. 37-43.
105. Wilkowska-Trojniel, M., B. Zdrodowska-Stefanow, I. Ostaszewska-Puchalska, M. Zbucka, S. Wolczynski, C. Grygoruk, W. Kuczynski, and M. Zdrodowski, *Chlamydia trachomatis urogenital infection in women with infertility.* Adv Med Sci, 2009. **54**(1): p. 82-5.
106. McCormack, W.M., *Pelvic inflammatory disease.* N Engl J Med, 1994. **330**(2): p. 115-9.
107. *Pelvic Inflammatory Disease*. 2012 [cited 2013 15/01/2013]; Available from: http://www.betterhealth.vic.gov.au/bhcv2/bhcarticles.nsf/pages/Pelvic_inflammatory_disease.
108. Sweet, R.L., *Treatment of acute pelvic inflammatory disease.* Infect Dis Obstet Gynecol, 2011. **2011**: p. 561909.
109. Rodriguez-Cerdeira, C., E. Sanchez-Blanco, A. Molares-Vila, and A. Alba, *Unveiling New Molecular Factors Useful for Detection of Pelvic Inflammatory Disease due to Chlamydia trachomatis Infection.* ISRN Obstet Gynecol, 2012. **2012**: p. 581725.
110. Prevention., C.f.D.C.a. *Sexually transmitted disease surveillance*. 2010 March 2012]; Available from: http://www.cdc.gov/std/pid/STDFact-PID.htm.
111. Davies, B., K. Turner, and H. Ward, *Risk of pelvic inflammatory disease after Chlamydia infection in a prospective cohort of sex workers.* Sex Transm Dis, 2013. **40**(3): p. 230-4.
112. Scholes, D., C.L. Satterwhite, O. Yu, D. Fine, H. Weinstock, and S. Berman, *Long-term trends in Chlamydia trachomatis infections and related outcomes in a U.S. managed care population.* Sex Transm Dis, 2012. **39**(2): p. 81-8.
113. Herzog, S.A., J.C. Heijne, P. Scott, C.L. Althaus, and N. Low, *Direct and indirect effects of screening for Chlamydia trachomatis on the prevention of pelvic inflammatory disease: a mathematical modeling study.* Epidemiology, 2013. **24**(6): p. 854-62.
114. Osser, S., K. Persson, and P. Liedholm, *Tubal infertility and silent chlamydial salpingitis.* Human reproduction, 1989. **4**(3): p. 280-284.
115. Paavonen, J., K. Teisala, P.K. Heinonen, R. Aine, S. Laine, M. Lehtinen, A. Miettinen, R. Punnonen, and P. GrÖNroos, *Microbiological and histopathological findings in acute pelvic inflammatory disease.* BJOG: An International Journal of Obstetrics & Gynaecology, 1987. **94**(5): p. 454-460.
116. Jaiyeoba, O. and D.E. Soper, *Cervicitis and Pelvic Inflammatory Disease.* Sexually Transmitted Diseases, 2012. **16**: p. 14.
117. Sweet, R.L., M. Blankfort-Doyle, M.O. Robbie, and J. Schacter, *The occurrence of chlamydial and gonococcal salpingitis during the menstrual*

cycle. JAMA: the journal of the American Medical Association, 1986. **255**(15): p. 2062-2064.
118. Rank, R. and M. Sanders, *Pathogenesis of endometritis and salpingitis in a guinea pig model of chlamydial genital infection.* The American journal of pathology, 1992. **140**(4): p. 927.
119. Aboulghar, M., R. Mansour, and G. Serour, *Controversies in the modern management of hydrosalpinx.* Human Reproduction Update, 1998. **4**(6): p. 882-890.
120. Treharne, J.D., K.T. Ripa, P.A. Mardh, L. Svensson, L. Westrom, and S. Darougar, *Antibodies to Chlamydia trachomatis in acute salpingitis.* Br J Vener Dis, 1979. **55**(1): p. 26-9.
121. Sweet, R.L., *Treatment of acute pelvic inflammatory disease.* Infectious diseases in obstetrics and gynecology, 2011. **2011**.
122. Malik, A., S. Jain, S. Hakim, I. Shukla, and M. Rizvi, *Chlamydia trachomatis infection & female infertility.* The Indian journal of medical research, 2006. **123**(6): p. 770.
123. Gnoth, C., E. Godehardt, P. Frank-Herrmann, K. Friol, J. Tigges, and G. Freundl, *Definition and prevalence of subfertility and infertility.* Human Reproduction, 2005. **20**(5): p. 1144-1147.
124. Walker, J., S.N. Tabrizi, C.K. Fairley, M.Y. Chen, C.S. Bradshaw, J. Twin, N. Taylor, B. Donovan, J.M. Kaldor, and K. McNamee, *Chlamydia trachomatis Incidence and Re-Infection among Young Women–Behavioural and Microbiological Characteristics.* PloS one, 2012. **7**(5): p. e37778.
125. Morhason-Bello, I., O. Ojengbede, A. Oladokun, B. Adedokun, A. Ajayi, A. Adeyanju, O. Ogundepo, and O. Kareem, *The prevalence and outcome of asymptomatic chlamydial infection screening among infertile women attending gynecological clinic in ibadan, South west Nigeria.* Ann Med Health Sci Res, 2014. **4**(2): p. 253-7.
126. Hjelholt, A., G. Christiansen, T.G. Johannesson, H.J. Ingerslev, and S. Birkelund, *Tubal factor infertility is associated with antibodies against Chlamydia trachomatis heat shock protein 60 (HSP60) but not human HSP60.* Hum Reprod, 2011. **26**(8): p. 2069-76.
127. Machado, A.C., E.M. Guimaraes, E. Sakurai, F.C. Fioravante, W.N. Amaral, and M.F. Alves, *High titers of Chlamydia trachomatis antibodies in Brazilian women with tubal occlusion or previous ectopic pregnancy.* Infect Dis Obstet Gynecol, 2007. **2007**: p. 24816.
128. Nwankwo, E.O. and M.N. Sadiq, *Prevalence of Chlamydia trachomatis infection among patients attending infertility and sexually transmitted diseases clinic (STD) in Kano, North Western Nigeria.* Afr Health Sci, 2014. **14**(3): p. 672-8.
129. Mårdh, P.-A., *Influence of infection with Chlamydia trachomatis on pregnancy outcome, infant health and life-long sequelae in infected offspring.* Best Practice & Research Clinical Obstetrics & Gynaecology, 2002. **16**(6): p. 847-864.
130. Gencay, M., M. Koskiniemi, P. ÄMmÄLÄ, V. Fellman, A. NÄRvÄNen, T. Wahlström, A. Vaheri, and M. Puolakkainen, *Chlamydia trachomatis seropositivity is associated both with stillbirth and preterm delivery.* Apmis, 2000. **108**(9): p. 584-588.

131. Coste, J., B. Laumon, A. Bremond, P. Collet, and N. Job-Spira, *Sexually transmitted diseases as major causes of ectopic pregnancy: results from a large case-control study in France.* Fertil Steril, 1994. **62**(2): p. 289-95.
132. Svensson, L., P. Mårdh, M. Ahlgren, and F. Nordenskjöld, *Ectopic pregnancy and antibodies to Chlamydia trachomatis.* Fertility and Sterility, 1985. **44**(3): p. 313.
133. Sziller, I., S. Witkin, M. Ziegert, Z. Csapo, A. Ujhazy, and Z. Papp, *Serological responses of patients with ectopic pregnancy to epitopes of the Chlamydia trachomatis 60 kDa heat shock protein.* Human Reproduction, 1998. **13**(4): p. 1088-1093.
134. Agholor, K., L. Omo-Aghoja, and F. Okonofua, *Association of anti-Chlamydia antibodies with ectopic pregnancy in Benin city, Nigeria: a case-control study.* Afr Health Sci, 2013. **13**(2): p. 430-40.
135. Sziller, I., P. Fedorcsak, Z. Csapo, K. Szirmai, I.M. Linhares, Z. Papp, and S.S. Witkin, *Circulating antibodies to a conserved epitope of the Chlamydia trachomatis 60-kDa heat shock protein is associated with decreased spontaneous fertility rate in ectopic pregnant women treated by salpingectomy.* Am J Reprod Immunol, 2008. **59**(2): p. 99-104.
136. Lan, J., A.J. van den Brule, D.J. Hemrika, E.K. Risse, J.M. Walboomers, M.E. Schipper, and C.J. Meijer, *Chlamydia trachomatis and ectopic pregnancy: retrospective analysis of salpingectomy specimens, endometrial biopsies, and cervical smears.* Journal of Clinical Pathology, 1995. **48**(9): p. 815-9.
137. Mol, F., N.M. van Mello, A. Strandell, K. Strandell, D. Jurkovic, J. Ross, K.T. Barnhart, T.M. Yalcinkaya, H.R. Verhoeve, G.C.M. Graziosi, C.A.M. Koks, I. Klinte, L. Hogström, I.C.A.H. Janssen, H. Kragt, A. Hoek, T.C.M. Trimbos-Kemper, F.J.M. Broekmans, W.N.P. Willemsen, W.M. Ankum, B.W. Mol, M. van Wely, F. van der Veen, and P.J. Hajenius, *Salpingotomy versus salpingectomy in women with tubal pregnancy (ESEP study): an open-label, multicentre, randomised controlled trial.* The Lancet. **383**(9927): p. 1483-1489.
138. Khin Nwe, O., A. Wah Wah, T. Moe, W. Khin Thet, Y. Hta Hta, and M. Win Win, *Case-control study of ectopic pregnancies in Myanmar: infectious etiological factors.* Southeast Asian J Trop Med Public Health, 2011. **42**(2): p. 347-54.
139. Barlow, R.E., I.D. Cooke, O. Odukoya, M.K. Heatley, J. Jenkins, G. Narayansingh, S.S. Ramsewak, and A. Eley, *The prevalence of Chlamydia trachomatis in fresh tissue specimens from patients with ectopic pregnancy or tubal factor infertility as determined by PCR and in-situ hybridisation.* J Med Microbiol, 2001. **50**(10): p. 902-8.
140. Mardh, P.A., B.R. Moller, H.J. Ingerselv, E. Nussler, L. Westrom, and P. Wolner-Hanssen, *Endometritis caused by Chlamydia trachomatis.* Br J Vener Dis, 1981. **57**(3): p. 191-5.
141. Mardh, P.A. and P. Wolner-Hanssen, *Three novel manifestations of chlamydia trachomatis infection- endometritis, perihepatitis and meningoencephalitis.* Infection, 1982. **10 Suppl 1**: p. S57-60.
142. Tait, I.A., S.J. Duthie, and D. Taylor-Robinson, *Silent upper genital tract chlamydial infection and disease in women.* Int J STD AIDS, 1997. **8**(5): p. 329-31.

143. Persson, K. and S. Osser, *Lack of evidence of a relationship between genital symptoms, cervicitis and salpingitis and different serovars of Chlamydia trachomatis.* Eur J Clin Microbiol Infect Dis, 1993. **12**(3): p. 195-9.
144. Wolner-Hanssen, P., P.A. Mardh, L. Svensson, and L. Westrom, *Laparoscopy in women with chlamydial infection and pelvic pain: a comparison of patients with and without salpingitis.* Obstet Gynecol, 1983. **61**(3): p. 299-303.
145. Ness, R.B., D.E. Soper, H.E. Richter, H. Randall, J.F. Peipert, D.B. Nelson, D. Schubeck, S.G. McNeeley, W. Trout, D.C. Bass, K. Hutchison, K. Kip, and R.C. Brunham, *Chlamydia antibodies, chlamydia heat shock protein, and adverse sequelae after pelvic inflammatory disease: the PID Evaluation and Clinical Health (PEACH) Study.* Sex Transm Dis, 2008. **35**(2): p. 129-35.
146. Westr¨om, L., R. Joesoef, G. Reynolds, A. Hadgu, and S.E. Tompson, *Pelvic inflammatory disease and fertility. A cohort study of 1844 women with laparoscopically verified disease and 657 control women with normal laparoscopic results.* Sex Trans Dis 1992. **19**: p. 185-192.
147. Korn, A.P., N.A. Hessol, N.S. Padian, G.A. Bolan, E. Donegan, D.V. Landers, and J. Schachter, *Risk factors for plasma cell endometritis among women with cervical Neisseria gonorrhoeae, cervical Chlamydia trachomatis, or bacterial vaginosis.* Am J Obstet Gynecol, 1998. **178**(5): p. 987-90.
148. Den Hartog, J., S. Morre, and J. Land, *Chlamydia trachomatis-associated tubal factor subfertility: immunogenetic aspects and serological screening.* Human reproduction update, 2006. **12**(6): p. 719-730.
149. Whittington, W.L.H., C. Kent, P. Kissinger, M.K. Oh, J.D. Fortenberry, S.E. Hillis, B. Litchfield, G.A. Bolan, M.E.S.T. LOUIS, and T.A. Farley, *Determinants of persistent and recurrent Chlamydia trachomatis infection in young women: results of a multicenter cohort study.* Sexually Transmitted Diseases, 2001. **28**(2): p. 117-123.
150. Dreses-Werringloer, U., I. Padubrin, B. Jürgens-Saathoff, A.P. Hudson, H. Zeidler, and L. Köhler, *Persistence of Chlamydia trachomatis is induced by ciprofloxacin and ofloxacin in vitro.* Antimicrobial Agents and Chemotherapy, 2000. **44**(12): p. 3288-3297.
151. Beatty, W.L., T.A. Belanger, A.A. Desai, R.P. Morrison, and G.I. Byrne, *Tryptophan depletion as a mechanism of gamma interferon-mediated chlamydial persistence.* Infection and Immunity, 1994. **62**(9): p. 3705-3711.
152. Ramsey, K.H., G.S. Miranpuri, I.M. Sigar, S. Ouellette, and G.I. Byrne, *Chlamydia trachomatis persistence in the female mouse genital tract: inducible nitric oxide synthase and infection outcome.* Infection and Immunity, 2001. **69**(8): p. 5131-5137.
153. Beatty, W.L., G.I. Byrne, and R.P. Morrison, *Morphologic and antigenic characterization of interferon gamma-mediated persistent Chlamydia trachomatis infection in vitro.* Proceedings of the National Academy of Sciences, 1993. **90**(9): p. 3998-4002.
154. Raulston, J.E., *Response of Chlamydia trachomatis serovar E to iron restriction in vitro and evidence for iron-regulated chlamydial proteins.* Infection and Immunity, 1997. **65**(11): p. 4539-4547.
155. Harper, A., C.I. Pogson, M.L. Jones, and J.H. Pearce, *Chlamydial development is adversely affected by minor changes in amino acid supply,*

blood plasma amino acid levels, and glucose deprivation. Infection and Immunity, 2000. **68**(3): p. 1457-1464.
156. Ibana, J.A., R.J. Belland, A.H. Zea, D.J. Schust, T. Nagamatsu, Y.M. AbdelRahman, D.J. Tate, W.L. Beatty, A.A. Aiyar, and A.J. Quayle, *Inhibition of indoleamine 2,3-dioxygenase activity by levo-1-methyl tryptophan blocks gamma interferon-induced Chlamydia trachomatis persistence in human epithelial cells.* Infect Immun, 2011. **79**(11): p. 4425-37.
157. Leonhardt, R.M., S.J. Lee, P.B. Kavathas, and P. Cresswell, *Severe tryptophan starvation blocks onset of conventional persistence and reduces reactivation of Chlamydia trachomatis.* Infection and Immunity, 2007. **75**(11): p. 5105-5117.
158. Singla, M., *Role of Tryptophan supplementation in the treatment of Chlamydia.* Medical hypotheses, 2007. **68**(2): p. 278-280.
159. Reveneau, N., D.D. Crane, E. Fischer, and H.D. Caldwell, *Bactericidal activity of first-choice antibiotics against gamma interferon-induced persistent infection of human epithelial cells by Chlamydia trachomatis.* Antimicrobial Agents and Chemotherapy, 2005. **49**(5): p. 1787-1793.
160. Kintner, J., D. Lajoie, J. Hall, J. Whittimore, and R.V. Schoborg, *Commonly prescribed beta-lactam antibiotics induce C. trachomatis persistence/stress in culture at physiologically relevant concentrations.* Front Cell Infect Microbiol, 2014. **4**: p. 44.
161. Gérard, H.C., L. Köhler, P.J. Branigan, H. Zeidler, H.R. Schumacher, and A.P. Hudson, *Viability and gene expression in Chlamydia trachomatis during persistent infection of cultured human monocytes.* Medical microbiology and immunology, 1998. **187**(2): p. 115-120.
162. Dean, D., R.J. Suchland, and W.E. Stamm, *Evidence for long-term cervical persistence of Chlamydia trachomatis by omp1 genotyping.* Journal of Infectious Diseases, 2000. **182**(3): p. 909-916.
163. Koehler, L., E. Nettelnbreker, A. Hudson, N. Ott, H. Gerard, P. Branigan, H. Schumacher, W. Drommer, and H. Zeidler, *Ultrastructural and molecular analyses of the persistence of Chlamydia trachomatis (serovar K) in human monocytes.* Microbial Pathogenesis, 1997. **22**(3): p. 133-142.
164. Hogan, R., S.A. Mathews, S. Mukhopadhyay, J.T. Summersgill, and P. Timms, *Chlamydial persistence: beyond the biphasic paradigm.* Infection and Immunity, 2004. **72**: p. 1843-1855.
165. Arsovic, A., A. Nikolov, P. Sazdanovic, S. Popovic, and D. Baskic, *Prevalence and diagnostic significance of specific IgA and anti-heat shock protein 60 Chlamydia trachomatis antibodies in subfertile women.* Eur J Clin Microbiol Infect Dis, 2014. **33**(5): p. 761-6.
166. Bjartling, C., S. Osser, and K. Persson, *The frequency of salpingitis and ectopic pregnancy as epidemiologic markers of Chlamydia trachomatis.* Acta Obstet Gynecol Scand, 2000. **79**(2): p. 123-8.
167. Campbell, L.A., D.L. Patton, D.E. Moore, A.L. Cappuccio, B.A. Mueller, and S.P. Wang, *Detection of Chlamydia trachomatis deoxyribonucleic acid in women with tubal infertility.* Fertil Steril, 1993. **59**(1): p. 45-50.
168. Patton, D.L., M. Askienazy-Elbhar, J. Henry-Suchet, L.A. Campbell, A. Cappuccio, W. Tannous, S.P. Wang, and C.C. Kuo, *Detection of Chlamydia trachomatis in fallopian tube tissue in women with postinfectious tubal infertility.* Am J Obstet Gynecol, 1994. **171**(1): p. 95-101.

169. Lewis, M.E., R.J. Belland, Y.M. AbdelRahman, W.L. Beatty, A.A. Aiyar, A.H. Zea, S.J. Greene, L. Marrero, L.R. Buckner, D.J. Tate, C.L. McGowin, P.A. Kozlowski, M. O'Brien, R.A. Lillis, D.H. Martin, and A.J. Quayle, *Morphologic and molecular evaluation of Chlamydia trachomatis growth in human endocervix reveals distinct growth patterns.* Front Cell Infect Microbiol, 2014. **4**: p. 71.
170. Bavoil, P.M., *What's in a word: the use, misuse, and abuse of the word "persistence" in Chlamydia biology.* Front Cell Infect Microbiol, 2014. **4**: p. 27.
171. Stephens, R.S., *The cellular paradigm of chlamydial pathogenesis.* TRENDS in Microbiology, 2003. **11**: p. 44-51.
172. Kinnunen, A.H., H.M. Surcel, M. Lehtinen, J. Karhukorpi, A. Tiitinen, M. Halttunen, A. Bloigu, R.P. Morrison, R. Karttunen, and J. Paavonen, *HLA DQ alleles and interleukin-10 polymorphism associated with Chlamydia trachomatis-related tubal factor infertility: a case-control study.* Hum Reprod, 2002. **17**(8): p. 2073-8.
173. Hvid, M., A. Baczynska, B. Deleuran, J. Fedder, H.J. Knudsen, G. Christiansen, and S. Birkelund, *Interleukin□1 is the initiator of Fallopian tube destruction during Chlamydia trachomatis infection.* Cellular Microbiology, 2007. **9**(12): p. 2795-2803.
174. Hillis, S.D., L.M. Owens, P.A. Marchbanks, L.F. Amsterdam, and W.R. Mac Kenzie, *Recurrent chlamydial infections increase the risks of hospitalization for ectopic pregnancy and pelvic inflammatory disease.* Am J Obstet Gynecol, 1997. **176**(1 Pt 1): p. 103-7.
175. Yang, X., *Distinct function of Th1 and Th2 type delayed type hypersensitivity: protective and pathological reactions to chlamydial infection.* Microsc Res Tech, 2001. **53**(4): p. 273-7.
176. Watkins, N.G., W.J. Hadlow, A.B. Moos, and H.D. Caldwell, *Ocular delayed hypersensitivity: a pathogenetic mechanism of chlamydial-conjunctivitis in guinea pigs.* Proceedings of the National Academy of Sciences, 1986. **83**(19): p. 7480.
177. Yang, X., J. Gartner, L. Zhu, S. Wang, and R.C. Brunham, *IL-10 gene knockout mice show enhanced Th1-like protective immunity and absent granuloma formation following Chlamydia trachomatis lung infection.* The Journal of Immunology, 1999. **162**(2): p. 1010.
178. Witkin, S.S., M. Askienazy-Elbhar, J. Henry-Suchet, J. Belaisch-Allart, J. Tort-Grumbach, and K. Sarjdine, *Circulating antibodies to a conserved epitope of the Chlamydia trachomatis 60 kDa heat shock protein (hsp60) in infertile couples and its relationship to antibodies to C.trachomatis surface antigens and the Escherichia coli and human HSP60.* Human Reproduction, 1998. **13**(5): p. 1175-9.
179. Spandorfer, S.D., A. Neuer, D. LaVerda, G. Byrne, H.C. Liu, Z. Rosenwaks, and S.S. Witkin, *Previously undetected Chlamydia trachomatis infection, immunity to heat shock proteins and tubal occlusion in women undergoing in-vitro fertilization.* Hum Reprod, 1999. **14**(1): p. 60-4.
180. Witkin, S.S. and I.M. Linhares, *Chlamydia trachomatis in subfertile women undergoing uterine instrumentation - An alternative to direct microbial testing or prophylactic antibiotic treatment.* Human Reproduction, 2002. **17**(8): p. 1938-1941.

181. Morrison, R.P., K. Lyng, and H.D. Caldwell, *Chlamydial disease pathogenesis. Ocular hypersensitivity elicited by a genus-specific 57-kD protein.* J Exp Med, 1989. **169**(3): p. 663-75.
182. Rank, R.G., C. Dascher, A.K. Bowlin, and P.M. Bavoil, *Systemic immunization with Hsp60 alters the development of chlamydial ocular disease.* Invest Ophthalmol Vis Sci, 1995. **36**(7): p. 1344-51.
183. Javaloy, J., C. Ferrer, M.T. Vidal, and J.L. Alio, *Follicular conjunctivitis caused by Chlamydia trachomatis in an infant Saharan population: molecular and clinical diagnosis.* Br J Ophthalmol, 2003. **87**(2): p. 142-6.
184. Jakus, S., A. Neuer, S. Dieterle, A.M. Bongiovanni, and S.S. Witkin, *Antibody to the Chlamydia trachomatis 60 kDa heat shock protein in follicular fluid and in vitro fertilization outcome.* Am J Reprod Immunol, 2008. **59**(2): p. 85-9.
185. Keltz, M.D., M.T. Sauerbrun-Cutler, M.S. Durante, E. Moshier, D.E. Stein, and E. Gonzales, *Positive Chlamydia trachomatis serology result in women seeking care for infertility is a negative prognosticator for intrauterine pregnancy.* Sex Transm Dis, 2013. **40**(11): p. 842-5.
186. de Barbeyrac, B., A. Papaxanthos-Roche, C. Mathieu, C. Germain, J.L. Brun, M. Gachet, G. Mayer, C. Bebear, G. Chene, and C. Hocke, *Chlamydia trachomatis in subfertile couples undergoing an in vitro fertilization program: a prospective study.* Eur J Obstet Gynecol Reprod Biol, 2006. **129**(1): p. 46-53.
187. Hosenfeld, C.B., K.A. Workowski, S. Berman, A. Zaidi, J. Dyson, D. Mosure, G. Bolan, and H.M. Bauer, *Repeat infection with Chlamydia and gonorrhea among females: a systematic review of the literature.* Sex Transm Dis, 2009. **36**(8): p. 478-89.
188. Batteiger, B.E., W. Tu, S. Ofner, B. Van Der Pol, D.R. Stothard, D.P. Orr, B.P. Katz, and J.D. Fortenberry, *Repeated Chlamydia trachomatis genital infections in adolescent women.* J Infect Dis, 2010. **201**(1): p. 42-51.
189. Brunham, R.C., B. Pourbohloul, S. Mak, R. White, and M.L. Rekart, *The unexpected impact of a Chlamydia trachomatis infection control program on susceptibility to reinfection.* J Infect Dis, 2005. **192**(10): p. 1836-44.
190. Scott Lamontagne, D., K. Baster, L. Emmett, T. Nichols, S. Randall, L. McLean, P. Meredith, V. Harindra, J.M. Tobin, G.S. Underhill, W. Graham Hewitt, J. Hopwood, T. Gleave, A.K. Ghosh, H. Mallinson, A.R. Davies, G. Hughes, K.A. Fenton, and G. Chlamydia Recall Study Advisory, *Incidence and reinfection rates of genital chlamydial infection among women aged 16-24 years attending general practice, family planning and genitourinary medicine clinics in England: a prospective cohort study by the Chlamydia Recall Study Advisory Group.* Sex Transm Infect, 2007. **83**(4): p. 292-303.
191. Low, N., M. Egger, J.A. Sterne, R.M. Harbord, F. Ibrahim, B. Lindblom, and B. Herrmann, *Incidence of severe reproductive tract complications associated with diagnosed genital chlamydial infection: the Uppsala Women's Cohort Study.* Sexually Transmitted Infections, 2006. **82**(3): p. 212-8.
192. Horner, P.J., *Azithromycin antimicrobial resistance and genital Chlamydia trachomatis infection: duration of therapy may be the key to improving efficacy.* Sex Transm Infect, 2012. **88**(3): p. 154-6.

193. Dreses-Werringloer, U., I. Padubrin, H. Zeidler, and L. Kohler, *Effects of azithromycin and rifampin on Chlamydia trachomatis infection in vitro.* Antimicrob Agents Chemother, 2001. **45**(11): p. 3001-8.
194. Brunham, R.C. and M.L. Rekart, *The arrested immunity hypothesis and the epidemiology of Chlamydia control.* Sex. Transm. Dis., 2008. **35**: p. 53-54.
195. Brunham, R.C. and J. Rey-Ladino, *Immunology of Chlamydia infection: implications for a Chlamydia trachomatis vaccine.* Nature Reviews Immunology, 2005. **5**(2): p. 149-161.
196. Dicker, L.W., D.J. Mosure, S.M. Berman, and W.C. Levine, *Gonorrhea prevalence and coinfection with chlamydia in women in the United States, 2000.* Sex Transm Dis, 2003. **30**(5): p. 472-6.
197. De Muylder, X., M. Laga, C. Tennstedt, E. Van Dyck, G.N. Aelbers, and P. Piot, *The role of Neisseria gonorrhoeae and Chlamydia trachomatis in pelvic inflammatory disease and its sequelae in Zimbabwe.* J Infect Dis, 1990. **162**(2): p. 501-5.
198. Wiesenfeld, H.C., S.L. Hillier, M.A. Krohn, D.V. Landers, and R.L. Sweet, *Bacterial vaginosis is a strong predictor of Neisseria gonorrhoeae and Chlamydia trachomatis infection.* Clin Infect Dis, 2003. **36**(5): p. 663-8.
199. Gong, Z., Y. Luna, P. Yu, and H. Fan, *Lactobacilli inactivate Chlamydia trachomatis through lactic acid but not H2O2.* PLoS One, 2014. **9**(9): p. e107758.
200. Caldwell, H.D., H. Wood, C. Crane, R. Bailey, R.B. Jones, D. Mabey, I. Maclean, Z. Mohammed, R. Peeling, C. Roshick, J. Schachter, A.W. Solomon, W.E. Stamm, R.J. Suchland, L. Taylor, S.K. West, T.C. Quinn, R.J. Belland, and G. McClarty, *Polymorphisms in Chlamydia trachomatis tryptophan synthase genes differentiate between genital and ocular isolates.* The Journal of Clinical Investigation, 2003. **111**: p. 1757-1769.
201. Nelson, D.E., D.P. Virok, H. Wood, C. Roshick, R.M. Johnson, W.M. Whitmire, D.D. Crane, O. Steele-Mortimer, L. Kari, G. McClarty, and H.D. Caldwell, *Chlamydial IFN-gamma immune evasion is linked to host infection tropism.* Proc Natl Acad Sci U S A, 2005. **102**(30): p. 10658-63.
202. Taylor, B.D., T. Darville, and C.L. Haggerty, *Does bacterial vaginosis cause pelvic inflammatory disease?* Sex Transm Dis, 2013. **40**(2): p. 117-22.
203. Herzog, S.A., C.L. Althaus, J.C. Heijne, P. Oakeshott, S. Kerry, P. Hay, and N. Low, *Timing of progression from Chlamydia trachomatis infection to pelvic inflammatory disease: a mathematical modelling study.* BMC Infect Dis, 2012. **12**: p. 187.
204. Gaudoin, M., P. Rekha, A. Morris, J. Lynch, and U. Acharya, *Bacterial vaginosis and past chlamydial infection are strongly and independently associated with tubal infertility but do not affect in vitro fertilization success rates.* Fertil Steril, 1999. **72**(4): p. 730-2.
205. Arno, J.N., B.P. Katz, R. McBride, G.A. Carty, B.E. Batteiger, V.A. Caine, and R.B. Jones, *Age and clinical immunity to infections with Chlamydia trachomatis.* Sex Transm Dis, 1994. **21**(1): p. 47-52.
206. Price, M.J., A.E. Ades, D. de Angelis, N.J. Welton, J. Macleod, K. Turner, and P.J. Horner, *Incidence of Chlamydia trachomatis infection in women in England: two methods of estimation.* Epidemiol Infect, 2014. **142**(3): p. 562-76.
207. Bender, N., B. Herrmann, B. Andersen, J.S. Hocking, J. van Bergen, J. Morgan, I.V. van den Broek, M. Zwahlen, and N. Low, *Chlamydia infection,*

pelvic inflammatory disease, ectopic pregnancy and infertility: cross-national study. Sex Transm Infect, 2011. **87**(7): p. 601-8.
208. Barnes, R.C., B.P. Katz, R.T. Rolfs, B. Batteiger, V. Caine, and R.B. Jones, *Quantitative culture of endocervical Chlamydia trachomatis.* J Clin Microbiol, 1990. **28**(4): p. 774-80.
209. Macleod, J., C. Salisbury, N. Low, A. McCarthy, J.A. Sterne, A. Holloway, R. Patel, E. Sanford, A. Morcom, P. Horner, G. Davey Smith, S. Skidmore, A. Herring, O. Caul, F.D. Hobbs, and M. Egger, *Coverage and uptake of systematic postal screening for genital Chlamydia trachomatis and prevalence of infection in the United Kingdom general population: cross sectional study.* BMJ, 2005. **330**(7497): p. 940.
210. Sonnenberg, P., S. Clifton, S. Beddows, N. Field, K. Soldan, C. Tanton, C.H. Mercer, F.C. da Silva, S. Alexander, A.J. Copas, A. Phelps, B. Erens, P. Prah, W. Macdowall, K. Wellings, C.A. Ison, and A.M. Johnson, *Prevalence, risk factors, and uptake of interventions for sexually transmitted infections in Britain: findings from the National Surveys of Sexual Attitudes and Lifestyles (Natsal).* Lancet, 2013. **382**(9907): p. 1795-806.
211. Skjeldestad, F.E., M.A. Marsico, H.L. Sings, S.A. Nordbo, and G. Storvold, *Incidence and risk factors for genital Chlamydia trachomatis infection: a 4-year prospective cohort study.* Sex Transm Dis, 2009. **36**(5): p. 273-9.
212. Okumu, C.V., R.K. Kamau, and K.O. Rogo, *Past reproductive and sexual characteristics of women with tubal infertility at Kenyatta National Hospital.* East Afr Med J, 1990. **67**(12): p. 864-72.
213. Mutsaerts, M.A., H. Groen, H.G. Huiting, W.K. Kuchenbecker, P.J. Sauer, J.A. Land, R.P. Stolk, and A. Hoek, *The influence of maternal and paternal factors on time to pregnancy--a Dutch population-based birth-cohort study: the GECKO Drenthe study.* Hum Reprod, 2012. **27**(2): p. 583-93.
214. Cabrera-Leon, A., V. Lopez-Villaverde, M. Rueda, and M.N. Moya-Garrido, *Calibrated prevalence of infertility in 30- to 49-year-old women according to different approaches: a cross-sectional population-based study.* Hum Reprod, 2015.
215. Kessler, L.M., B.M. Craig, S.M. Plosker, D.R. Reed, and G.P. Quinn, *Infertility evaluation and treatment among women in the United States.* Fertil Steril, 2013. **100**(4): p. 1025-32.
216. Tilly, J.L. and D.A. Sinclair, *Germline energetics, aging, and female infertility.* Cell Metab, 2013. **17**(6): p. 838-50.
217. Selesniemi, K., H.J. Lee, A. Muhlhauser, and J.L. Tilly, *Prevention of maternal aging-associated oocyte aneuploidy and meiotic spindle defects in mice by dietary and genetic strategies.* Proc Natl Acad Sci U S A, 2011. **108**(30): p. 12319-24.
218. Kort, J.D., C. Winget, S.H. Kim, and R.B. Lathi, *A retrospective cohort study to evaluate the impact of meaningful weight loss on fertility outcomes in an overweight population with infertility.* Fertil Steril, 2014. **101**(5): p. 1400-3.
219. Clark, A.M., B. Thornley, L. Tomlinson, C. Galletley, and R.J. Norman, *Weight loss in obese infertile women results in improvement in reproductive outcome for all forms of fertility treatment.* Hum Reprod, 1998. **13**(6): p. 1502-5.
220. van der Steeg, J.W., P. Steures, M.J. Eijkemans, J.D. Habbema, P.G. Hompes, J.M. Burggraaff, G.J. Oosterhuis, P.M. Bossuyt, F. van der Veen,

and B.W. Mol, *Obesity affects spontaneous pregnancy chances in subfertile, ovulatory women.* Hum Reprod, 2008. **23**(2): p. 324-8.
221. Lin, X.H., M.E. Liu, H.Y. Xu, X.J. Chen, H. Wang, S. Tian, J.Z. Sheng, and H.F. Huang, *Leptin down-regulates gamma-ENaC expression: a novel mechanism involved in low endometrial receptivity.* Fertil Steril, 2015. **103**(1): p. 228-35.e3.
222. Caserta, D., G. Bordi, N. Di Segni, A. D'Ambrosio, M. Mallozzi, and M. Moscarini, *The influence of cigarette smoking on a population of infertile men and women.* Arch Gynecol Obstet, 2013. **287**(4): p. 813-8.
223. Zenzes, M.T., P. Wang, and R.F. Casper, *Cigarette smoking may affect meiotic maturation of human oocytes.* Hum Reprod, 1995. **10**(12): p. 3213-17.
224. Van Voorhis, B.J., J.D. Dawson, D.W. Stovall, A.E. Sparks, and C.H. Syrop, *The effects of smoking on ovarian function and fertility during assisted reproduction cycles.* Obstet Gynecol, 1996. **88**(5): p. 785-91.
225. Wei, M., M.P. Stern, and S.M. Haffner, *Serum leptin levels in Mexican Americans and non-Hispanic whites: association with body mass index and cigarette smoking.* Ann Epidemiol, 1997. **7**(2): p. 81-6.
226. Donahue, R.P., P. Zimmet, J.A. Bean, M. Decourten, R.A. DeCarlo Donahue, G. Collier, R.B. Goldberg, R.J. Prineas, J. Skyler, and N. Schneiderman, *Cigarette smoking, alcohol use, and physical activity in relation to serum leptin levels in a multiethnic population: The Miami Community Health Study.* Ann Epidemiol, 1999. **9**(2): p. 108-13.
227. Bolumar, F., J. Olsen, M. Rebagliato, I. Saez-Lloret, and L. Bisanti, *Body mass index and delayed conception: a European Multicenter Study on Infertility and Subfecundity.* Am J Epidemiol, 2000. **151**(11): p. 1072-9.
228. Hakim, R.B., R.H. Gray, and H. Zacur, *Alcohol and caffeine consumption and decreased fertility.* Fertil Steril, 1998. **70**(4): p. 632-7.
229. Klonoff-Cohen, H., P. Lam-Kruglick, and C. Gonzalez, *Effects of maternal and paternal alcohol consumption on the success rates of in vitro fertilization and gamete intrafallopian transfer.* Fertil Steril, 2003. **79**(2): p. 330-9.
230. Wdowiak, A., M. Sulima, M. Sadowska, B. Grzegorz, and I. Bojar, *Alcohol consumption and quality of embryos obtained in programmes of in vitro fertilization.* Ann Agric Environ Med, 2014. **21**(2): p. 450-3.
231. Gormack, A.A., J.C. Peek, J.G. Derraik, P.D. Gluckman, N.L. Young, and W.S. Cutfield, *Many women undergoing fertility treatment make poor lifestyle choices that may affect treatment outcome.* Hum Reprod, 2015. **30**(7): p. 1617-24.
232. Brabin, L., E. Fairbrother, D. Mandal, S.A. Roberts, S.P. Higgins, S. Chandiok, P. Wood, G. Barnard, and H.C. Kitchener, *Biological and hormonal markers of chlamydia, human papillomavirus, and bacterial vaginosis among adolescents attending genitourinary medicine clinics.* Sex Transm Infect, 2005. **81**(2): p. 128-32.
233. Farrow, A., M.G. Hull, K. Northstone, H. Taylor, W.C. Ford, and J. Golding, *Prolonged use of oral contraception before a planned pregnancy is associated with a decreased risk of delayed conception.* Hum Reprod, 2002. **17**(10): p. 2754-61.
234. Azenabor, A.A., P. Kennedy, and S. Balistreri, *Chlamydia trachomatis infection of human trophoblast alters estrogen and progesterone*

biosynthesis: an insight into role of infection in pregnancy sequelae. Int J Med Sci, 2007. **4**(4): p. 223-31.
235. Keay, S.D., R. Barlow, A. Eley, G.M. Masson, F.W. Anthony, and J.M. Jenkins, *The relation between immunoglobulin G antibodies to Chlamydia trachomatis and poor ovarian response to gonadotropin stimulation before in vitro fertilization.* Fertil Steril, 1998. **70**(2): p. 214-8.
236. Rosenthal, G.E. and C.S. Landefeld, *The relation of chlamydial infection of the cervix to time elapsed from the onset of menses.* J Clin Epidemiol, 1990. **43**(1): p. 15-20.
237. Geisler, W.M., S.Y. Lensing, C.G. Press, and E.W. Hook, 3rd, *Spontaneous resolution of genital Chlamydia trachomatis infection in women and protection from reinfection.* J Infect Dis, 2013. **207**(12): p. 1850-6.
238. Lal, J.A., J. Malogajski, S.P. Verweij, P. de Boer, E. Ambrosino, A. Brand, S. Ouburg, and S.A. Morre, *Chlamydia trachomatis infections and subfertility: opportunities to translate host pathogen genomic data into public health.* Public Health Genomics, 2013. **16**(1-2): p. 50-61.
239. Yu, L., L. Wang, and S. Chen, *Toll-like receptors, inflammation and tumor in the human female reproductive tract.* Am J Reprod Immunol, 2009. **62**(1): p. 1-8.
240. Laisk, T., M. Peters, M. Saare, K. Haller-Kikkatalo, H. Karro, and A. Salumets, *Association of CCR5, TLR2, TLR4 and MBL genetic variations with genital tract infections and tubal factor infertility.* J Reprod Immunol, 2010. **87**(1-2): p. 74-81.
241. Karimi, O., S. Ouburg, H.J. de Vries, A.S. Pena, J. Pleijster, J.A. Land, and S.A. Morre, *TLR2 haplotypes in the susceptibility to and severity of Chlamydia trachomatis infections in Dutch women.* Drugs Today (Barc), 2009. **45 Suppl B**: p. 67-74.
242. Taylor, B.D., T. Darville, R.E. Ferrell, C.M. Kammerer, R.B. Ness, and C.L. Haggerty, *Variants in toll-like receptor 1 and 4 genes are associated with Chlamydia trachomatis among women with pelvic inflammatory disease.* J Infect Dis, 2012. **205**(4): p. 603-9.
243. den Hartog, J.E., S. Ouburg, J.A. Land, J.M. Lyons, J.I. Ito, A.S. Pena, and S.A. Morre, *Do host genetic traits in the bacterial sensing system play a role in the development of Chlamydia trachomatis-associated tubal pathology in subfertile women?* BMC Infect Dis, 2006. **6**: p. 122.
244. Wang, W., F.R. Stassen, H.M. Surcel, H. Ohman, A. Tiitinen, J. Paavonen, H.J. de Vries, R. Heijmans, J. Pleijster, S.A. Morre, and S. Ouburg, *Analyses of polymorphisms in the inflammasome-associated NLRP3 and miRNA-146A genes in the susceptibility to and tubal pathology of Chlamydia trachomatis infection.* Drugs Today (Barc), 2009. **45 Suppl B**: p. 95-103.
245. Laisk, T., M. Peters, and A. Salumets, *Mannose-binding lectin genotypes: potential role in tubal damage and adverse IVF outcome.* J Reprod Immunol, 2011. **92**(1-2): p. 62-7.
246. Cohen, C.R., J. Gichui, R. Rukaria, S.S. Sinei, L.K. Gaur, and R.C. Brunham, *Immunogenetic correlates for Chlamydia trachomatis-associated tubal infertility.* Obstet Gynecol, 2003. **101**(3): p. 438-44.
247. Wang, C., J. Tang, W.M. Geisler, P.A. Crowley-Nowick, C.M. Wilson, and R.A. Kaslow, *Human leukocyte antigen and cytokine gene variants as predictors of recurrent Chlamydia trachomatis infection in high-risk adolescents.* J Infect Dis, 2005. **191**(7): p. 1084-92.

248. Ohman, H., A. Tiitinen, M. Halttunen, J. Paavonen, and H.M. Surcel, *Cytokine gene polymorphism and Chlamydia trachomatis-specific immune responses.* Hum Immunol, 2011. **72**(3): p. 278-82.
249. Ohman, H., A. Tiitinen, M. Halttunen, M. Lehtinen, J. Paavonen, and H.M. Surcel, *Cytokine polymorphisms and severity of tubal damage in women with Chlamydia-associated infertility.* J Infect Dis, 2009. **199**(9): p. 1353-9.
250. Ohman, H., R. Bailey, A. Natividad, J. Ragoussis, L.L. Johnson, A. Tiitinen, M. Halttunen, J. Paavonen, and H.M. Surcel, *Effect of IL12A and IL12B polymorphisms on the risk of Chlamydia trachomatis-induced tubal factor infertility and disease severity.* Hum Reprod, 2012. **27**(7): p. 2217-23.
251. Murillo, L.S., J.A. Land, J. Pleijster, C.A. Bruggeman, A.S. Pena, and S.A. Morre, *Interleukin-1B (IL-1B) and interleukin-1 receptor antagonist (IL-1RN) gene polymorphisms are not associated with tubal pathology and Chlamydia trachomatis-related tubal factor subfertility.* Hum Reprod, 2003. **18**(11): p. 2309-14.
252. Eng, H.L., C.H. Wang, C.H. Chen, M.H. Chou, C.T. Cheng, and T.M. Lin, *A CD14 promoter polymorphism is associated with CD14 expression and Chlamydia-stimulated TNF alpha production.* Genes Immun, 2004. **5**(5): p. 426-30.
253. Eng, H.L., C.H. Chen, C.C. Kuo, J.S. Wu, C.H. Wang, and T.M. Lin, *Association of CD14 promoter gene polymorphism and Chlamydia pneumoniae infection.* J Infect Dis, 2003. **188**(1): p. 90-7.
254. Muraille, E. and O. Leo, *Revisiting the Th1/Th2 paradigm.* Scand J Immunol, 1998. **47**(1): p. 1-9.
255. Mascellino, M.T., P. Boccia, and A. Oliva, *Immunopathogenesis in Chlamydia trachomatis Infected Women.* ISRN Obstet Gynecol, 2011. **2011**: p. 436936.
256. Perry, L.L., K. Feilzer, and H.D. Caldwell, *Immunity to Chlamydia trachomatis is mediated by T helper 1 cells through IFN-gamma-dependent and -independent pathways.* J Immunol, 1997. **158**(7): p. 3344-52.
257. Khader, S.A. and R. Gopal, *IL-17 in protective immunity to intracellular pathogens.* Virulence, 2010. **1**(5): p. 423-7.
258. Arno, J.N., V.A. Ricker, B.E. Batteiger, B.P. Katz, V.A. Caine, and R.B. Jones, *Interferon-gamma in endocervical secretions of women infected with Chlamydia trachomatis.* J Infect Dis, 1990. **162**(6): p. 1385-9.
259. Spear, G.T., S.R. Kendrick, H.Y. Chen, T.T. Thomas, M. Bahk, R. Balderas, S. Ghosh, A. Weinberg, and A.L. Landay, *Multiplex immunoassay of lower genital tract mucosal fluid from women attending an urban STD clinic shows broadly increased IL1ss and lactoferrin.* PloS one, 2011. **6**(5): p. e19560.
260. Agrawal, T., V. Vats, S. Salhan, and A. Mittal, *Mucosal and peripheral immune responses to chlamydial heat shock proteins in women infected with Chlamydia trachomatis.* Clin Exp Immunol, 2007. **148**(3): p. 461-8.
261. Jha, R., P. Srivastava, S. Salhan, A. Finckh, C. Gabay, A. Mittal, and S. Bas, *Spontaneous secretion of interleukin-17 and -22 by human cervical cells in Chlamydia trachomatis infection.* Microbes Infect, 2011. **13**(2): p. 167-78.
262. Kelly, K.A., D. Wiley, E. Wiesmeier, M. Briskin, A. Butch, and T. Darville, *The combination of the gastrointestinal integrin (alpha4beta7) and selectin ligand enhances T-Cell migration to the reproductive tract during infection with Chlamydia trachomatis.* Am J Reprod Immunol, 2009. **61**(6): p. 446-52.

263. Agrawal, T., A.R. Bhengraj, V. Vats, S. Salhan, and A. Mittal, *Expression of TLR 2, TLR 4 and iNOS in cervical monocytes of Chlamydia trachomatis-infected women and their role in host immune response.* Am J Reprod Immunol, 2011. **66**(6): p. 534-43.
264. Gupta, R., H. Vardhan, P. Srivastava, S. Salhan, and A. Mittal, *Modulation of cytokines and transcription factors (T-Bet and GATA3) in CD4 enriched cervical cells of Chlamydia trachomatis infected fertile and infertile women upon stimulation with chlamydial inclusion membrane proteins B and C.* Reprod Biol Endocrinol, 2009. **7**: p. 84.
265. Balasubamaniam, E.S., S. Van Noorden, and M. El-Bahrawy, *The expression of interleukin (IL)-6, Il-8 and their receptors in fallopian tubes with ectopic tubal gestation.* Fertility and Sterility, 2012. **98**: p. 898-894.
266. Hvid, M., A. Baczynska, B. Deleuran, J. Fedder, H.J. Knudsen, G. Christiansen, and S. Birkelund, *Interleukin-1 is the initiator of Fallopian tube destruction during Chlamydia trachomatis infection.* Cell Microbiol, 2007. **9**(12): p. 2795-803.
267. Shaw, J.L., G.S. Wills, K.F. Lee, P.J. Horner, M.O. McClure, V.M. Abrahams, N. Wheelhouse, H.N. Jabbour, H.O. Critchley, G. Entrican, and A.W. Horne, *Chlamydia trachomatis infection increases fallopian tube PROKR2 via TLR2 and NFkappaB activation resulting in a microenvironment predisposed to ectopic pregnancy.* Am J Pathol, 2011. **178**(1): p. 253-60.
268. Kinnunen, A., P. Molander, A. Laurila, I. Rantala, R. Morrison, M. Lehtinen, R. Karttunen, A. Tiitinen, J. Paavonen, and H.M. Surcel, *Chlamydia trachomatis reactive T lymphocytes from upper genital tract tissue specimens.* Hum Reprod, 2000. **15**(7): p. 1484-9.
269. Rasmussen, S.J., L. Eckmann, A.J. Quayle, L. Shen, Y.-X. Zhang, D.J. Anderson, J. Fierer, R.S. Stephens, and M.F. Kagnoff, *Secretion of proinflammatory cytokines by epithelial cells in response to Chlamydia infection suggest a central role for eptihelial cells in chlamydial pathogenesis.* The Journal of Clinical Investigation, 1997. **99**: p. 77-87.
270. Kinnunen, A., H.M. Surcel, M. Halttunen, A. Tiitinen, R.P. Morrison, S.G. Morrison, P. Koskela, M. Lehtinen, and J. Paavonen, *Chlamydia trachomatis heat shock protein-60 induced interferon-gamma and interleukin-10 production in infertile women.* Clin Exp Immunol, 2003. **131**(2): p. 299-303.
271. Cohen, C.R., K.M. Koochesfahani, A.S. Meier, C. Shen, K. Karunakaran, B. Ondondo, T. Kinyari, N.R. Mugo, R. Nguti, and R.C. Brunham, *Immunoepidemiologic profile of Chlamydia trachomatis infection: importance of heat-shock protein 60 and interferon- gamma.* J Infect Dis, 2005. **192**(4): p. 591-9.
272. Schrader, S., A. Klos, S. Hess, H. Zeidler, J.G. Kuipers, and M. Rihl, *Expression of imflammatory host genes in Chlamydia trachomatis-infected human monocytes.* Arthritis Research and Therapy, 2007. **9**: p. 54-62.
273. Debattista, J., P. Timms, J. Allan, and J. Allan, *Reduced levels of gamma-interferon secretion in response to chlamydial 60 kDa heat shock protein amongst women with pelvic inflammatory disease and a history of repeated Chlamydia trachomatis infections.* Immunol Lett, 2002. **81**(3): p. 205-10.
274. Debattista, J., C.M. Gazzard, R. Wood, J.A. Allan, J.M. Allan, A. Scarman, M. Mortlock, P. Timms, and C.L. Knox, *Interactions of microbiology and*

pathology in women undergoing investigations for infertility. Infectious diseases, Obstetrics and Gynecology, 2004. **12**: p. 135-145.
275. Gervassi, A., M.R. Alderson, R. Suchland, J.F. Maisonneuve, K.H. Grabstein, and P. Probst, *Differential regulation of inflammatory cytokine secretion by human dendritic cells upon Chlamydia trachomatis infection.* Infect Immun, 2004. **72**(12): p. 7231-9.
276. Matyszak, M.K., J.L. Young, and J.S. Gaston, *Uptake and processing of Chlamydia trachomatis by human dendritic cells.* Eur J Immunol, 2002. **32**(3): p. 742-51.
277. Hook, C.E., N. Telyatnikova, J.C. Goodall, V.M. Braud, A.J. Carmichael, M.R. Wills, and J.S. Gaston, *Effects of Chlamydia trachomatis infection on the expression of natural killer (NK) cell ligands and susceptibility to NK cell lysis.* Clin Exp Immunol, 2004. **138**(1): p. 54-60.
278. Cunningham, K., S.H. Stansfield, P. Patel, S. Menon, V. Kienzle, J.A. Allan, and W.M. Huston, *The IL-6 response to Chlamydia from primary reproductive epithelial cells is highly variable and may be involved in differential susceptibility to the immunopathological consequences of chlamydial infection.* BMC Immunol, 2013. **14**: p. 50.
279. Cheng, W., P. Shivshankar, Y. Zhong, D. Chen, Z. Li, and G. Zhong, *Intracellular interleukin-1alpha mediates interleukin-8 production induced by Chlamydia trachomatis infection via a mechanism independent of type I interleukin-1 receptor.* Infect Immun, 2008. **76**(3): p. 942-51.
280. Mpiga, P., S. Mansour, R. Morisset, R. Beaulieu, and M. Ravaoarinoro, *Sustained interleukin-6 and interleukin-8 expression following infection with Chlamydia trachomatis serovar L2 in a HeLa/THP-1 cell co-culture model.* Scand J Immunol, 2006. **63**(3): p. 199-207.
281. Dessus-Babus, S., T.L. Darville, F.P. Cuozzo, K. Ferguson, and P.B. Wyrick, *Differences in innate immune responses (in vitro) to HeLa cells infected with nondisseminating serovar E and disseminating serovar L2 of Chlamydia trachomatis.* Infection and Immunity, 2002. **70**(6): p. 3234-48.
282. Humphrys, M.S., T. Creasy, Y. Sun, A.C. Shetty, M.C. Chibucos, E.F. Drabek, C.M. Fraser, U. Farooq, N. Sengamalay, S. Ott, H. Shou, P.M. Bavoil, A. Mahurkar, and G.S. Myers, *Simultaneous transcriptional profiling of bacteria and their host cells.* PLoS One, 2013. **8**(12): p. e80597.
283. Shemer-Avni, Y., D. Wallach, and I. Sarov, *Inhibition of Chlamydia trachomatis growth by recombinant tumor necrosis factor.* Infect Immun, 1988. **56**(9): p. 2503-6.
284. Lu, H., C. Shen, and R.C. Brunham, *Chlamydia trachomatis infection of epithelial cells induces the activation of caspase-1 and release of mature IL-18.* J Immunol, 2000. **165**(3): p. 1463-9.
285. Reddy, B.S., S. Rastogi, B. Das, S. Salhan, S. Verma, and A. Mittal, *Cytokine expression pattern in the genital tract of Chlamydia trachomatis positive infertile women - implication for T-cell responses.* Clin Exp Immunol, 2004. **137**(3): p. 552-8.
286. Mylonas, I., *Female genital Chlamydia trachomatis infection: where are we heading?* Archives of Gynecology and Obstetrics, 2012. **285**(5): p. 1271-1285.
287. Xiong, L., F. Kong, H. Zhou, and G.L. Gilbert, *Use of PCR and reverse line blot hybridization assay for rapid simultaneous detection and serovar*

identification of Chlamydia trachomatis. J Clin Microbiol, 2006. **44**(4): p. 1413-8.
288. Jurstrand, M., L. Falk, H. Fredlund, M. Lindberg, P. Olcen, S. Andersson, K. Persson, J. Albert, and A. Backman, *Characterization of Chlamydia trachomatis omp1 genotypes among sexually transmitted disease patients in Sweden.* J Clin Microbiol, 2001. **39**(11): p. 3915-9.
289. Gao, X., X.S. Chen, Y.P. Yin, M.Y. Zhong, M.Q. Shi, W.H. Wei, Q. Chen, R.W. Peeling, and D. Mabey, *Distribution study of Chlamydia trachomatis serovars among high-risk women in China performed using PCR-restriction fragment length polymorphism genotyping.* J Clin Microbiol, 2007. **45**(4): p. 1185-9.
290. Quint, K.D., L.J. van Doorn, B. Kleter, M.N. de Koning, H.A. van den Munckhof, S.A. Morre, B. ter Harmsel, E. Weiderpass, G. Harbers, W.J. Melchers, and W.G. Quint, *A highly sensitive, multiplex broad-spectrum PCR-DNA-enzyme immunoassay and reverse hybridization assay for rapid detection and identification of Chlamydia trachomatis serovars.* J Mol Diagn, 2007. **9**(5): p. 631-8.
291. Walker, J., S.N. Tabrizi, C.K. Fairley, M.Y. Chen, C.S. Bradshaw, J. Twin, N. Taylor, B. Donovan, J.M. Kaldor, K. McNamee, E. Urban, S. Walker, M. Currie, H. Birden, F. Bowden, J. Gunn, M. Pirotta, L. Gurrin, V. Harindra, S.M. Garland, and J.S. Hocking, *Chlamydia trachomatis incidence and re-infection among young women--behavioural and microbiological characteristics.* PLoS One, 2012. **7**(5): p. e37778.
292. Lan, J., I. Melgers, C.J. Meijer, J.M. Walboomers, R. Roosendaal, C. Burger, O.P. Bleker, and A.J. van den Brule, *Prevalence and serovar distribution of asymptomatic cervical Chlamydia trachomatis infections as determined by highly sensitive PCR.* J Clin Microbiol, 1995. **33**(12): p. 3194-7.
293. Verweij, S.P., E. Lanjouw, C.J. Bax, K.D. Quint, P.M. Oostvogel, P.J. Dorr, J. Pleijster, H.J. de Vries, R.P. Peters, S. Ouburg, and S.A. Morre, *Serovar D and E of serogroup B induce highest serological responses in urogenital Chlamydia trachomatis infections.* BMC Infect Dis, 2014. **14**: p. 3.
294. Morre, S.A., A.J. van den Brule, L. Rozendaal, A.J. Boeke, F.J. Voorhorst, S. de Blok, and C.J. Meijer, *The natural course of asymptomatic Chlamydia trachomatis infections: 45% clearance and no development of clinical PID after one-year follow-up.* Int J STD AIDS, 2002. **13 Suppl 2**: p. 12-8.
295. Workowski, K.A., C.E. Stevens, R.J. Suchland, K.K. Holmes, D.A. Eschenbach, M.B. Pettinger, and W.E. Stamm, *Clinical manifestations of genital infection due to Chlamydia trachomatis in women: differences related to serovar.* Clin Infect Dis, 1994. **19**(4): p. 756-60.
296. Risser, W.L. and J.M. Risser, *The incidence of pelvic inflammatory disease in untreated women infected with Chlamydia trachomatis: a structured review.* Int J STD AIDS, 2007. **18**(11): p. 727-31.
297. Simms, I. and P. Horner, *Has the incidence of pelvic inflammatory disease following chlamydial infection been overestimated?* Int J STD AIDS, 2008. **19**(4): p. 285-6.
298. Morrison, R.P., *Differential sensitivities of Chlamydia trachomatis strains to inhibitory effects of gamma interferon.* Infect Immun, 2000. **68**(10): p. 6038-40.

299. Borges, V., A. Nunes, R. Ferreira, M.J. Borrego, and J.P. Gomes, *Directional evolution of Chlamydia trachomatis towards niche-specific adaptation.* J Bacteriol. , 2012. **194**: p. 6143-53.
300. Jeffrey, B.M., R.J. Suchland, K.L. Quinn, J.R. Davidson, W.E. Stamm, and D.D. Rockey, *Genome sequencing of recent clinical Chlamydia trachomatis strains identifies loci associated with tissue tropism and regions of apparent recombination.* Infect Immun, 2010. **78**(6): p. 2544-53.
301. Zugel, U. and S.H. Kaufmann, *Role of heat shock proteins in protection from and pathogenesis of infectious diseases.* Clin Microbiol Rev, 1999. **12**(1): p. 19-39.
302. Kinnunen, A., P. Molander, R. Morrison, M. Lehtinen, R. Karttunen, A. Tiitinen, J. Paavonen, and H.M. Surcel, *Chlamydial heat shock protein 60--specific T cells in inflamed salpingeal tissue.* Fertil Steril, 2002. **77**(1): p. 162-6.
303. Srivastava, P., R. Jha, S. Bas, S. Salhan, and A. Mittal, *In infertile women, cells from Chlamydia trachomatis infected sites release higher levels of interferon-gamma, interleukin-10 and tumor necrosis factor-alpha upon heat-shock-protein stimulation than fertile women.* Reprod Biol Endocrinol, 2008. **6**: p. 20.
304. Betsou, F., J.M. Sueur, and J. Orfila, *Serological investigation of Chlamydia trachomatis heat shock protein 10.* Infect Immun, 1999. **67**(10): p. 5243-6.
305. Chen, A.L., K.A. Johnson, J.K. Lee, C. Sutterlin, and M. Tan, *CPAF: a Chlamydial protease in search of an authentic substrate.* PLoS Pathog, 2012. **8**(8): p. e1002842.
306. Sharma, J., A.M. Bosnic, J.M. Piper, and G. Zhong, *Human antibody responses to a Chlamydia-secreted protease factor.* Infect Immun, 2004. **72**(12): p. 7164-71.
307. Murthy, A.K., J.P. Chambers, P.A. Meier, G. Zhong, and B.P. Arulanandam, *Intranasal vaccination with a secreted chlamydial protein enhances resolution of genital Chlamydia muridarum infection, protects against oviduct pathology, and is highly dependent upon endogenous gamma interferon production.* Infect Immun, 2007. **75**(2): p. 666-76.
308. Zhong, G., P. Fan, H. Ji, F. Dong, and Y. Huang, *Identification of a chlamydial protease-like activity factor responsible for the degradation of host transcription factors.* J Exp Med, 2001. **193**(8): p. 935-42.
309. Rödel, J., C. Große, H. Yu, K. Wolf, G.P. Otto, E. Liebler-Tenorio, V. Forsbach-Birk, and E. Straube, *Persistent Chlamydia trachomatis infection of HeLa cells mediates apoptosis resistance through a Chlamydia protease-like activity factor-independent mechanism and induces high mobility group box 1 release.* Infection and Immunity, 2012. **80**(1): p. 195-205.
310. Giles, D.K. and P.B. Wyrick, *Trafficking of chlamydial antigens to the endoplasmic reticulum of infected epithelial cells.* Microbes Infect, 2008. **10**(14-15): p. 1494-503.
311. Gupta, R., P. Srivastava, H. Vardhan, S. Salhan, and A. Mittal, *Host immune responses to chlamydial inclusion membrane proteins B and C in Chlamydia trachomatis infected women with or without fertility disorders.* Reproductive Biology and Endocrinology, 2009. **7**(1): p. 38.
312. Gottlieb, S.L., S.M. Berman, and N. Low, *Screening and treatment to prevent sequelae in women with Chlamydia trachomatis genital infection: how much*

do we know? Journal of Infectious Diseases, 2010. **201**(Supplement 2): p. S156-S167.
313. Tang, F., H. Chang, Y. Huang, and K. Wang, *Studies on the etiology of trachoma with special reference to isolation of the virus in chick embryo.* Chinese medical journal, 1957. **75**(6): p. 429.
314. Barnes, R.C., *Laboratory diagnosis of human chlamydial infections.* Clinical microbiology reviews, 1989. **2**(2): p. 119-136.
315. Hodinka, R.L., C. Davis, J. Choong, and P.B. Wyrick, *Ultrastructural study of endocytosis of Chlamydia trachomatis by McCoy cells.* Infection and Immunity, 1988. **56**(6): p. 1456-1463.
316. Chan, S.W. and A.L. Cunningham, *Comparison of Giemsa and fluorescent monoclonal antibody staining of inoculated cell cultures for diagnosis of Chlamydia trachomatis.* Pathology, 1994. **26**(2): p. 194-7.
317. Black, C.M., *Current methods of laboratory diagnosis of Chlamydia trachomatis infections.* Clinical microbiology reviews, 1997. **10**(1): p. 160-184.
318. Stary, A. and G. Stary, *Chlamydia trachomatis: Diagnostic Procedures.* Sexually Transmitted Infections and Sexually Transmitted Diseases, 2011: p. 111.
319. Uyeda, C.T., P. Welborn, N. Ellison-Birang, K. Shunk, and B. Tsaouse, *Rapid diagnosis of chlamydial infections with the MicroTrak direct test.* Journal of clinical microbiology, 1984. **20**(5): p. 948-950.
320. Dhawan, B., J. Rawre, A. Ghosh, N. Malhotra, M.M. Ahmed, V. Sreenivas, and R. Chaudhry, *Diagnostic efficacy of a real time-PCR assay for Chlamydia trachomatis infection in infertile women in north India.* Indian J Med Res, 2014. **140**(2): p. 252-61.
321. Rahm, V.A., H. Gnarpe, and V. Odlind, *Evaluation of a direct fluorescence assay as a screening method in asymptomatic young women.* Sex Transm Dis, 1992. **19**(2): p. 84-7.
322. Thejls, H., J. Gnarpe, O. Lundkvist, G. Heimer, G. Larsson, and A. Victor, *Diagnosis and prevalence of persistent chlamydia infection in infertile women: tissue culture, direct antigen detection, and serology.* Fertil Steril, 1991. **55**(2): p. 304-10.
323. Bébéar, C. and B. De Barbeyrac, *Genital Chlamydia trachomatis infections.* Clinical Microbiology and Infection, 2009. **15**(1): p. 4-10.
324. Geisler, W.M., J.M. Chow, J. Schachter, and W.M. Mccormack, *Pelvic examination findings and Chlamydia trachomatis infection in asymptomatic young women screened with a nucleic acid amplification test.* Sexually transmitted diseases, 2007. **34**(6): p. 335.
325. Schachter, J., W.M. McCormack, M.A. Chernesky, D.H. Martin, B. Van Der Pol, P.A. Rice, E.W. Hook III, W.E. Stamm, T.C. Quinn, and J.M. Chow, *Vaginal swabs are appropriate specimens for diagnosis of genital tract infection with Chlamydia trachomatis.* Journal of clinical microbiology, 2003. **41**(8): p. 3784-3789.
326. Van Dyck, E., M. Ieven, S. Pattyn, L. Van Damme, and M. Laga, *Detection of Chlamydia trachomatis andNeisseria gonorrhoeae by Enzyme Immunoassay, Culture, and Three Nucleic Acid Amplification Tests.* Journal of clinical microbiology, 2001. **39**(5): p. 1751-1756.

327. Forward, K.R., *The impact of switching to polymerase chain reaction for the diagnosis of Chlamydia trachomatis infections in women.* Canadian journal of public health, 2003. **94**(3): p. 229-232.
328. Chernesky, M., D. Jang, J. Gilchrist, T. Hatchette, A. Poirier, J.F. Flandin, M. Smieja, and S. Ratnam, *Head-to-head comparison of second-generation nucleic acid amplification tests for detection of Chlamydia trachomatis and Neisseria gonorrhoeae on urine samples from female subjects and self-collected vaginal swabs.* J Clin Microbiol, 2014. **52**(7): p. 2305-10.
329. Geelen, T.H., J.W. Rossen, A.M. Beerens, L. Poort, S.A. Morré, W.S. Ritmeester, H.E. van Kruchten, M.M. van de Pas, and P.H.M. Savelkoul, *Performance of cobas® 4800 and m2000 real-time™ assays for detection of Chlamydia trachomatis and Neisseria gonorrhoeae in rectal and self-collected vaginal specimen.* Diagnostic Microbiology and Infectious Disease, 2013. **77**(2): p. 101-105.
330. Klint, M., R. Hadad, L. Christerson, B. Lore, C. Anagrius, A. Osterlund, I. Larsson, S. Sylvan, H. Fredlund, M. Unemo, and B. Herrmann, *Prevalence trends in Sweden for the new variant of Chlamydia trachomatis.* Clin Microbiol Infect, 2011. **17**(5): p. 683-9.
331. Moller, J.K., L.N. Pedersen, and K. Persson, *Comparison of Gen-probe transcription-mediated amplification, Abbott PCR, and Roche PCR assays for detection of wild-type and mutant plasmid strains of Chlamydia trachomatis in Sweden.* J Clin Microbiol, 2008. **46**(12): p. 3892-5.
332. Persson, K., B. Hammas, H. Janson, C. Bjartling, J. Dillner, and L. Dillner, *Decline of the new Swedish variant of Chlamydia trachomatis after introduction of appropriate testing.* Sex Transm Infect, 2012. **88**(6): p. 451-5.
333. van Dommelen, L., F.H. van Tiel, S. Ouburg, E.E.H.G. Brouwers, P.H.W. Terporten, P.H.M. Savelkoul, S.A. Morré, C.A. Bruggeman, and C.J.P.A. Hoebe, *Alarmingly poor performance in Chlamydia trachomatis point-of-care testing.* Sexually transmitted infections, 2010. **86**(5): p. 355-359.
334. van der Helm, J.J., L.O.A. Sabajo, A.W. Grunberg, S.A. Morré, A.G.C.L. Speksnijder, and H.J.C. de Vries, *Point-of-Care Test for Detection of Urogenital Chlamydia in Women Shows Low Sensitivity. A Performance Evaluation Study in Two Clinics in Suriname.* PLoS ONE, 2012. **7**(2): p. e32122.
335. Bally, F., A. Quach, G. Greub, K. Jaton, C. Petignat, C. Ambord, J. Fellay, E. Masserey, and B. Spencer, *Opportunistic testing for urogenital infection with Chlamydia trachomatis in south-western Switzerland, 2012: a feasibility study.* Euro Surveill, 2015. **20**(9).
336. Naderi, T., F. Kazerani, and A. Bahraminpoor, *Comparison of chlamydia infection prevalence between patients with and without ectopic pregnancy using the PCR method.* Ginekol Pol, 2012. **83**(11): p. 819-21.
337. Sulaiman, S., P.P. Chong, R. Mokhtarudin, M.S. Lye, and W.H. Wan Hassan, *Comparison of nested and ELISA based polymerase chain reaction assays for detecting Chlamydia trachomatis in pregnant women with preterm complications.* Trop Biomed, 2014. **31**(1): p. 36-45.
338. Dirks, J.A., P.F. Wolffs, N.H. Dukers-Muijrers, A.A. Brink, A.G. Speksnijder, and C.J. Hoebe, *Chlamydia trachomatis load in population-based screening and STI-clinics: implications for screening policy.* PLoS One, 2015. **10**(3): p. e0121433.

339. Dukers-Muijrers, N.H., A.G. Speksnijder, S.A. Morre, P.F. Wolffs, M.A. van der Sande, A.A. Brink, I.V. van den Broek, M.I. Werner, and C.J. Hoebe, *Detection of anorectal and cervicovaginal Chlamydia trachomatis infections following azithromycin treatment: prospective cohort study with multiple time-sequential measures of rRNA, DNA, quantitative load and symptoms.* PLoS One, 2013. **8**(11): p. e81236.
340. Gomes, J.P., M.J. Borrego, B. Atik, I. Santo, J. Azevedo, A. Brito de Sa, P. Nogueira, and D. Dean, *Correlating Chlamydia trachomatis infectious load with urogenital ecological success and disease pathogenesis.* Microbes Infect, 2006. **8**(1): p. 16-26.
341. Jalal, H., H. Stephen, M.D. Curran, J. Burton, M. Bradley, and C. Carne, *Development and validation of a rotor-gene real-time PCR assay for detection, identification, and quantification of Chlamydia trachomatis in a single reaction.* J Clin Microbiol, 2006. **44**(1): p. 206-13.
342. Bromhead, C., A. Miller, M. Jones, and D. Whiley, *Comparison of the cobas 4800 CT/NG test with culture for detecting Neisseria gonorrhoeae in genital and nongenital specimens in a low-prevalence population in New Zealand.* J Clin Microbiol, 2013. **51**(5): p. 1505-9.
343. Dize, L., S. West, J.A. Williams, B. Van Der Pol, T.C. Quinn, and C.A. Gaydos, *Comparison of the Abbott m2000 RealTime CT assay and the Cepheid GeneXpert CT/NG assay to the Roche Amplicor CT assay for detection of Chlamydia trachomatis in ocular samples from Tanzania.* J Clin Microbiol, 2013. **51**(5): p. 1611-3.
344. Gaydos, C.A., B. Van Der Pol, M. Jett-Goheen, M. Barnes, N. Quinn, C. Clark, G.E. Daniel, P.B. Dixon, and E.W. Hook, 3rd, *Performance of the Cepheid CT/NG Xpert Rapid PCR Test for Detection of Chlamydia trachomatis and Neisseria gonorrhoeae.* J Clin Microbiol, 2013. **51**(6): p. 1666-72.
345. Upton, A., C. Bromhead, and D.M. Whiley, *Neisseria gonorrhoeae false-positive result obtained from a pharyngeal swab by using the Roche cobas 4800 CT/NG assay in New Zealand in 2012.* J Clin Microbiol, 2013. **51**(5): p. 1609-10.
346. Van Der Pol, B., *Cobas(R) 4800: a fully automated system for the detection of Chlamydia trachomatis and Neisseria gonorrhoeae.* Expert Rev Mol Diagn, 2013. **13**(2): p. 131-40.
347. Marashi, S.M., Z. Moulana, A.A. Imani Fooladi, and M. Mashhadi Karim, *Comparison of Genital Chlamydia trachomatis Infection Incidence Between Women With Infertility and Healthy Women in Iran Using PCR and Immunofluorescence Methods.* Jundishapur J Microbiol, 2014. **7**(4): p. e9450.
348. de Lima Freitas, N.S., C.M. Borborema-Santos, D. Barroso Serrao das Neves, C.M. Costa de Oliveira, J.R. Dutra Ferreira, and S. Astolfi-Filho, *High prevalence detection of Chlamydia trachomatis by polymerase chain reaction in endocervical samples of infertile women attending university hospital in Manaus-Amazonas, Brazil.* Gynecol Obstet Invest, 2011. **72**(4): p. 220-6.
349. Tabrizi, S.N., M. Unemo, D. Golparian, J. Twin, A.E. Limnios, M. Lahra, and R. Guy, *Analytical evaluation of GeneXpert CT/NG, the first genetic point-of-care assay for simultaneous detection of Neisseria gonorrhoeae and Chlamydia trachomatis.* J Clin Microbiol, 2013. **51**(6): p. 1945-7.
350. Causer, L.M., B. Hengel, L. Natoli, A. Tangey, S.G. Badman, S.N. Tabrizi, D. Whiley, J. Ward, J.M. Kaldor, and R.J. Guy, *A field evaluation of a new*

molecular-based point-of-care test for chlamydia and gonorrhoea in remote Aboriginal health services in Australia. Sex Health, 2014.
351. Pearce, D., D. Shenton, J. Holden, and C. Gaydos, *Evaluation of a novel electrochemical detection method for Chlamydia trachomatis: application for point-of-care diagnostics.* Biomedical Engineering, IEEE Transactions on, 2011(99): p. 1-1.
352. Ham, J.Y., J. Jung, B.G. Hwang, W.J. Kim, Y.S. Kim, E.J. Kim, M.Y. Cho, M.S. Hwang, D.I. Won, and J.S. Suh, *Highly sensitive and novel point-of-care system, aQcare Chlamydia TRF kit for detecting Chlamydia trachomatis by using europium (Eu) (III) chelated nanoparticles.* Ann Lab Med, 2015. **35**(1): p. 50-6.
353. Forsbach□Birk, V., U. Simnacher, K.I. Pfrepper, E. Soutschek, A. Kiselev, M. Lampe, T. Meyer, E. Straube, and A. Essig, *Identification and evaluation of a combination of chlamydial antigens to support the diagnosis of severe and invasive Chlamydia trachomatis infections.* Clinical Microbiology and Infection, 2010. **16**(8): p. 1237-1244.
354. Wilkowska-Trojniel, M., B. Zdrodowska-Stefanow, I. Ostaszewska-Puchalska, M. Zbucka, S. Wołczyński, C. Grygoruk, W. Kuczyński, and M. Zdrodowski, *Chlamydia trachomatis urogenital infection in women with infertility.* Advances in Medical Sciences, 2009. **54**(1): p. 82-85.
355. Piura, B., L. Sarov, B. Sarov, D. Kleinman, W. Chaim, and V. Insler, *Serum IgG and IgA antibodies specific for Chlamydia trachomatis in salpingitis patients as determined by the immunoperoxidase assay.* European journal of epidemiology, 1985. **1**(2): p. 110-116.
356. Den Hartog, J., J. Land, F. Stassen, A. Kessels, and C. Bruggeman, *Serological markers of persistent C. trachomatis infections in women with tubal factor subfertility.* Human reproduction, 2005. **20**(4): p. 986-990.
357. van den Broek, I.V., J.A. Land, J.E. van Bergen, S.A. Morre, and M.A. van der Sande, *Chlamydia trachomatis Antibody Testing in Vaginal Mucosal Material versus Blood Samples of Women Attending a Fertility Clinic and an STI Clinic.* Obstet Gynecol Int, 2014. **2014**: p. 601932.
358. Puolakkainen, M., E. Vesterinen, E. Purola, P. Saikku, and J. Paavonen, *Persistence of chlamydial antibodies after pelvic inflammatory disease.* J Clin Microbiol, 1986. **23**(5): p. 924-8.
359. Muvunyi, C.M., L. Claeys, T. De Sutter, P. De Sutter, M. Temmerman, L. Van Renterghem, G. Claeys, and E. Padalko, *Comparison of four serological assays for the diagnosis of Chlamydia trachomatis in subfertile women.* J Infect Dev Ctries, 2012. **6**(5): p. 396-402.
360. Machado, A., E. Guimarães, E. Sakurai, F. Fioravante, W. Amaral, and M. Alves, *High Titers ofChlamydia trachomatis Antibodies in Brazilian Women with Tubal Occlusion or Previous Ectopic Pregnancy.* Infectious diseases in obstetrics and gynecology, 2007. **2007**.
361. Eggert-Kruse, W., G. Rohr, T. Demirakca, R. Rusu, H. Naher, D. Petzoldt, and B. Runnebaum, *Chlamydial serology in 1303 asymptomatic subfertile couples.* Hum Reprod, 1997. **12**(7): p. 1464-75.
362. Akande, V.A., L.P. Hunt, D.J. Cahill, E.O. Caul, W.C. Ford, and J.M. Jenkins, *Tubal damage in infertile women: prediction using chlamydia serology.* Hum Reprod, 2003. **18**(9): p. 1841-7.

363. Sonmez, S., E. Sonmez, L. Yasar, F. Aydin, A. Coskun, and N. Sut, *Can screening Chlamydia trachomatis by serological tests predict tubal damage in infertile patients?* New Microbiol, 2008. **31**(1): p. 75-9.
364. Keltz, M.D., P.S. Gera, and M. Moustakis, *Chlamydia serology screening in infertility patients.* Fertility and Sterility, 2006. **85**(3): p. 752-754.
365. Muvunyi, C.M., N. Dhont, R. Verhelst, M. Temmerman, G. Claeys, and E. Padalko, *Chlamydia trachomatis infection in fertile and subfertile women in Rwanda: prevalence and diagnostic significance of IgG and IgA antibodies testing.* Hum Reprod, 2011. **26**(12): p. 3319-26.
366. Wang, S. and J.T. Grayston, *Human serology in Chlamydia trachomatis infection with microimmunofluorescence.* Journal of Infectious Diseases, 1974. **130**(4): p. 388-397.
367. Bas, S., P. Muzzin, and T.L. Vischer, *Chlamydia trachomatis serology: diagnostic value of outer membrane protein 2 compared with that of other antigens.* J Clin Microbiol, 2001. **39**(11): p. 4082-5.
368. Coppus, S.F., B.C. Opmeer, S. Logan, F. van der Veen, S. Bhattacharya, and B.W. Mol, *The predictive value of medical history taking and Chlamydia IgG ELISA antibody testing (CAT) in the selection of subfertile women for diagnostic laparoscopy: a clinical prediction model approach.* Hum Reprod, 2007. **22**(5): p. 1353-8.
369. Sharma, J., Y. Zhong, F. Dong, J. Piper, G. Wang, and G. Zhong, *Profiling of human antibody responses to Chlamydia trachomatis urogenital tract infection using microplates arrayed with 156 chlamydial fusion proteins.* Infect Immun, 2006. **74**(3): p. 1490 - 1499.
370. Donati, M., K. Laroucau, E. Storni, C. Mazzeo, S. Magnino, A. Di Francesco, R. Baldelli, L. Ceglie, M. Renzi, and R. Cevenini, *Serological response to pgp3 protein in animal and human chlamydial infections.* Veterinary microbiology, 2009. **135**(1-2): p. 181-185.
371. Bas, S., P. Muzzin, B. Ninet, J.E. Bornand, C. Scieux, and T.L. Vischer, *Chlamydial serology: comparative diagnostic value of immunoblotting, microimmunofluorescence test, and immunoassays using different recombinant proteins as antigens.* J Clin Microbiol, 2001. **39**(4): p. 1368-77.
372. Gijsen, A.P., J.A. Land, V.J. Goossens, M.E. Slobbe, and C.A. Bruggeman, *Chlamydia antibody testing in screening for tubal factor subfertility: the significance of IgG antibody decline over time.* Hum Reprod, 2002. **17**(3): p. 699-703.
373. Gijsen, A.P., J.A. Land, V.J. Goossens, P. Leffers, C.A. Bruggeman, and J.L. Evers, *Chlamydia pneumoniae and screening for tubal factor subfertility.* Hum Reprod, 2001. **16**(3): p. 487-91.
374. Broeze, K., B. Opmeer, S. Coppus, N. Van Geloven, M. Alves, G. Ånestad, S. Bhattacharya, J. Allan, M. Guerra-Infante, and J. Den Hartog, *Chlamydia antibody testing and diagnosing tubal pathology in subfertile women: an individual patient data meta-analysis.* Human reproduction update, 2011. **17**(3): p. 301-310.
375. Jones, C.S., P.A. Maple, N.J. Andrews, I.D. Paul, and E.O. Caul, *Measurement of IgG antibodies to Chlamydia trachomatis by commercial enzyme immunoassays and immunofluorescence in sera from pregnant women and patients with infertility, pelvic inflammatory disease, ectopic pregnancy, and laboratory diagnosed Chlamydia psittaci/Chlamydia pneumoniae infection.* J Clin Pathol, 2003. **56**(3): p. 225-9.

376. Land, J.A., A.P. Gijsen, A.G.H. Kessels, M.E.P. Slobbe, and C.A. Bruggeman, *Performance of five serological chlamydia antibody tests in subfertile women.* Human Reproduction, 2003. **18**(12): p. 2621-2627.
377. Bax, C.J., J.A. Mutsaers, C.L. Jansen, J.B. Trimbos, P.J. Dorr, and P.M. Oostvogel, *Comparison of serological assays for detection of Chlamydia trachomatis antibodies in different groups of obstetrical and gynecological patients.* Clin Diagn Lab Immunol, 2003. **10**(1): p. 174-6.
378. Dutta, R., R. Jha, S. Salhan, and A. Mittal, *Chlamydia trachomatis-specific heat shock proteins 60 antibodies can serve as prognostic marker in secondary infertile women.* Infection, 2008. **36**(4): p. 374-378.
379. Toye, B., C. Laferriere, P. Claman, P. Jessamine, and R. Peeling, *Association between antibody to the chlamydial heat-shock protein and tubal infertility.* J Infect Dis, 1993. **168**(5): p. 1236-40.
380. Claman, P., L. Honey, R.W. Peeling, P. Jessamine, and B. Toye, *The presence of serum antibody to the chlamydial heat shock protein (CHSP60) as a diagnostic test for tubal factor infertility.* Fertil Steril, 1997. **67**(3): p. 501-4.
381. Tiitinen, A., H.M. Surcel, M. Halttunen, S. Birkelund, A. Bloigu, G. Christiansen, P. Koskela, S.G. Morrison, R.P. Morrison, and J. Paavonen, *Chlamydia trachomatis and chlamydial heat shock protein 60-specific antibody and cell-mediated responses predict tubal factor infertility.* Human Reproduction, 2006. **21**(6): p. 1533-8.
382. Collet, T., T. Macnaughton, T. Walsh, J. Debattista, and P. Timms, *Identification of novel markers for uncomplicated lower genital tract infections and upper genital tract pathology due to Chlamydia trachomatis.* International Journal of Infectious Diseases, 2011. **15**(4): p. e257-e266.
383. LaVerda, D., L.N. Albanese, P.E. Ruther, S.G. Morrison, R.P. Morrison, K.A. Ault, and G.I. Byrne, *Seroreactivity to Chlamydia trachomatis Hsp10 correlates with severity of human genital tract disease.* Infect Immun, 2000. **68**(1): p. 303-9.
384. Persson, K., S. Osser, S. Birkelund, G. Christiansen, and H. Brade, *Antibodies to Chlamydia trachomatis heat shock proteins in women with tubal factor infertility are associated with prior infection by C. trachomatis but not by C. pneumoniae.* Hum Reprod, 1999. **14**(8): p. 1969-73.
385. Rodgers, A.K., N.M. Budrys, S. Gong, J. Wang, A. Holden, R.S. Schenken, and G. Zhong, *Genome-wide identification of Chlamydia trachomatis antigens associated with tubal factor infertility.* Fertil Steril, 2011. **96**(3): p. 715-21.
386. Rodgers, A.K., J. Wang, Y. Zhang, A. Holden, B. Berryhill, N.M. Budrys, R.S. Schenken, and G. Zhong, *Association of tubal factor infertility with elevated antibodies to Chlamydia trachomatis caseinolytic protease P.* American journal of obstetrics and gynecology, 2010. **203**(5): p. 494. e7-494. e14.
387. Budrys, N.M., S. Gong, A.K. Rodgers, J. Wang, C. Louden, R. Shain, R.S. Schenken, and G. Zhong, *Chlamydia trachomatis antigens recognized in women with tubal factor infertility, normal fertility, and acute infection.* Obstet Gynecol, 2012. **119**(5): p. 1009-16.
388. Qi, M., S. Gong, L. Lei, Q. Liu, and G. Zhong, *A Chlamydia trachomatis OmcB C-terminal fragment is released into the host cell cytoplasm and is immunogenic in humans.* Infect Immun, 2011. **79**(6): p. 2193-203.

389. Frikha-Gargouri, O., R. Gdoura, A. Znazen, J. Gargouri, and A. Hammami, *Diagnostic value of enzyme-linked immunosorbent assays using hypothetical proteins CT226 and CT795 as antigens in Chlamydia trachomatis serodiagnosis.* Diagn Microbiol Infect Dis, 2009. **65**(3): p. 224-31.
390. Wills, G.S., P.J. Horner, R. Reynolds, A.M. Johnson, D.A. Muir, D.W. Brown, A. Winston, A.J. Broadbent, D. Parker, and M.O. McClure, *Pgp3 antibody enzyme-linked immunosorbent assay, a sensitive and specific assay for seroepidemiological analysis of Chlamydia trachomatis infection.* Clin Vaccine Immunol, 2009. **16**(6): p. 835-43.
391. Ghaem-Maghami, S., G. Ratti, M. Comanducci, P. Hay, R. Bailey, D. Mabey, H. Whittle, M. Ward, and D. Lewis, *Mucosal and systemic immune responses to plasmid protein pgp3 in patients with genital and ocular Chlamydia trachomatis infection.* Clinical & Experimental Immunology, 2003. **132**(3): p. 436-442.
392. Donati, M., V. Sambri, M. Comanducci, K. Di Leo, E. Storni, L. Giacani, G. Ratti, and R. Cevenini, *DNA immunization with pgp3 gene of Chlamydia trachomatis inhibits the spread of chlamydial infection from the lower to the upper genital tract in C3H/HeN mice.* Vaccine, 2003. **21**(11-12): p. 1089-1093.
393. Liu , X., M. Afrane, D.E. Clemmer, G. Zhong, and D.E. Nelson, *Identification of Chlamydia trachomatis Outer Membrane Complex Proteins by Differential Proteomics.* Journal of Bacteriology, 2010. **192**(11): p. 2852-2860.
394. Greub, G., C. Kebbi-Beghdadi, C. Bertelli, F. Collyn, B.M. Riederer, C. Yersin, A. Croxatto, and D. Raoult, *High throughput sequencing and proteomics to identify immunogenic proteins of a new pathogen: the dirty genome approach.* PLoS ONE, 2009. **4**(12): p. e8423.
395. Inhorn, M.C. and P. Patrizio, *Infertility around the globe: new thinking on gender, reproductive technologies and global movements in the 21st century.* Hum Reprod Update, 2015. **21**(4): p. 411-26.
396. Balsak, D., F. Uysal, S. Sadik, A. Guler, S. Tinar, and O. Taskin, *Comparison of hysterosalpingography and transvaginal hydrolaparoscopy in patients with tubal factor infertility: a prospective cohort study.* Wideochir Inne Tech Maloinwazyjne, 2014. **9**(2): p. 190-5.
397. Logan, S., R. Gazvani, H. McKenzie, A. Templeton, and S. Bhattacharya, *Can history, ultrasound, or ELISA chlamydial antibodies, alone or in combination, predict tubal factor infertility in subfertile women?* Hum Reprod, 2003. **18**(11): p. 2350-6.
398. Broeze, K.A., B.C. Opmeer, N. Van Geloven, S.F. Coppus, J.A. Collins, J.E. Den Hartog, P.J. Van der Linden, P. Marianowski, E.H. Ng, J.W. Van der Steeg, P. Steures, A. Strandell, F. Van der Veen, and B.W. Mol, *Are patient characteristics associated with the accuracy of hysterosalpingography in diagnosing tubal pathology? An individual patient data meta-analysis.* Hum Reprod Update, 2011. **17**(3): p. 293-300.
399. Swart, P., B.W. Mol, F. van der Veen, M. van Beurden, W.K. Redekop, and P.M. Bossuyt, *The accuracy of hysterosalpingography in the diagnosis of tubal pathology: a meta-analysis.* Fertil Steril, 1995. **64**(3): p. 486-91.
400. Thomas, K., L. Coughlin, P.T. Mannion, and N.G. Haddad, *The value of Chlamydia trachomatis antibody testing as part of routine infertility investigations.* Hum Reprod, 2000. **15**(5): p. 1079-82.

401. den Hartog, J.E., C.M. Lardenoije, J.L. Severens, J.A. Land, J.L. Evers, and A.G. Kessels, *Screening strategies for tubal factor subfertility.* Hum Reprod, 2008. **23**(8): p. 1840-8.
402. Mol, B.W.J., B. Dijkman, P. Wertheim, J. Lijmer, F. van der Veen, and P.M.M. Bossuyt, *The accuracy of serum chlamydial antibodies in the diagnosis of tubal pathology: a meta-analysis.* Fertility and Sterility. **67**(6): p. 1031-1037.
403. Dabekausen, Y.A., J.L. Evers, J.A. Land, and F.S. Stals, *Chlamydia trachomatis antibody testing is more accurate than hysterosalpingography in predicting tubal factor infertility.* Fertil Steril, 1994. **61**(5): p. 833-7.
404. Johnson, N.P., K. Taylor, A.A. Nadgir, D.J. Chinn, and P.J. Taylor, *Can diagnostic laparoscopy be avoided in routine investigation for infertility?* Bjog, 2000. **107**(2): p. 174-8.
405. Salmani, M.P., P.B. Mindolli, and G. Vishwanath, *Seroprevalence of Chlamydia trachomatis infection in women with bad obstetric history and infertility.* J Commun Dis, 2011. **43**(4): p. 243-7.
406. Malik, A., S. Jain, M. Rizvi, I. Shukla, and S. Hakim, *Chlamydia trachomatis infection in women with secondary infertility.* Fertil Steril, 2009. **91**(1): p. 91-5.
407. Collins, J.A., *An international survey of the health economics of IVF and ICSI.* Human Reproduction Update, 2002. **8**(3): p. 265-277.
408. Baud, D., L. Regan, and G. Greub, *Comparison of five commercial serological tests for the detection of anti-Chlamydia trachomatis antibodies.* European Journal of Clinical Microbiology & Infectious Diseases, 2010. **29**(6): p. 669-675.
409. Obstetrie, N.V.v.O.e., *Orienterend fertiliteitsonderzoek [NVOG guideline diagnostic fertility workup]*. 2004.
410. Land, J.A., A.P. Gijsen, A.G. Kessels, M.E. Slobbe, and C.A. Bruggeman, *Performance of five serological chlamydia antibody tests in subfertile women.* Hum Reprod, 2003. **18**(12): p. 2621-7.
411. Stansfield, S.H., P. Patel, J. Debattista, C.W. Armitage, K. Cunningham, P. Timms, J. Allan, A. Mittal, and W.M. Huston, *Proof of concept: A bioinformatic and serological screening method for identifying new peptide antigens for Chlamydia trachomatis related sequelae in women.* Results in Immunology, 2013. **3**(0): p. 33-39.
412. Jacobson, R., *Principles of validation of diagnostic assays for infectious diseases.* Manual of standards for diagnostic tests and vaccines, 3rd ed. Paris: Office International de Epizooties, 1996: p. 8-15.
413. Viechtbauer, W. *Conducting meta-analyses in R with the metafor package*. Journal of Statistical Software, 2010. **36**, 1-48.
414. Pinheiro, J., D. Bates, S. DebRoy, and D. Sarkar, *Linear and nonlinear mixed effects models.* R package version, 2007. **3**: p. 57.
415. Svensson, L., P.A. Mardh, and L. Westrom, *Infertility after acute salpingitis with special reference to Chlamydia trachomatis.* Fertil Steril, 1983. **40**(3): p. 322-9.
416. Land, J.A., J.E. Van Bergen, S.A. Morre, and M.J. Postma, *Epidemiology of Chlamydia trachomatis infection in women and the cost-effectiveness of screening.* Hum Reprod Update, 2010. **16**(2): p. 189-204.
417. Evers, J.L., *Female subfertility.* Lancet, 2002. **360**(9327): p. 151-9.

418. Heinonen, P.K. and A. Miettinen, *Laparoscopic study on the microbiology and severity of acute pelvic inflammatory disease.* Eur J Obstet Gynecol Reprod Biol, 1994. **57**(2): p. 85-9.
419. Eckert, L.O., S.E. Hawes, P.K. Wolner-Hanssen, N.B. Kiviat, J.N. Wasserheit, J.A. Paavonen, D.A. Eschenbach, and K.K. Holmes, *Endometritis: the clinical-pathologic syndrome.* Am J Obstet Gynecol, 2002. **186**(4): p. 690-5.
420. Lavy, Y., A. Lev-Sagie, H. Holtzer, A. Revel, and A. Hurwitz, *Should laparoscopy be a mandatory component of the infertility evaluation in infertile women with normal hysterosalpingogram or suspected unilateral distal tubal pathology?* European Journal of Obstetrics & Gynecology and Reproductive Biology, 2004. **114**(1): p. 64-68.
421. Tanahatoe, S., C. Lambalk, J. McDonnell, J. Dekker, V. Mijatovic, and P. Hompes, *Diagnostic laparoscopy is needed after abnormal hysterosalpingography to prevent over-treatment with IVF.* Reproductive BioMedicine Online, 2008. **16**(3): p. 410-415.
422. Bonneau, C., O. Chanelles, C. Sifer, and C. Poncelet, *Use of laparoscopy in unexplained infertility.* Eur J Obstet Gynecol Reprod Biol, 2012. **163**(1): p. 57-61.
423. Fiddelers, A.A., J.A. Land, G. Voss, A.G. Kessels, and J.L. Severens, *Cost-effectiveness of Chlamydia antibody tests in subfertile women.* Hum Reprod, 2005. **20**(2): p. 425-32.
424. Jones, R.B., B.R. Ardery, S.L. Hui, and R.E. Cleary, *Correlation between serum antichlamydial antibodies and tubal factor as a cause of infertility.* Fertil Steril, 1982. **38**(5): p. 553-8.
425. Sellors, J.W., J.B. Mahony, M.A. Chernesky, and D.J. Rath, *Tubal factor infertility: an association with prior chlamydial infection and asymptomatic salpingitis.* Fertil Steril, 1988. **49**(3): p. 451-7.
426. Shaw, A.C., M. Rossel Larsen, P. Roepstorff, J. Justesen, G. Christiansen, and S. Birkelund, *Mapping and identification of interferon gamma-regulated HeLa cell proteins separated by immobilized pH gradient two-dimensional gel electrophoresis.* Electrophoresis, 1999. **20**(4-5): p. 984-93.
427. Sanchez-Campillo, M., L. Bini, M. Comanducci, R. Raggiaschi, B. Marzocchi, V. Pallini, and G. Ratti, *Identification of immunoreactive proteins of Chlamydia trachomatis by Western blot analysis of a two-dimensional electrophoresis map with patient sera.* Electrophoresis, 1999. **20**(11): p. 2269-79.
428. Forsbach-Birk, V., U. Simnacher, K.I. Pfrepper, E. Soutschek, A.O. Kiselev, M.F. Lampe, T. Meyer, E. Straube, and A. Essig, *Identification and evaluation of a combination of chlamydial antigens to support the diagnosis of severe and invasive Chlamydia trachomatis infections.* Clin Microbiol Infect, 2010. **16**(8): p. 1237-44.
429. Zhu, S., J. Chen, M. Zheng, W. Gong, X. Xue, W. Li, and L. Zhang, *Identification of immunodominant linear B-cell epitopes within the major outer membrane protein of Chlamydia trachomatis.* Acta Biochim Biophys Sin (Shanghai), 2010. **42**(11): p. 771-8.
430. Li, Z.Y., Q.L. Huang, S.M. Su, G.M. Zhong, and Y.M. Wu, *Development of ELISAs for the detection of urogenital chlamydia trachomatis infection targeting the pORF5 protein.* Biomed Environ Sci, 2013. **26**(3): p. 169-75.

431. Greiner, M., D. Pfeiffer, and R.D. Smith, *Principles and practical application of the receiver-operating characteristic analysis for diagnostic tests.* Prev Vet Med, 2000. **45**(1-2): p. 23-41.
432. Parikh, R., A. Mathai, S. Parikh, G. Chandra Sekhar, and R. Thomas, *Understanding and using sensitivity, specificity and predictive values.* Indian Journal of Ophthalmology, 2008. **56**(1): p. 45-50.
433. Rodgers, A.K., N.M. Budrys, S. Gong, J. Wang, A. Holden, R.S. Schenken, and G. Zhong, *Genome-wide identification of Chlamydia trachomatis antigens associated with tubal factor infertility.* Fertility and sterility, 2011.
434. Buderer, N.M., *Statistical methodology: I. Incorporating the prevalence of disease into the sample size calculation for sensitivity and specificity.* Acad Emerg Med, 1996. **3**: p. 895-900.
435. Banoo, S., D. Bell, P. Bossuyt, A. Herring, D. Mabey, F. Poole, P.G. Smith, N. Sriram, C. Wongsrichanalai, and R. Linke, *Evaluation of diagnostic tests for infectious diseases: general principles.* Nature Reviews Microbiology, 2008. **8**: p. S16-S28.
436. Glas, A.S., J.G. Lijmer, M.H. Prins, G.J. Bonsel, and P.M. Bossuyt, *The diagnostic odds ratio: a single indicator of test performance.* J Clin Epidemiol, 2003. **56**(11): p. 1129-35.
437. Mouton, J.W., M.F. Peeters, J.H. van Rijssort-Vos, and R.P. Verkooyen, *Tubal factor pathology caused by Chlamydia trachomatis: the role of serology.* Int J STD AIDS, 2002. **13 Suppl 2**: p. 26-9.
438. Assis, L.M., J.R. Sousa, N.F. Pinto, A.A. Silva, A.F. Vaz, P.P. Andrade, E.M. Carvalho, and M.A. De Melo, *B-cell epitopes of antigenic proteins in Leishmania infantum: an in silico analysis.* Parasite Immunol, 2014. **36**(7): p. 313-23.
439. Pal, S., X. Cheng, E.M. Peterson, and L.M. de la Maza, *Mapping of a surface-exposed B-cell epitope to the variable sequent 3 of the major outer-membrane protein of Chlamydia trachomatis.* J Gen Microbiol, 1993. **139**(7): p. 1565-70.
440. den Hartog, J.E., J.A. Land, F.R. Stassen, M.E. Slobbe-van Drunen, A.G. Kessels, and C.A. Bruggeman, *The role of chlamydia genus-specific and species-specific IgG antibody testing in predicting tubal disease in subfertile women.* Hum Reprod, 2004. **19**(6): p. 1380-4.
441. Tasdemir, I., M. Tasdemir, H. Kodama, K. Sekine, and T. Tanaka, *Effect of chlamydial antibodies on the outcome of in vitro fertilization (IVF) treatment.* J Assist Reprod Genet, 1994. **11**(2): p. 104-6.
442. Claman, P., M.N. Amimi, R.W. Peeling, B. Toye, and P. Jessamine, *Does serologic evidence of remote Chlamydia trachomatis infection and its heat shock protein (CHSP 60) affect in vitro fertilization-embryo transfer outcome?* Fertil Steril, 1996. **65**(1): p. 146-9.
443. Hess, S., J. Peters, G. Bartling, C. Rheinheimer, P. Hegde, M. Magid-Slav, R. Tal-Singer, and A. Klos, *More than just innate immunity: comparative analysis of Chlamydophila pneumoniae and Chlamydia trachomatis effects on host-cell gene regulation.* Cell Microbiol, 2003. **5**(11): p. 785-95.
444. Buchholz, K.R. and R.S. Stephens, *Activation of the host cell proinflammatory interleukin-8 response by Chlamydia trachomatis.* Cell Microbiol, 2006. **8**(11): p. 1768-79.
445. Zhou, H., Q. Huang, Z. Li, Y. Wu, X. Xie, K. Ma, W. Cao, Z. Zhou, C. Lu, and G. Zhong, *PORF5 plasmid protein of Chlamydia trachomatis induces*

MAPK-mediated pro-inflammatory cytokines via TLR2 activation in THP-1 cells. Sci China Life Sci, 2013. **56**(5): p. 460-6.
446. Nagarajan, U.M., D.M. Ojcius, L. Stahl, R.G. Rank, and T. Darville, *Chlamydia trachomatis induces expression of IFN-gamma-inducible protein 10 and IFN-beta independent of TLR2 and TLR4, but largely dependent on MyD88.* J Immunol, 2005. **175**(1): p. 450-60.
447. Roth, A., P. Konig, G. van Zandbergen, M. Klinger, T. Hellwig-Burgel, W. Daubener, M.K. Bohlmann, and J. Rupp, *Hypoxia abrogates antichlamydial properties of IFN-gamma in human fallopian tube cells in vitro and ex vivo.* Proc Natl Acad Sci U S A, 2010. **107**(45): p. 19502-7.
448. Vicetti Miguel, R.D., S.A. Harvey, W.A. LaFramboise, S.D. Reighard, D.B. Matthews, and T.L. Cherpes, *Human female genital tract infection by the obligate intracellular bacterium Chlamydia trachomatis elicits robust Type 2 immunity.* PLoS One, 2013. **8**(3): p. e58565.
449. Vicetti Miguel, R.D., S.A. Maryak, and T.L. Cherpes, *Brefeldin A, but not monensin, enables flow cytometric detection of interleukin-4 within peripheral T cells responding to ex vivo stimulation with Chlamydia trachomatis.* J Immunol Methods, 2012. **384**(1-2): p. 191-5.
450. Khamesipour, A., S. Pal, E.M. Peterson, and L.M. de la Maza, *Induction of infertility by the Chlamydia trachomatis mouse pneumonitis biovar in strains of mice that differ in their response to the 60 kDa heat shock protein.* Journal of Reproduction and Fertility, 1994. **101**(2): p. 287-294.
451. Pal, S., T.J. Fielder, E.M. Peterson, and L.M. de la Maza, *Protection against infertility in a BALB/c mouse salpingitis model by intranasal immunization with the mouse pneumonitis biovar of Chlamydia trachomatis.* Infect Immun, 1994. **62**(8): p. 3354-62.
452. Pal, S., J. Rangel, E.M. Peterson, and L.M. de la Maza, *Immunogenic and protective ability of the two developmental forms of Chlamydiae in a mouse model of infertility.* Vaccine, 1999. **18**(7-8): p. 752-61.
453. Loomis, W.P. and M.N. Starnbach, *Chlamydia trachomatis infection alters the development of memory CD8+ T cells.* J Immunol, 2006. **177**(6): p. 4021-7.
454. Frazer, L.C., A.M. Scurlock, M.A. Zurenski, M.M. Riley, M. Mintus, D.A. Pociask, J.E. Sullivan, C.W. Andrews, Jr., and T. Darville, *IL-23 induces IL-22 and IL-17 production in response to Chlamydia muridarum genital tract infection, but the absence of these cytokines does not influence disease pathogenesis.* Am J Reprod Immunol, 2013. **70**(6): p. 472-84.
455. Scurlock, A.M., L.C. Frazer, C.W. Andrews, Jr., C.M. O'Connell, I.P. Foote, S.L. Bailey, K. Chandra-Kuntal, J.K. Kolls, and T. Darville, *Interleukin-17 contributes to generation of Th1 immunity and neutrophil recruitment during Chlamydia muridarum genital tract infection but is not required for macrophage influx or normal resolution of infection.* Infect Immun, 2011. **79**(3): p. 1349-62.
456. Balasubramaniam, E.S., S. Van Noorden, and M. El-Bahrawy, *The expression of interleukin (IL)-6, IL-8, and their receptors in fallopian tubes with ectopic tubal gestation.* Fertil Steril, 2012. **98**(4): p. 898-904.
457. Johnson, R.M., M.S. Kerr, and J.E. Slaven, *An atypical CD8 T-cell response to Chlamydia muridarum genital tract infections includes T cells that produce interleukin-13.* Immunology, 2014. **142**(2): p. 248-57.

458. Gondek, D.C., A.J. Olive, G. Stary, and M.N. Starnbach, *CD4+ T cells are necessary and sufficient to confer protection against Chlamydia trachomatis infection in the murine upper genital tract.* J Immunol, 2012. **189**(5): p. 2441-9.
459. Witkin, S.S., J. Jeremias, M. Toth, and W.J. Ledger, *Cell-mediated immune response to the recombinant 57-kDa heat-shock protein of Chlamydia trachomatis in women with salpingitis.* J Infect Dis, 1993. **167**(6): p. 1379-83.
460. Paavonen, J., K. Teisala, P.K. Heinonen, R. Aine, S. Laine, M. Lehtinen, A. Miettinen, R. Punnonen, and P. Gronroos, *Microbiological and histopathological findings in acute pelvic inflammatory disease.* BJ Obstet Gynaecol, 1987. **94**(5): p. 454-60.
461. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
462. [Internet], T.N.h. *The NCBI handbook [Internet]*. 2002 Oct; Available from: http://www.ncbi.nlm.nih.gov/books/NBK21101.
463. Rutz, S., C. Eidenschenk, and W. Ouyang, *IL-22, not simply a Th17 cytokine.* Immunol Rev, 2013. **252**(1): p. 116-32.
464. de Jesus De Haro-Cruz, M., I. Deleon-Rodriguez, M.R. Escobedo-Guerra, M. Lopez-Hurtado, G. Arteaga-Troncoso, F.J. Ortiz-Ibarra, and F.M. Guerra-Infante, *Genotyping of Chlamydia trachomatis from endocervical specimens of infertile Mexican women.* Enferm Infecc Microbiol Clin, 2011. **29**(2): p. 102-8.
465. Gaur, L.K., R.W. Peeling, M. Cheang, J. Kimani, J. Bwayo, F. Plummer, and R.C. Brunham, *Association of Chlamydia trachomatis heat-shock protein 60 antibody and HLA class II DQ alleles.* J Infect Dis, 1999. **180**(1): p. 234-7.
466. Kimani, J., I.W. Maclean, J.J. Bwayo, K. MacDonald, J. Oyugi, G.M. Maitha, R.W. Peeling, M. Cheang, N.J. Nagelkerke, F.A. Plummer, and R.C. Brunham, *Risk factors for Chlamydia trachomatis pelvic inflammatory disease among sex workers in Nairobi, Kenya.* J Infect Dis, 1996. **173**(6): p. 1437-44.
467. Kim, S.K., L. Devine, M. Angevine, R. DeMars, and P.B. Kavathas, *Direct detection and magnetic isolation of Chlamydia trachomatis major outer membrane protein-specific CD8+ CTLs with HLA class I tetramers.* J Immunol, 2000. **165**(12): p. 7285-92.
468. Costantini, S., R. Raucci, T. De Vero, G. Castello, and G. Colonna, *Common structural interactions between the receptors CXCR3, CXCR4 and CXCR7 complexed with their natural ligands, CXCL11 and CXCL12, by a modeling approach.* Cytokine, 2013. **64**(1): p. 316-21.
469. Belay, T., F.O. Eko, G.A. Ananaba, S. Bowers, T. Moore, D. Lyn, and J.U. Igietseme, *Chemokine and chemokine receptor dynamics during genital chlamydial infection.* Infect Immun, 2002. **70**(2): p. 844-50.
470. Buckner, L.R., M.E. Lewis, S.J. Greene, T.P. Foster, and A.J. Quayle, *Chlamydia trachomatis infection results in a modest pro-inflammatory cytokine response and a decrease in T cell chemokine secretion in human polarized endocervical epithelial cells.* Cytokine, 2013. **63**(2): p. 151-65.
471. Caldwell, H.D., H.K. Maxion, and K.A. Kelly, *Chemokine expression patterns differ within anatomically distinct regions of the genital tract during Chlamydia trachomatis infection.* Infect Immun, 2002. **70**(3): p. 1538-46.

472. Porcella, S.F., J.H. Carlson, D.E. Sturdevant, G.L. Sturdevant, K. Kanakabandi, K. Virtaneva, H. Wilder, W.M. Whitmire, and L. Song, *Transcriptional profiling of human epithelial cells infected with plasmid-bearing and plasmid-deficient Chlamydia trachomatis.* 2015. **83**(2): p. 534-43.
473. Sakthivel, S.K., U.P. Singh, S. Singh, D.D. Taub, J.U. Igietseme, and J.W. Lillard, Jr., *CCL5 regulation of mucosal chlamydial immunity and infection.* BMC Microbiol, 2008. **8**: p. 136.
474. Sellami, H., N. Said-Sadier, A. Znazen, R. Gdoura, D.M. Ojcius, and A. Hammami, *Chlamydia trachomatis infection increases the expression of inflammatory tumorigenic cytokines and chemokines as well as components of the Toll-like receptor and NF-kappaB pathways in human prostate epithelial cells.* Mol Cell Probes, 2014. **28**(4): p. 147-54.
475. Shibahara, H., Y. Hirano, Ayustawati, K. Kikuchi, A. Taneichi, H. Fujiwara, S. Takamizawa, and I. Sato, *Chemokine bioactivity of RANTES is elevated in the sera of infertile women with past Chlamydia trachomatis infection.* Am J Reprod Immunol, 2003. **49**(3): p. 169-73.
476. Maxion, H.K. and K.A. Kelly, *Chemokine expression patterns differ within anatomically distinct regions of the genital tract during Chlamydia trachomatis infection.* Infect Immun, 2002. **70**(3): p. 1538-46.
477. Kaplan, A.P., *Chemokines, chemokine receptors and allergy.* Int Arch Allergy Immunol, 2001. **124**(4): p. 423-31.
478. Wan, C., J.L. Latter, A. Amirshahi, I. Symonds, J. Finnie, N. Bowden, R.J. Scott, K.A. Cunningham, P. Timms, and K.W. Beagley, *Progesterone activates multiple innate immune pathways in Chlamydia trachomatis-infected endocervical cells.* Am J Reprod Immunol, 2014. **71**(2): p. 165-77.
479. Osawa, Y., M. Nagaki, Y. Banno, D.A. Brenner, T. Asano, Y. Nozawa, H. Moriwaki, and S. Nakashima, *Tumor necrosis factor alpha-induced interleukin-8 production via NF-kappaB and phosphatidylinositol 3-kinase/Akt pathways inhibits cell apoptosis in human hepatocytes.* Infect Immun, 2002. **70**(11): p. 6294-301.
480. Darville, T., C.W. Andrews, Jr., and R.G. Rank, *Does inhibition of tumor necrosis factor alpha affect chlamydial genital tract infection in mice and guinea pigs?* Infect Immun, 2000. **68**(9): p. 5299-305.
481. Murthy, A.K., W. Li, B.K. Chaganty, S. Kamalakaran, M.N. Guentzel, J. Seshu, T.G. Forsthuber, G. Zhong, and B.P. Arulanandam, *Tumor necrosis factor alpha production from CD8+ T cells mediates oviduct pathological sequelae following primary genital Chlamydia muridarum infection.* Infect Immun, 2011. **79**(7): p. 2928-35.
482. Manam, S., B.J. Nicholson, and A.K. Murthy, *OT-1 mice display minimal upper genital tract pathology following primary intravaginal Chlamydia muridarum infection.* Pathog Dis, 2013. **67**(3): p. 221-4.
483. Sharara, F.I. and J.T. Queenan, Jr., *Elevated serum Chlamydia trachomatis IgG antibodies. Association with decreased implantation rates in GIFT.* J Reprod Med, 1999. **44**(7): p. 581-6.
484. Coppus, S.F., J.A. Land, B.C. Opmeer, P. Steures, M.J. Eijkemans, P.G. Hompes, P.M. Bossuyt, F. van der Veen, B.W. Mol, and J.W. van der Steeg, *Chlamydia trachomatis IgG seropositivity is associated with lower natural conception rates in ovulatory subfertile women without visible tubal pathology.* Hum Reprod, 2011. **26**(11): p. 3061-7.

485. Mascellino, M.T., M.R. Ciardi, A. Oliva, F. Cecinato, M.P. Hassemer, and L. Borgese, *Chlamydia trachomatis detection in a population of asymptomatic and symptomatic women: Correlation with the presence of serological markers for this infection.* The new microbiologica, 2008. **31**(2): p. 249.
486. Malik, A., S. Jain, S. Hakim, I. Shukla, and M. Rizvi, *Chlamydia trachomatis infection & female infertility.* Indian J Med Res, 2006. **123**(6): p. 770-5.
487. Patel, A., D. Sachdev, P. Nagpal, U. Chaudhry, S. Sonkar, S. Mendiratta, and D. Saluja, *Prevalence of Chlamydia infection among women visiting a gynaecology outpatient department: evaluation of an in-house PCR assay for detection of Chlamydia trachomatis.* Annals of Clinical Microbiology and Antimicrobials, 2010. **9**(1): p. 1-10.
488. Adams, E.J., A. Charlett, W.J. Edmunds, and G. Hughes, *Chlamydia trachomatis in the United Kingdom: a systematic review and analysis of prevalence studies.* Sex Transm Infect, 2004. **80**(5): p. 354-62.
489. Marangoni, A., C. Foschi, P. Nardini, A. D'Antuono, N. Banzola, A. Di Francesco, F. Ostanello, I. Russo, M. Donati, and R. Cevenini, *Chlamydia trachomatis serovar distribution and other sexually transmitted coinfections in subjects attending an STD outpatients clinic in Italy.* New Microbiol, 2012. **35**(2): p. 215-9.
490. Kohli, R., W.P. Konya, T. Obura, W. Stones, and G. Revathi, *Prevalence of genital Chlamydia infection in urban women of reproductive age, Nairobi, Kenya.* BMC Res Notes, 2013. **6**: p. 44.
491. Ngandjio, A., M. Clerc, M.C. Fonkoua, J. Thonnon, F. Njock, R. Pouillot, F. Lunel, C. Bebear, B. De Barbeyrac, and A. Bianchi, *Screening of volunteer students in Yaounde (Cameroon, Central Africa) for Chlamydia trachomatis infection and genotyping of isolated C. trachomatis strains.* J Clin Microbiol, 2003. **41**(9): p. 4404-7.
492. Datta, S.D., E. Torrone, D. Kruszon-Moran, S. Berman, R. Johnson, C.L. Satterwhite, J. Papp, and H. Weinstock, *Chlamydia trachomatis trends in the United States among persons 14 to 39 years of age, 1999-2008.* Sex Transm Dis, 2012. **39**(2): p. 92-6.
493. Satterwhite, C.L., A.M. Gray, S. Berman, H. Weinstock, D. Kleinbaum, and P.P. Howards, *Chlamydia trachomatis infections among women attending prenatal clinics: United States, 2004-2009.* Sex Transm Dis, 2012. **39**(6): p. 416-20.
494. Haar, K., V. Bremer, C. Houareau, T. Meyer, S. Desai, M. Thamm, and O. Hamouda, *Risk factors for Chlamydia trachomatis infection in adolescents: results from a representative population-based survey in Germany, 2003-2006.* Euro Surveill, 2013. **18**(34).
495. McMillan, H.M., H. O'Carroll, J.S. Lambert, K.B. Grundy, M. O'Reilly, B. Lennon, C. Collins, T.A. Walsh, M.P. Geary, and M.T. Cafferkey, *Screening for Chlamydia trachomatis in asymptomatic women attending outpatient clinics in a large maternity hospital in Dublin, Ireland.* Sex Transm Infect, 2006. **82**(6): p. 503-5.
496. Gravningen, K., A.S. Furberg, G.S. Simonsen, and T. Wilsgaard, *Early sexual behaviour and Chlamydia trachomatis infection - a population based cross-sectional study on gender differences among adolescents in Norway.* BMC Infect Dis, 2012. **12**: p. 319.
497. van Aar, F., M. de Moraes, S.A. Morre, J.E. van Bergen, F.R. van der Klis, J.A. Land, M.A. van der Sande, and I.V. van den Broek, *Chlamydia*

trachomatis IgG seroprevalence in the general population of the Netherlands in 1996 and in 2007: differential changes by gender and age. Sex Transm Infect, 2014. **90**(5): p. 434-40.
498. Mania-Pramanik, J., S. Kerkar, S. Sonawane, P. Mehta, and V. Salvi, *Current Chlamydia trachomatis Infection, A Major Cause of Infertility.* J Reprod Infertil, 2012. **13**(4): p. 204-10.
499. Abdella, R.M., H.I. Abdelmoaty, R.H. Elsherif, A.M. Sayed, N.A. Sherif, H.M. Gouda, A. El Lithy, M. Almohamady, M. Abdelbar, A.N. Hosni, A. Magdy, and Y. Ma, *Screening for Chlamydia trachomatis in Egyptian women with unexplained infertility, comparing real-time PCR techniques to standard serology tests: case control study : Chlamydia trachomatis and unexplained infertility.* BMC Womens Health, 2015. **15**(1): p. 45.
500. Ikeme, A.C., H.U. Ezegwui, L.C. Ikeako, I. Agbata, and E. Agbata, *Seroprevalence of Chlamydia trachomatis in Enugu, Nigeria.* Niger J Clin Pract, 2011. **14**(2): p. 176-80.
501. Jobe, K.A., R.F. Downey, D. Hammar, L. Van Slyke, and T.A. Schmidt, *Epidemiology of sexually transmitted infections in rural southwestern Haiti: the Grand'Anse Women's Health Study.* Am J Trop Med Hyg, 2014. **91**(5): p. 881-6.
502. Fernandez-Benitez, C., P. Mejuto-Lopez, L. Otero-Guerra, M.J. Margolles-Martins, P. Suarez-Leiva, and F. Vazquez, *Prevalence of genital Chlamydia trachomatis infection among young men and women in Spain.* BMC Infect Dis, 2013. **13**: p. 388.
503. Gyaneshwar, R., H. Nsanze, K.P. Singh, S. Pillay, and I. Seruvatu, *The prevalence of sexually transmitted disease agents in pregnant women in Suva.* Aust N Z J Obstet Gynaecol, 1987. **27**(3): p. 213-5.
504. Afrasiabi, S., R. Moniri, M. Samimi, A. Khorshidi, and S.G. Mousavi, *The Prevalence of Endocervical Chlamydia trachomatis Infection Among Young Females in Kashan, Iran.* Jundishapur J Microbiol, 2015. **8**(4): p. e15576.
505. Cliffe, S.J., S. Tabrizi, and E.A. Sullivan, *Chlamydia in the Pacific region, the silent epidemic.* Sex Transm Dis, 2008. **35**(9): p. 801-6.
506. Sullivan, E.A., S. Koro, S. Tabrizi, J. Kaldor, G. Poumerol, S. Chen, M. O'Leary, and S.M. Garland, *Prevalence of sexually transmitted diseases and human immunodeficiency virus among women attending prenatal services in Apia, Samoa.* Int J STD AIDS, 2004. **15**(2): p. 116-9.
507. Fresse, A.S., J.M. Sueur, and F. Hamdad, *Diagnosis and follow-up of genital chlamydial infection by direct methods and by detection of serum IgG, IgA and secretory IgA.* Indian J Med Microbiol, 2010. **28**(4): p. 326-31.
508. Jha, R., H. Vardhan, S. Bas, S. Salhan, and A. Mittal, *Chlamydia trachomatis heat shock proteins 60 and 10 induce apoptosis in endocervical epithelial cells.* Inflamm Res, 2011. **60**(1): p. 69-78.
509. Agrawal, T., V. Vats, S. Salhan, and A. Mittal, *Local markers for prediction of women at higher risk of developing sequelae to Chlamydia trachomatis infection.* Am J Reprod Immunol, 2007. **57**(2): p. 153-9.
510. Karinen, L., A. Pouta, A.L. Hartikainen, A. Bloigu, M. Paldanius, M. Leinonen, P. Saikku, and M.R. Jarvelin, *Antibodies to Chlamydia trachomatis heat shock proteins Hsp60 and Hsp10 and subfertility in general population at age 31.* Am J Reprod Immunol, 2004. **52**(5): p. 291-7.
511. Pasternack, R., A. Mustila, P. Vuorinen, P.K. Heinonen, and A. Miettinen, *Polymerase Chain Reaction Assay With Urine Specimens in the Diagnosis of*

Acute Chlamydia trachomatis Infection in Women. Infectious Diseases in Obstetrics and Gynecology, 1996. **4**(5): p. 276-280.
512. Dietrich, W., M. Rath, G. Stanek, P. Apfalter, J.C. Huber, and C. Tempfer, *Multiple site sampling does not increase the sensitivity of Chlamydia trachomatis detection in infertility patients.* Fertil Steril, 2010. **93**(1): p. 68-71.
513. Idahl, A., J. Boman, U. Kumlin, and J.I. Olofsson, *Demonstration of Chlamydia trachomatis IgG antibodies in the male partner of the infertile couple is correlated with a reduced likelihood of achieving pregnancy.* Hum Reprod, 2004. **19**(5): p. 1121-6.
514. Siemer, J., O. Theile, Y. Larbi, P.A. Fasching, K.A. Danso, R. Kreienberg, and A. Essig, *Chlamydia trachomatis infection as a risk factor for infertility among women in Ghana, West Africa.* Am J Trop Med Hyg, 2008. **78**(2): p. 323-7.
515. Mehanna, M.T., M.A. Rizk, N.Y. Eweiss, M. Ramadan, S.A. Zaki, A. Sadek, J.M. Chow, and J. Schachter, *Chlamydial serology among patients with tubal factor infertility and ectopic pregnancy in Alexandria, Egypt.* Sex Transm Dis, 1995. **22**(5): p. 317-21.
516. Walsh, M.S., E. Hope, L. Isaia, A. Righarts, T. Niupulusu, S.V. Temese, L. Iosefa-Siitia, L. Auvaa, S.A. Tapelu, M.F. Motu, C. Edwards, M. Wernick, W.M. Huston, T. Suaalii-Sauni, and P.C. Hill, *Prevalence of Chlamydia trachomatis infection in Samoan women aged 18 to 29 and assessment of possible risk factors: a community-based study.* Trans R Soc Trop Med Hyg, 2015. **109**(4): p. 245-51.
517. *Samoa Demographic and Health Survey 2009*. 2010: Apia, Samoa.
518. den Hartog, J.E., J.A. Land, F.R.M. Stassen, A.G.H. Kessels, and C.A. Bruggeman, *Serological markers of persistent C. trachomatis infections in women with tubal factor subfertility.* Human Reproduction, 2005. **20**(4): p. 986-990.
519. Hoque, S.M., M.A. Hossain, S.K. Paul, M.C. Mahmud, S. Ahmed, N.U. Mahmud, E.R. Khan, M.A. Sakib, S. Ghosh, and N. Kobayashi, *Detection of Chlamydia trachomatis by immunological and genetic methods in female sex workers and the local female population of reproductive age in Mymensingh, Bangladesh.* Jpn J Infect Dis, 2013. **66**(3): p. 256-9.
520. Jeremiah, I., O. Okike, and C. Akani, *The prevalence of serum immunoglobulin g antibody to Chlamydia trachomatis in subfertile women presenting at the university of port harcourt teaching hospital, Nigeria.* Int J Biomed Sci, 2011. **7**(2): p. 120-4.
521. Hajikhani, B., T. Motallebi, J. Norouzi, A. Bahador, R. Bagheri, S. Asgari, and L. Chamani-Tabriz, *Classical and Molecular Methods for Evaluation of Chlamydia trachomatis Infection in Women with Tubal Factor Infertility.* J Reprod Infertil, 2013. **14**(1): p. 29-33.
522. Mueller, B.A., M. Luz-Jimenez, J.R. Daling, D.E. Moore, B. McKnight, and N.S. Weiss, *Risk factors for tubal infertility. Influence of history of prior pelvic inflammatory disease.* Sex Transm Dis, 1992. **19**(1): p. 28-34.
523. Iris, A., D. Aydogan Kirmizi, and C.E. Taner, *Effects of infertility and infertility duration on female sexual functions.* Arch Gynecol Obstet, 2013. **287**(4): p. 809-12.
524. Kortekangas-Savolainen, O., J. Makinen, K. Koivusalo, and K. Mattila, *Hospital-diagnosed late sequelae after female Chlamydia trachomatis*

infections in 1990-2006 in Turku, Finland. Gynecol Obstet Invest, 2012. **73**(4): p. 299-303.
525. Eggert, J., H. Theobald, and P. Engfeldt, *Effects of alcohol consumption on female fertility during an 18-year period.* Fertil Steril, 2004. **81**(2): p. 379-83.
526. Grodstein, F., M.B. Goldman, L. Ryan, and D.W. Cramer, *Relation of female infertility to consumption of caffeinated beverages.* Am J Epidemiol, 1993. **137**(12): p. 1353-60.
527. Porras, C., M. Safaeian, P. Gonzalez, A. Hildesheim, S. Silva, M. Schiffman, A.C. Rodriguez, S. Wacholder, E. Freer, K. Quint, C. Bratti, A. Espinoza, B. Cortes, and R. Herrero, *Epidemiology of genital Chlamydia trachomatis infection among young women in Costa Rica.* Sex Transm Dis, 2008. **35**(5): p. 461-8.
528. Komoda, T., *Kinetic study of antibodies (IgG, IgA) to Chlamydia trachomatis: importance of IgA antibody in screening test for C. trachomatis infection by peptide-based enzyme immunosorbent assay.* Jpn J Infect Dis, 2007. **60**(6): p. 347-51.
529. Ushijima, H., M. Dairaku, H. Honma, K. Sato, K. Sugiyama, T. Kitamura, and N. Inaba, *Incidence of antibodies against human immunodeficiency virus, human T-cell lymphotropic virus type 1, hepatitis B virus, hemorrhagic fever with renal syndrome virus and Chlamydia in Tonga and western Samoa.* Jpn J Med Sci Biol, 1990. **43**(4): p. 101-9.
530. Contini, C., S. Seraceni, R. Cultrera, M. Castellazzi, E. Granieri, and E. Fainardi, *Chlamydophila pneumoniae Infection and Its Role in Neurological Disorders.* Interdisciplinary Perspectives on Infectious Diseases, 2010. **2010**: p. 18.
531. Kuo, C.C., L.A. Jackson, L.A. Campbell, and J.T. Grayston, *Chlamydia pneumoniae (TWAR).* Clin Microbiol Rev, 1995. **8**(4): p. 451-61.
532. Lees, J., J. McCool, and A. Woodward, *Eye health outreach services in the Pacific Islands region: an updated profile.* N Z Med J, 2015. **128**(1420): p. 25-33.
533. Morre, S.A., C. Munk, K. Persson, S. Kruger-Kjaer, R. van Dijk, C.J. Meijer, and A.J. van Den Brule, *Comparison of three commercially available peptide-based immunoglobulin G (IgG) and IgA assays to microimmunofluorescence assay for detection of Chlamydia trachomatis antibodies.* J Clin Microbiol, 2002. **40**(2): p. 584-7.
534. Frikha-Gargouri, O., R. Gdoura, A. Znazen, B. Gargouri, J. Gargouri, A. Rebai, and A. Hammami, *Evaluation of an in silico predicted specific and immunogenic antigen from the OmcB protein for the serodiagnosis of Chlamydia trachomatis infections.* BMC Microbiol, 2008. **8**: p. 217.
535. Bax, C.J., P.J. Dorr, J.B. Trimbos, J. Spaargaren, P.M. Oostvogel, A.S. Pena, and S.A. Morre, *Chlamydia trachomatis heat shock protein 60 (cHSP60) antibodies in women without and with tubal pathology using a new commercially available assay.* Sex Transm Infect, 2004. **80**(5): p. 415-6.
536. Freidank, H.M., A. Clad, A.S. Herr, M. Wiedmann-Al-Ahmad, and B. Jung, *Immune response to Chlamydia trachomatis heat-shock protein in infertile female patients and influence of Chlamydia pneumoniae antibodies.* Eur J Clin Microbiol Infect Dis, 1995. **14**(12): p. 1063-9.
537. Gorini, G., F. Milano, P. Olliaro, A. Regazzetti, and E.G. Rondanelli, *Chlamydia trachomatis infection in primary unexplained infertility.* Eur J Epidemiol, 1990. **6**(3): p. 335-8.

538. Allahbadia, G.N. and R. Merchant, *Fallopian tube recanalization: lessons learnt and future challenges.* Women's Health, 2010. **6**(4): p. 531-549.
539. Burian, K., V. Endresz, J. Deak, Z. Kormanyos, A. Pal, D. Nelson, and D.P. Virok, *Transcriptome Analysis Indicates an Enhanced Activation of Adaptive and Innate Immunity by Chlamydia-Infected Murine Epithelial Cells Treated with Interferon γ.* The Journal of Infectious Diseases, 2010. **202**(9): p. 1405-1414.
540. Darville, T. and T.J. Hiltke, *Pathogenesis of genital tract disease due to Chlamydia trachomatis.* J Infect Dis, 2010. **201 Suppl 2**: p. S114-25.
541. den Hartog, J.E., S.A. Morre, and J.A. Land, *Chlamydia trachomatis-associated tubal factor subfertility: Immunogenetic aspects and serological screening.* Hum Reprod Update, 2006. **12**(6): p. 719-30.
542. Nachtigall, R.D., *International disparities in access to infertility services.* Fertil Steril, 2006. **85**(4): p. 871-5.
543. Morgan, J., C. Colonne, and A. Bell, *Trends of reported chlamydia infections and related complications in New Zealand, 1998-2008.* Sex Health, 2011. **8**(3): p. 412-8.
544. Kennedy, E., N. Gray, P. Azzopardi, and M. Creati, *Adolescent fertility and family planning in East Asia and the Pacific: a review of DHS reports.* Reprod Health, 2011. **8**: p. 11.