Chlamydia trachomatis diversity and pathogenesis in young South African women, related to HPV prevalence and risk

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LIST OF ABBREVIATIONS

AGYW	Adolescent girls and young women
BV	Bacterial vaginosis
CCVR	Combined contraceptive vaginal ring
CDS	Coding sequences
COC	Combined oral contraceptive pill
COPD	Chronic obstructive pulmonary disease
CPAF	Chlamydial protease-like factor
CT	*Chlamydia trachomatis*
CT–	CT negative
CT+	CT positive
EBs	Elementary bodies
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papillomavirus
HR-HPV	High-risk Human papillomavirus
HSP-60	Heat shock protein 60
IFN-γ	interferon gamma
IFN-γR$^{-/-}$	IFN-γ receptor knockout mice
IP-10	Interferon-inducible protein 10
LGV	Lymphogranuloma venereum
LPS	Lipopolysaccharide
LR-HPV	Low-risk Human papillomavirus
MACPF	Chlamydial membrane attack complex/perforin
MAPK	Mitogen-activated protein kinases
MG	*Mycoplasma genitalium*
MIP-2	Macrophage inflammatory protein-2
MLST	Multilocus sequence typing
MOMP	Major outer membrane protein
MTB	*Mycobacterium tuberculosis*
NAATs	Nucleic acid amplification tests

NG	*Neisseria gonorrhoea*
OmcB	Outer membrane complex B
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
Pgp	Plasmid gene proteins
PID	Pelvic inflammatory disease
PMP	Polymorphic membrane protein
POC	Point-of-care
pORF	Plasmid open reading frame
qPCR	quantitative PCR
RBs	Reticulate bodies
STI	Sexually transmitted infection
TARP	Translocated actin-recruiting phosphoprotein
TLRs	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
TV	*Trichomoniasis vaginalis*
UCT	University of Cape Town
WGS	Whole genome sequencing
WHO	World Health Organisation

KEYWORDS

Adolescent girls young women

Asymptomatic

Bacterial load

Chlamydia

Chlamydia trachomatis

Cytokine

High-Risk HPV

High-resolution MLST

Human immunodeficiency virus

Human papillomavirus

Inflammation

Major outer membrane protein

Plasmid copy number

Prevalence

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ABSTRACT

Sexually transmitted infections (STIs) pose a significant threat to women's reproductive health and significantly increase the risk of acquiring human immunodeficiency virus (HIV). *Chlamydia trachomatis* (CT) and human papillomavirus (HPV) infections are particularly prevalent among younger demographics, with young women experiencing higher rates of infection compared to men. Due to these often being asymptomatic, both CT and HPV infections are challenging to detect in the general population, unless specifically tested for, leading to chronic, persistent infections.

The aim of this dissertation was to investigate the impact of urogenital CT infection and bacterial genetic diversity on protective and pathogenic immune responses in the lower reproductive tract of South African adolescent girls and young women (AGYW), and risks associated with HPV co-infection.

Using a multiplex quantitative PCR (qPCR) assay, CT bacterial loads and plasmid copy number were measured in CT-positive (CT+) cervicovaginal samples from AGYW (15-19 years) who participated in the uCHOOSE study, a randomized cross-over study investigating the effect of hormonal contraceptives on biomarkers of genital tract inflammation. Luminex multiplex assays were used to measure cytokines, including interleukin (IL)-1β, IL-6, IL-17A, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, and IL-10, all of which have previously been linked to CT protection or pathogenesis. Cervical cytobrush T-cell activation and CCR5 expression were determined using a 8-colour flow cytometry panel (including CD3, CD4, CCR5, CD38, HLA-DR, and Ki67; CD19 and CD14 [dump channel]). Genotyping of the CT *ompA* gene and high resolution multilocus sequence typing (hr-MLST) were performed to understand CT genetic diversity. HPV typing was performed by Linear Array Genotyping, which measured 36 HPV types.

Prevalence and persistence of CT infection was high in this cohort of AGYW, with 21.5% (28/130) being CT+ at baseline, 8.4% (9/107) at crossover and 15.2% (14/92) at exit having CT infections. Despite all CT infections being asymptomatic, the median CT bacterial loads for the cohort were 454 *omcB* copies/µl (interquartile range [IQR], 52-1,349), and all CT strains contained a median of 9 plasmids/bacteria (IQR, 7-13). CT MOMP type was inferred from *ompA* sequencing, with genovar E being the most common (accounting for 39.5%; 17/43 of

infections), followed by genovar D (27.9%; 12/43 of infections). Using hr-MLST, a total of 27 CT sequence types (STs) were identified, including 18 novel STs. ST3 (8/44) and ST12 (6/44) were the most common ST found circulating.

CT-infected AGYW had higher concentrations of IL-1β, IFN-γ, and IL-17A in cervicovaginal secretions than CT-negative (CT–) counterparts. TNF-α and IL-6 levels were particularly elevated in those with higher CT bacterial loads. No significant differences in cervical $CD4^+$ T cell activation or CCR5 expression was evident between CT-infected and uninfected AGYW, although both markers were generally expressed at high frequencies. Although all CT infections were asymptomatic, CT infection in this cohort was associated with elevated inflammatory cytokines, suggesting that inflammatory biomarkers are a more sensitive measure of mucosal tissue inflammation than clinical symptoms. A significant difference in IFN-γ concentrations was noted between CT *ompA* genovars, using one-way ANOVA ($P = 0.0079$), with genovar F being lower. Similarly, $CD4^+CD38^+$ expression differed significantly by *ompA* genovar ($p = 0.0438$), with Ia being lower.

Similarly to CT prevalence, AGYW had high prevalence of HPV overall, with 94.4% (102/108) being infected with any HPV at study baseline and 90.5% (86/95) being infected at crossover. HPV-35, HPV-52 and HPV 62/81 were the most common HPVs detected. About 80% of AGYW were infected with HR-HPV types, and over 80% had persistent infections lasting more than four months. Instances of multiple HPV types were more frequent than single infections. Every AGYW in this cohort infected with CT also had an HPV infection, and HPV infections, especially with high-risk HPV-16, was more prevalent in CT+ than CT– AGYW, as was rates of multiple HPV infections.

The high prevalence and persistence of CT and HR HPV infections in these AGYW, both of which were asymptomatic despite being associated with evident inflammatory cytokine changes, may increase HIV acquisition and transmission risk. Data presented in this dissertation argues for an urgent need for policy change in South Africa away from World Health Organization (WHO) syndromic management of STIs, which lacks sensitivity and specificity. Furthermore, while AGYW are currently being vaccinated using Cervarix® vaccine for HPV-16 and HPV-18, the non-HPV-16/18 types commonly found in this cohort argue for the need for a broader vaccination strategy (eg. Gardasil®9). Public health initiatives

are needed to be implemented to reduce the burden of clinically asymptomatic but inflammation causing infections with CT among young women by wider roll-out of near point-of-care (POC) or true POC STI screening tests.

CHAPTER 1: Literature Review

1.1 Introduction

Chlamydia trachomatis (CT) is the most prevalent bacterial sexually transmitted infection (STI) in the world, with high epidemic potential because it is frequently asymptomatic, particularly in women, and therefore not treated (Newman *et al.*, 2015; Reekie *et al.*, 2018a; WHO, 2018). Even if asymptomatic, untreated CT can cause pelvic inflammatory disease (PID), poor birth outcomes (preterm birth, stillbirth, small for gestational age infants), increased risk of Human immunodeficiency virus (HIV) transmission (in those already HIV infected) and acquisition (in those not yet infected with HIV) (Rowley *et al.*, 2019; Smith *et al.*, 2021). The World Health Organisation (WHO) estimates that the annual cost of treating *Chlamydia* infection, particularly adolescents, is around $10 billion (Chiaradonna, 2008). The cost of untreated *Chlamydia* and associated sequalae has not been estimated.

1.2 Surveillance and Epidemiology of CT

Annual global surveillance is critical for significantly reducing the global burden of CT, (WHO, 2016a). Information regarding CT clonal diversification, genovar prevalence, and the role of co-infections with multiple CT strains, is important for informing international CT surveillance programs, building more effective global STI surveillance networks, and designing CT vaccines (WHO, 2012; Chesson *et al.*, 2017). However, many low- and middle-income countries lack or have limited surveillance systems for tracking STI prevalence, resulting in limited data on the burden of CT infections and their health consequences.

In Sub-Saharan Africa and South Africa, the incidence of curable STIs is among the highest in the world, and these are a significant threat to reproductive health and HIV infection in women in this region (Joseph Davey *et al.*, 2019; Nyemba *et al.*, 2021; Nyemba *et al.*, 2022). Sub-Saharan Africa and South Africa also bear a heavy HIV burden (Nxasana *et al.*, 2022; UNAIDS 2022; Moyo *et al.*, 2023), with an estimated 8.45 million people living with HIV in South Africa in 2022 (https://www.gov.za/about-sa/people-south-africa-0). High HIV transmission rates are associated with untreated STIs in Sub-Saharan Africa and South Africa and these high rates of STI transmission have contributed to the high HIV prevalence in the region (Ginindza *et al.*, 2017). STIs have the highest prevalence in low-resource, low-income settings and vulnerable populations [men who have sex with men, adolescent girls and young women

(AGYW), and pregnant women]. Of the STIs circulating in Sub-Saharan Africa and South Africa, CT infection is the most common. Sub-Saharan countries estimates of CT incidence and prevalence are the highest in the world (Kenyon *et al.,* 2014; Hussen *et al.,* 2018; Armstrong-Mensah *et al.,* 2021). Data has demonstrated that the prevalence of CT varies significantly between regions, over time, research populations, study settings, and types of laboratory diagnostic methods (Crichton *et al.,* 2015; Hussen *et al.,* 2018). Importantly, the majority of all new CT infections are found among young people <24 years (Satterwhite *et al.,* 2013; Shannon and Klausner, 2018; Monteiro *et al.,* 2023), highlighting the urgency of prioritizing AGYW in CT screening programs.

Strain typing of CT is crucial to understanding the genetic population structure and is a useful tool in epidemiological studies, investigation of infection transmission or recurrence, sexual network analysis, and surveillance of emerging strains and is essential to inform on CT vaccine design (Herrmann, 2007). People with the same *Chlamydia* strain are thought to be more likely to be related epidemiologically than those with different strains (Gravningen *et al.,* 2012). The term "serotype" refers to strains identified solely through serotyping and genovar is used specifically to refer to strains identified based on a molecular approach.

1.3 *Chlamydia* infections

Chlamydia is a unique genus of bacteria that has the ability to infect a wide array of hosts, including humans and amoebae (Bachmann *et al.,* 2014; Collingro *et al.,* 2020; Marti and Jelocnik, 2022). In addition to CT which infects humans, certain strains of C*hlamydia* infect mice (*C. muridarum*), cows, sheep, koala's and other livestock (*C. abortus* and *C. pecorum*), birds (*C. avium, C. gallinacea* and *C. buteonis*), Guinea pigs (*C. caviae*), cats (*C. felis*), and pigs (*C. suis*) (Sachse *et al.,* 2015; Cheong *et al.,* 2019b; Zaręba-Marchewka *et al.,* 2021). *C. pneumoniae* infects a wide range of hosts, including humans, mice, pigs, marsupials, birds, cats, and livestock. Similarly, *C. psittaci* infects parrots, livestock and humans. The most prevalent chlamydial infections in humans are CT and *C. pneumoniae,* but zoonotic infections of *C. psittaci* and *C. abortus* in humans have also been described (Stewardson and Grayson, 2010; Burgener *et al.,* 2022; Turin *et al.,* 2022).

CT can infect both genital and non-genital sites, including the cervix, rectum, and eyes (Mishori *et al.,* 2012; Morrison *et al.,* 2020). The molecular mechanisms underlying tropism are still

poorly understood (O'Connell and Ferone, 2016). At present, 19 CT genovars have been described (Figure 1.1), which are primarily identified by analysing variations in the amino acid sequence of the major outer membrane protein (MOMP), which is encoded by the *omp1* gene (Geisler *et al.,* 2003). CT serotypes A to C have been shown to cause chronic inflammation of the conjunctiva, called trachoma or active trachoma, which is endemic in some regions of Africa, Asia, Australia and the Middle East (Taylor *et al.,* 2014; Lansingh, 2016). CT serovars D to K are the leading cause of bacteria STIs in humans, they infect the endocervical epithelia of women and the urethral epithelia in both sexes (Stamm and Batteiger, 2010; WHO, 2011; Newman *et al.,* 2015; WHO, 2018). Sexually transmitted serovars, L1, L2, and L3 causes Lymphogranuloma venereum (LGV), an infection of lymph nodes as well as genital and rectal tissue (Mabey and Peeling, 2002; Stoner and Cohen, 2015; Kleine and Stich, 2020). The LGV causing strains can cause severe infection if chronic, with possibly irreversible sequelae if left untreated (like lymphatic scarring, oedema and genital elephantiasis (Ceovic and Gulin, 2015; O'Byrne *et al.,* 2016).

Since more sensitive genomic based typing methods have been developed, however, recent CT typing approaches are genotypic.

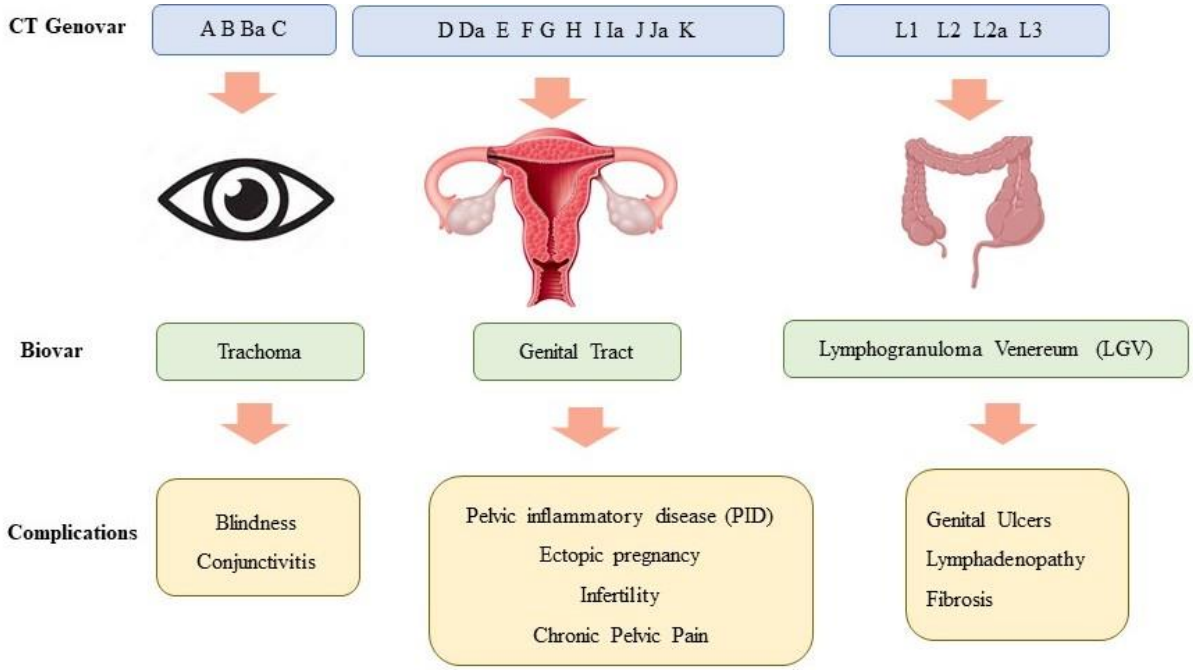

Figure 1.1. **Schematic showing CT can be classified into 19 genovars, their corresponding biovars, and associated complications**. CT biovars are further divided into genovars which are identified by the variable domains found on the surface protein MOMP. Those that infect the eye include genovars A, B, Ba, and C. Those that infect the reproductive tract include D, Da, E, F, G, H, I, Ia, J, Ja, and K. Those that cause lymphogranuloma venereum (LGV) include L1, L2, L2a, and L3. Sub-genovars are labelled with low case letters (Ba, Da, Ia, Ja and L2a). Adopted with permission from (Murray and McKay, 2021)

1.3.1 Symptoms and sequalae of CT infection in women

The genetic diversity within CT, among affected individuals and according to gender of those infected, can give rise to a range of clinical signs and symptoms (Land *et al.*, 2009; Schautteet *et al.*, 2011; Witkin *et al.*, 2017).

In symptomatic CT positive (CT+) women, genital infections can lead to changes in vaginal discharge (discharge can be white, yellow or green), and intermittent, intermenstrual, and/or post-coital bleeding (Stamm *et al.*, 1980; O'Connell and Ferone, 2016). Clinical examination may reveal further identify specific signs of CT infection, such as mucopurulent endocervical discharge evident by speculum examination and visual inspection of the cervix, easily induced endocervical bleeding, or edematous (abnormal swollen with fluid) ectopy (Marrazzo *et al.*, 2002). However, CT infections in women are frequently asymptomatic (Wiesenfeld *et al.*, 2012), particularly those affecting the upper reproductive tract, meaning that women may not self-identify symptoms or these are not identified by health care providers during routine reproductive health screening (Detels *et al.*, 2011; Rantsi *et al.*, 2018; Mohseni *et al.*, 2022).

If left untreated, CT infection can persist for up to four years (Molano *et al.,* 2005; Moazenchi *et al.,* 2018). Spontaneous clearance of CT infection has also been observed, suggesting a role for protective immunity (Geisler *et al.,* 2013). CT infection can lead to complications when it spreads beyond the cervix. For instance, CT can result in infertility, endometritis and inflammation of the fallopian tube when the infection ascends and persist. Women with chlamydial PID may present with symptoms such as pelvic or lower abdominal discomfort, cervical motion tenderness, uterine and adnexal tenderness (pain around women's pelvis) during clinical examination (Davies *et al.,* 2016). Studies have shown that 2-5% of women with untreated CT developed PID within a two-weeks of testing positive for CT, despite receiving treatment (Bachmann *et al.,* 1999; Geisler *et al.,* 2008). Repeat chlamydial infections, in particular, have been associated with reproductive sequelae, including higher risk for developing PID (Davies *et al.,* 2016; Reekie *et al.,* 2018a; den Heijer *et al.,* 2019). While the risk of infertility directly attributable to untreated CT infection has not been quantified, studies suggest that as many as 18% of women may experience infertility following symptomatic PID (Ness *et al.,* 2006; Hoenderboom *et al.,* 2019; Liu *et al.,* 2022a). While estimates of risk of reproductive difficulties following CT infection have varied by cohort and geographic location, PID remains the most common and significant avoidable cause of infertility and adverse reproductive health outcomes in women (Land *et al.,* 2010; Low *et al.,* 2016).

CT infection is also a recognized risk factor for infection with other bacterial and viral pathogens, including HIV, human papillomavirus (HPV) (Bellaminutti *et al.,* 2014; Escarcega-Tame *et al.,* 2020), *Mycoplasma genitalium* (MG) (Harrison *et al.,* 2019; Borgogna *et al.,* 2020) and *Neisseria gonorrhoeae* (NG) (Lim *et al.,* 2015; Seo *et al.,* 2021; Kirkoyun Uysal *et al.,* 2023). CT infection also increases risk of HPV-associated cervical intraepithelial neoplasia progression to cancer of the cervix (Lima *et al.,* 2018). The link between CT and other STIs may also be bidirectional, with CT infection increasing risk of bacterial vaginosis (BV), a dysbiosis of the vaginal microbiota, and infections with HIV and HPV, and vice versa (Johnson and Lewis, 2008; Cohen *et al.,* 2019; Kharsany *et al.,* 2020).

1.3.2 CT infection during pregnancy and birth

Untreated infections with CT may lead to various adverse pregnancy outcomes, including premature rupture of membranes (Mullick *et al.,* 2005; Adachi *et al.,* 2016a), preterm labour and delivery (Mullick *et al.,* 2005), chorioamnionitis,(polymicrobial bacterial infection of the

placenta and amniotic fluid) (Mullick *et al.*, 2005), low birth weight, (Darville 2005; Adachi *et al.*, 2016a), congenital infections (Darville 2005; Rours *et al.*, 2008; Adachi *et al.*, 2016b), stillbirth (Reekie *et al.*, 2018b; Warr *et al.*, 2019) or increased neonatal mortality (Darville 2005; Rours *et al.*, 2008). CT infection can also lead to neonatal ophthalmia neonatorum (bacterial eye infection) and pneumonia in infants (Beem and Saxon, 1977; Yang *et al.*, 2023).

1.4 Diagnosis of CT infections

The diagnosis of CT infections is crucial for effective management and prevention of complications. Historically, serological assays were fundamental for CT diagnosis and typing, where specific antibodies were used to classifying different CT strains. However, with advances in molecular techniques like Polymerase chain reaction (PCR), serological assays have largely disappeared in diagnostic prominence, giving way to more precise and direct methods for CT detection and typing. Measuring antibodies against CT are still useful to infer past exposure but are less useful for current infections. Typically, molecular methods like nucleic acid amplification tests (NAATs) have become the gold standard for diagnosing CT due to their accuracy.

1.4.1 CT culture

Culture-based methods for CT diagnosis involve isolating and growing the bacterium in cell culture, which have provided key insights into intracellular CT biology (Rockey and Matsumoto 1999). Some commonly employed cell lines used for CT culture include HeLa (human cervical cancer-derived epithelial cells) and McCoy cells (derived from African green monkey kidney cells). However, culture of CT has some limitations in that most clinical isolates of CT are difficult to culture *in vitro*, culture takes long, and CT has the propensity for entering a non-cultivable state (Gaydos *et al.*, 2021).

1.4.2 CT serotyping

As described previously in section 1.4, CT serotyping is a method which uses specific antibodies directed against the MOMP of CT. Based on serology to MOMP, CT was divided into 19 serovars (including subtypes): A, B, Ba, C, D, E, F, G, H, I, J, K, L1, L2 and L3 (Baehr *et al.*, 1988; Yuan *et al.*, 1989; Dean *et al.*, 1991). In addition to using MOMP as the basis for CT serotyping, other enzyme immunoassays were also developed that used the

lipopolysaccharide (LPS) genus-specific antigen for CT, which is more prevalent and soluble than MOMP. Rapid diagnostic tests have also been developed that use immunochromatography to detect chlamydial LPS antigen. However, these proved to have low sensitivity in clinical trials (Sabidó *et al.,* 2009). Furthermore, immune-based detection of CT LPS was shown to have higher rates of false-positives than detection of CT MOMP, due to CT LPS-specific antibodies' cross-reacting with antigenically similar LPS of other gram-negative bacteria.

1.4.3 Molecular tests for CT diagnosis and molecular typing

Various types of molecular tests for CT have been developed, including NAATs [including near point-of-care (POC) NAATs], multi-locus sequence typing (MLST), whole genome sequencing (WGS), restriction fragment length polymorphism, and DNA microarrays based on CT *ompA* gene (encoding MOMP) (Stothard 2001; Quint *et al.,* 2007; Pannekoek *et al.,* 2008; Pedersen *et al.,* 2008; Ruettger *et al.,* 2011; Gallo Vaulet *et al.,* 2016). Some of these molecular techniques have low discrimination power and several disadvantages, leading to them being replaced by other typing methods especially those based on sequencing, which are much more specific and enable CT intra-specific typing (de Vries *et al.,* 2015). Details of each of these is discussed in the following sections.

1.4.3.1 NAATs for diagnosis of CT

The development of NAATs in CT diagnostics transformed the field. Several different high-throughput CT-specific NAATs have been developed for large-scale testing for CT, including the COBAS® PCR assay (from Roche Molecular Systems, USA), a ligase chain reaction assay (from Abbott Diagnostics, USA), a strand displacement amplification assay (from ProbeTec ET, Becton Dickinson, USA), a transcription-mediated amplification assay (from Aptima, GenProbe, now Hologic, USA), a multiplex BD Max™ CT/GC/TV (MAX) assay (from BD Diagnostics, Franklin Lakes, NJ, USA; (Caruso *et al.,* 2021), and the Seegene platform (multiplex *PCR assay*)(Seegene Inc) (Cheng and Kirby 2014; Bristow *et al.,* 2017; Boiko *et al.,* 2019; Silveira *et al.,* 2020; Zhou *et al.,* 2021). Generally, many of these require large, expensive equipment, trained personnel and typically are offered by major diagnostic laboratories linked to hospitals.

Several rapid near POC tests have also been developed, including an AccuPower test (by Bioneer, Inc., Daejeon, Korea) and the Cepheid GeneXpert CT/NG assay that can be run with little infrastructure and at clinics with small on-site laboratories. The Cepheid GeneXpert platform is a well-established POC system in South Africa that is being used at both trial sites and at mobile laboratories (Rivard *et al.,* 2017; Shetty *et al.,* 2021). The Cepheid GeneXpert CT/NG assay detects the DNA of both NG and CT in female endocervical, vaginal, and urine specimens from both symptomatic and asymptomatic individuals (Gaydos *et al.,* 2013).

1.4.3.2 Multi-locus sequence typing of CT

Chlamydial MLST has been widely used in epidemiological studies of CT (Herrmann *et al.,* 2015; Danielewski *et al.,* 2017), with several different MLST approaches being developed over the past two decades (Klint *et al.,* 2007; Pannekoek *et al.,* 2008; Pedersen *et al.,* 2009; Christerson *et al.,* 2011b; Herrmann *et al.,* 2015; Isaksson *et al.,* 2016; Patino *et al.,* 2018; Pilo *et al.,* 2021). MLST was used in this dissertation to genotype circulating CT strains in AGYW. Table 1.1 compares three of the most widely used CT MLST schemes. Two are based on housekeeping genes: the scheme described by Pannekoek *et al*., (2008) includes housekeeping genes *gatA*, *oppA*, *hflX*, *gidA*, *enoA*, *hemN*, and *fumC*; while the scheme described by Dean *et al*., (2009) includes *glyA*, *mdhC*, *pdhA*, *yhbG*, *pykF*, *lysS*, and *leuS*. The third scheme, called the Uppsala MLST scheme (high resolution-MLST) described by Klint *et al*. (2007), is based on five highly variable genomic loci of non-housekeeping genes: *hctB* (CT046), CT058, CT144, CT172, and *pbpB* (CT682). Each of these MLST schemes serves a different purpose, with the Uppsala MLST scheme being considered the best for high-resolution typing, which is useful to discriminate in short-term clinical epidemiology and outbreak investigations, as will be described in this dissertation (Klint *et al*., 2007; Bom *et al*., 2011; Bom *et al*., 2013). Open access databases, such as PubMLST (Jolley *et al.,* 2018), can be used to screen for CT MLST sequences, allele profiles, and STs found globally.

Table 1.1. Comparison of CT MLST schemes

Typing system	**Pannekoek *et al*. (2008)**	**Dean** et al**. (2009)**	**Klint *et al*. (2007) "Uppsala"**
Number of genes:	7	7	5
Type of genes:	Housekeeping genes	Housekeeping genes	Non-housekeeping genes
Genes (and what they putatively encode):	*gatA* – serine hydroxymethyltransferase *oppA* – oligopeptide transporter *hflX* - ubiquitous GTPase; recycles ribosomes *gidA* - a tRNA modification enzyme *enoA* - alpha-enolase *hemN* - S-adenosylmethionine enzyme *fumC* - fumarase enzymes	*glyA* - serine hydroxymethyltransferase *mdhC* – malonate decarboxylase *pdhA* - pyruvate dehydrogenase *yhbG* - LPS export system ATP-binding protein *pykF* – pyruvate kinase *lysS* – lysyl-tRNA synthetase *leuS* – leucine-tRNA ligase	CT058 - inclusion membrane (Inc) protein CT144 – Type III secretion system CT172 – Unknown *hctB* - Histone-like protein Hc2 *pbpB*- penicillin-binding protein
Suitability For:	Evolutionary studies	Evolutionary studies	Short-term clinical epidemiology and outbreak investigation
Purpose:	Designed to analyse evolutionary changes over time and its usefulness for comparison of strains from different species	Designed for CT intra-taxa variability	Designed to discriminate CT strains for epidemiological purposes
CT sequence types (ST) identified:	75	44	520
Resolution:	Similar to that of *ompA* sequencing	Similar to that of *ompA* sequencing	Better than the other two schemes
Identification:	Inter-species identification	Intra-species identification	Intra-species identification
Nucleotide diversity index [a]:	Low (Low recombination)	Low (Low recombination)	High (High recombination)

a-Nucleotide diversity index- measures the degree of polymorphism within a gene

1.4.3.3 Whole genome sequencing of CT

For decades, CT strains were differentiated based on sequence analysis of the *ompA* gene alone (Dean *et al.,* 1992). However, recent CT whole genome studies have shown that *ompA* gene is not always an accurate epidemiological marker in isolation, because this CT protein is under significant immune pressure from the host and the gene is therefore subject to high degrees of recombination to escape immune detection (Harris *et al.,* 2012; Matičič *et al.,* 2016; Luu *et al.,* 2023). The first whole CT genome was sequenced in 1998, belonging to a genotype D urogenital (D/UW3) strain (Stephens *et al.,* 1998).

Chlamydial genomes, like those of other obligate intracellular bacteria, have a small, circularized chromosome (Collingro *et al.,* 2011; Pillonel *et al.,* 2018; Sigalova *et al.,* 2019). Sequencing of the whole genome of the urogenital strain CT genotype D (D/UW3) genome revealed a 1.04 mega (base pair) bp chromosome, encoding 894 proteins, and multiple copies of a 7.4 kbp plasmid. WGS of the CT genome revealed many surprises, including a novel pathway for peptidoglycan biosynthesis (Liechti *et al.,* 2014). In addition, several putative virulence factors were identified, including a complete type III secretion system and nine paralogous polymorphic membrane proteins (*Pmps*; encoded by *Pmps*), and a group of membrane bound surface-exposed chlamydial proteins that have been characterized as autotransporter adhesins, important in the initial phase of chlamydial infection. In addition, many genes and pathways that were thought to be essential in bacteria were missing, including those needed for essential amino acids and nucleotides synthesis, cell wall division, and DNA oxidative damage repair (Hadfield *et al.,* 2018).

WGS has been used to evaluate whether CT isolates with different disease pathologies were genetically distinct, and whether they could be differentiated based on non-synonymous mutations, pseudogenes, and gene gain/loss (Thomson *et al.,* 2008). However, alignment between LGV and urogenital CT isolates showed that no additional genes were present in LGV strains compared to urogenital isolates that could explain differences in disease outcome. Subsequently, pan-genome analysis of 227 chlamydial whole genomes demonstrated a high degree of similarity, with 81% of the CT genes universally or partially conserved, and 19% of the genes being unique to singular genomes (Luu *et al.,* 2023). The two major phylogenetic lineages of CT (LGV and trachoma), for example, could be separated by ~500 single nucleotide polymorphisms (Harris *et al.,* 2012).

Importantly, WGS and MLST-based phylogenetic trees for CT were shown to be equivalent in terms of the degree of incongruence (Herrmann *et al.*, 2015; Gupta *et al.*, 2017; Floridia-Yapur *et al.*, 2021). Based on the advantages and ease of use of the Uppsala MLST typing scheme and its agreement with WGS, this CT typing scheme was used in the current dissertation.

1.5 Antibiotic treatment guidelines for CT

For individuals infected with CT, the WHO STI guidelines recommend a one-time oral dose of 1g azithromycin to treat uncomplicated genital chlamydial infections in females, or an oral dose of 100 mg doxycycline twice daily for 7 days (WHO, 2021; Mohseni *et al.*, 2022). WHO syndromic management of STIs and BV is practised by South Africa and many LMICs, in which women with clinical signs and symptoms (like lower abdominal pain syndrome or vaginal discharge syndrome) are treated with broad-spectrum antibiotics without a laboratory diagnosis (WHO, 2016b; Garrett *et al.*, 2017; Nyemba *et al.*, 2021). (Table 1.2). For many years, the WHO promoted the syndromic management strategy because laboratory based STI testing were not widely available in many countries.

The latest WHO global strategy on HIV and STIs placed a much stronger emphasis on active surveillance and causative treatment, because of availability in many LMICs of near POC platforms (like the GeneXpert), in so doing moving away from syndromic management (WHO, 2022). In 2023, WHO released new guidelines for point-of-care diagnostic testing for STIs/HIV manual, STI landscape analysis and target product profiles for point-of-care tests for STIs (https://www.who.int/news/item/24-07-2023-who-releases-new-guidance-to-improve-testing-and-diagnosis-of-sexually-transmitted-infections)(Figure 1.2).

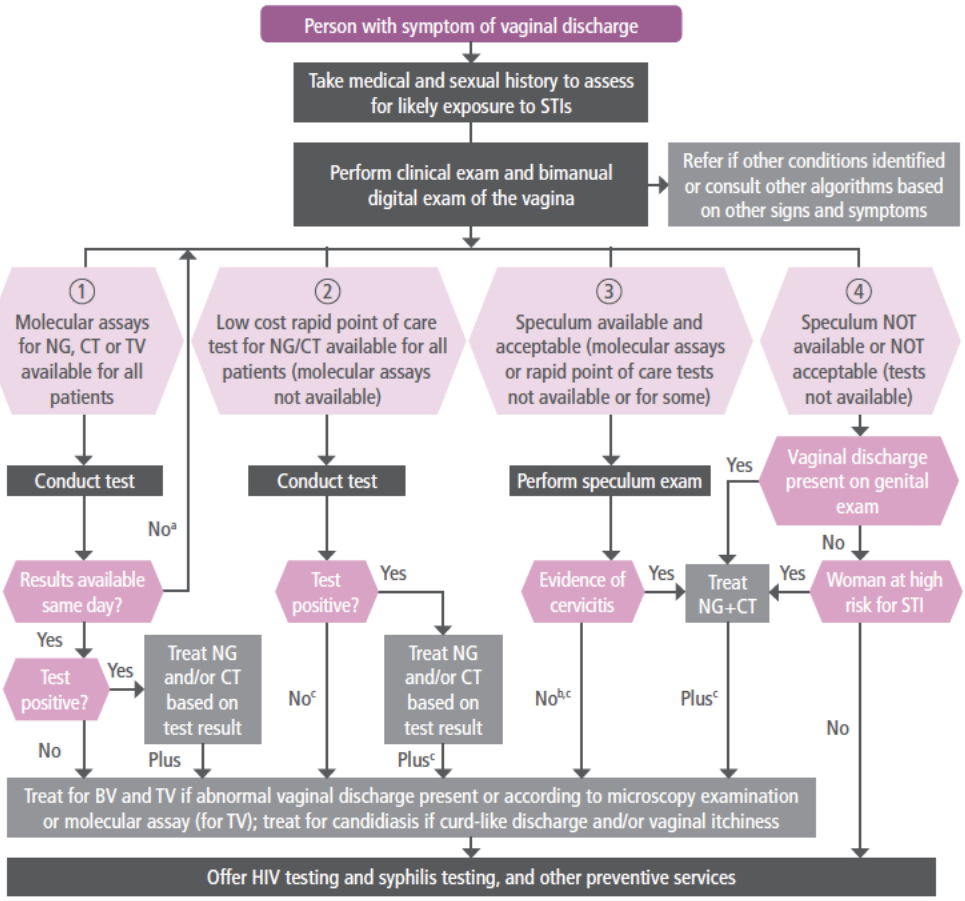

Figure 1.2. **WHO proposed a flow chart for health-care providers to manage people with symptoms of vaginal discharge**. This flow chart can be used as is or customised based on country-specific needs. Reproduced with permission from (WHO, 2021)

While syndromic management was created as a tool to enhance the case management of patients with symptomatic STIs, most cases of CT, particularly in women, are asymptomatic, so are often overlooked and untreated (Kaida *et al.*, 2018). Syndromic management was adopted in South Africa in the 1990's, as standards of care, which recommends treatment of CT with azithromycin (https://www.health. gov.za/wp-content/uploads/2020/11/sti-guidelines-27-08-19.pdf).

Table 1.2. **Comparison between South African and WHO syndromic treatment guidelines**

Syndrome	Main pathogens	WHO guideline recommendation	South Africa guideline recommendation
Vaginal discharge syndrome	CT NG TV BV Candida	*Treatment for vaginal infections:* Metronidazole 400 mg orally twice daily for seven days (alternative: 2 g orally single dose) PLUS miconazole vaginal pessary 200 mg inserted at night for three nights (alternatives: clotrimazole vaginal tablet 100 mg inserted at night for seven nights OR fluconazole 150 mg or 200 mg orally single dose). *Treatment for cervical infections:* Ceftriaxone IM 250 mg once daily PLUS azithromycin oral 1 g once daily. If you have recurring or persistent discharge, MG should be considered.	*Non sexually active women:* Clotrimazole 500 mg nocte stat or clotrimazole cream vaginally twice daily for 7 days if vulva scratched/red/curd-like discharge. If not, take metronidazole 2 g orally in a single dose. *Sexually active women*: Ceftriaxone IM 250 mg once-daily PLUS Azithromycin oral 1 g single dose PLUS metronidazole oral 2 g single dose. If candidiasis is suspected, consider adding clotrimazole.
PID	CT NG TV BV Other bacteria including Gram-negative rods and other mycoplasmas	Ceftriaxone IM 250 mg once daily PLUS azithromycin oral 1 g once daily PLUS doxycycline 100 mg orally twice daily for 14 days PLUS metronidazole oral 400 mg twice daily for 14 days. Note: If pregnancy or surgical pathology is suspected, or if you are severely ill, seek emergency care.	Ceftriaxone IM 250 mg single dose in addition to metronidazole oral 400 mg twice daily for seven days and azithromycin oral 1 g single dose. Note: If you are very ill or suspect surgical pathology or pregnancy, get emergency care.

According to the WHO guidelines, in cases of complicated PID (Table 1.2), treatment with intravenous ceftriaxone 250 mg every 24 hours, intravenous or oral doxycycline 100 mg every 12 hours combined with either metronidazole 400 mg oral or IV every 12 hours is recommended (Grygiel-Gorniak and Folga, 2023). Metronidazole can also be added to the PID regimen, to provide protection against other anaerobic organisms in the upper genital tract (Wiesenfeld *et al.,* 2020). For pregnant women infected with CT, one-time oral dose of 1 g azithromycin is recommended (Dionne-Odom *et al.,* 2020; Antonucci *et al.,* 2022). Other alternative treatment regimens include 500 mg oral amoxicillin three times daily for 7 days or 500 mg oral erythromycin twice daily for 7 days (Mohseni *et al.,* 2022).

1.6 Partner notification

Since CT is highly transmissible, with an estimated transmission efficacy from male-to-female partners ranging from 32-34% (Lewis *et al.,* 2021), the WHO and South African National guidelines for managing CT infections recommends that individual therapy must be combined with partner notification and treatment (where sexual partners are notified about STI exposure

and possible education and treatment options). These guidelines do not offer specific implementation advice and, as a result, partner notification is frequently not carried out (WHO, 2016b). Conversations with sexual partners about an STI diagnosis, particularly for younger people, since these relationships are commonly transient remains challenging. To illustrate this, a recent study in Zimbabwe that included individuals between 16–24 years, who were attending a community-based sexual and reproductive health clinic, where offered STI testing and treatment – the CHIEDZA trial (Lariat *et al.*, 2023). Of those who were diagnosed with an STI and counselled, 41.2% took partner notification slips, but only 5.7% of their partners returned for treatment at the clinic. Feedback from the young participants in this trial, partner notification posed serious social risks and jeopardized their physical and emotional safety.

1.7 CT intracellular lifecycle

CT is a highly evolved pathogen with a relatively small genome compared to other bacterial pathogens, that infects genital mucosal epithelial cells, promoting its own uptake into these non-phagocytic cells (Luján *et al.*, 2016). *Chlamydia* has a unique biphasic life cycle (Figure 1.3) that begins with contact of infectious, environmentally resistant, elementary bodies (EBs) with the apical surface of the epithelial cell. EBs enter mucosal cells into inclusions, a host membrane-bound compartment, where they differentiate into the metabolically active reticulate bodies (RBs). Several receptors, including the mannose receptor, the mannose 6-phosphate receptor, and the estrogen receptor, have been proposed to mediate the interaction between CT EBs and the host cell (Cocchiaro and Valdivia 2009). EBs attachment to host cells is aided by multiple bacterial adhesins and ligands, including glycosaminoglycan (Menozzi *et al.*, 2002), MOMP (Su *et al.*, 1996), outer membrane complex (encoded by *omcB;* (Fadel and Eley 2007), and *Pmp* (encoded by *pmpD;* (Mölleken *et al.*, 2010). Envelope protein *omcB* is surface exposed, CT envelope protein which binds to glycosaminoglycans on the host cell surface (Fadel and Eley, 2007; Moelleken and Hegemann, 2008; Liang *et al.*, 2021) and MOMP has been shown to potentially bind heparan sulfate receptors on the host cell (Su *et al.*, 1996). Given the significance of binding and entry to its intracellular developmental cycle, it is likely that chlamydial EBs combine and employ multiple mechanisms for adhesion (Hegemann and Moelleken, 2012; Romero *et al.*, 2020; Turman *et al.*, 2023b).

Bacterial effectors [like translocated actin-recruiting phosphoprotein (Tarp) (Clifton *et al.*, 2004; Ghosh *et al.*, 2020), early translocator phosphoprotein (Chen *et al.*, 2014), proteins

involved in CTs type III secretion system CT694 (Hower *et al.,* 2009) and CT695 (Mueller and Fields, 2015; McKuen *et al.,* 2017)] are secreted into the host cell cytosol when chlamydial EBs attach to the host cell. The infectious EBs enter the host cell as membrane-bound vesicles, which proceed in the direction of the perinucleus before joining together to form an inclusion, a single larger vacuole. After entering this altered phagosome, EBs undergo differentiation into RBs, which are the replicative bacterial form of CT and are metabolically active but not infectious. *Chlamydia* RBs can also differentiate into a non-replicating, persistent form known as aberrant bodies , in response to stress (Panzetta *et al.,* 2018). Within the boundaries of the expanding inclusion, RBs multiply asynchronously through binary fission. Following multiple replication cycles, RBs re-differentiate into infectious EBs so they can continue the infectious process (Elwell *et al.,* 2016; Gitsels *et al.,* 2019; Chiarelli *et al.,* 2020) (Figure 1.3). Since survival of these intracellular pathogen depends on their ability to establish an intracellular niche, disrupt host cellular processes, obtain nutrients from the host, and evade the host immune response, the chlamydial lifecycle plays a crucial role in their pathogenesis (Elwell *et al.,* 2016; Panzetta *et al.,* 2018; Gitsels *et al.,* 2019; Chiarelli *et al.,* 2020).

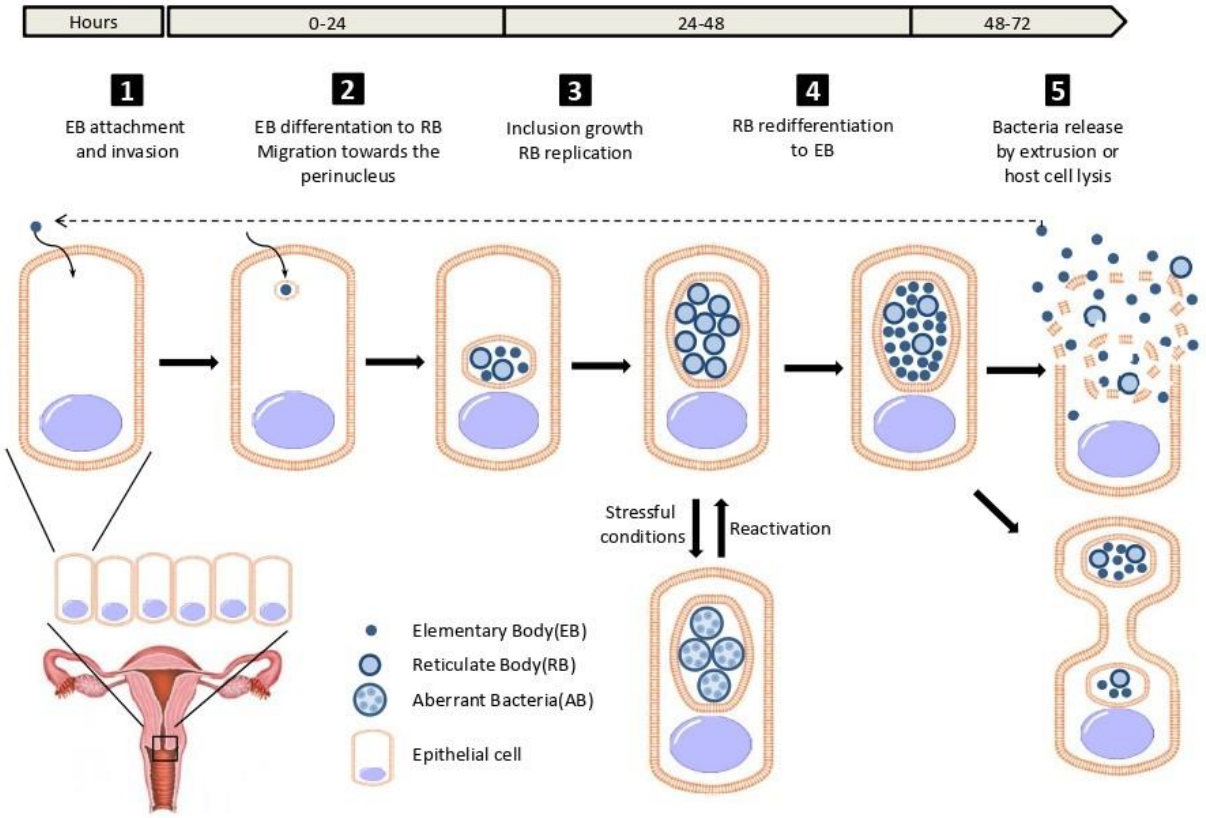

Figure 1.3. **Diagram showing the distinct biphasic phases of chlamydial intracellular life cycle.** EBs infect epithelial cells in the female reproductive tract, (1) following attachment and invasion. Once inside the host cells, within a vacuole, (2) the EB differentiates to a RBs and migrates to the perinuclear region (0-24 hours). (3) The RB replicates in inclusion by binary fusion (24-48 hours). (4) and (5) Around 24 hours post infection, RBs differentiate back into EBs and keep differentiating until lysis or release occurs. The entire cycle takes 40-72 hours to complete. Stressful environments cause RBs to go into a latent stage, which lasts until more ideal growing conditions. Reproduced with permission from (Luján *et al.*, 2016)

1.8 CT virulence factors and immunopathogenesis in women

The majority of women infected with CT develop acquired immunity (both CT-specific antibodies and T cells) (Mascellino *et al.*, 2011; Vasilevsky *et al.*, 2014). However, this acquired immunity does not always clear the infection, and some infected women will experience long-lasting CT infections if they do not receive treatment. These chronic CT infections increase risk of sequelae, including the development of severe health complications like PID, tubal scarring, ectopic pregnancy, and infertility (Hua *et al.*, 2015).

1.8.1 CT antigens and CT virulence factors

Several chlamydial antigens have been linked to CT pathogenesis, including MOMP, chlamydial protease-like factor (CPAF), and heat shock protein 60 (HSP60).

1.8.1.1 Major outer membrane protein

CT MOMP is a *Pmp* and transmembrane protein that consists of five conserved sequences in the periplasmic space and four variable domains in the extracellular space (Figure 1.4) (Baehr *et al.,* 1988; Kim and DeMars, 2001; Gitsels *et al.,* 2020). More than half of the biomass of the chlamydial outer membrane complex consists of MOMP, which is also an important immunodominant target for the host (Su *et al.,* 1990; Su and Caldwell, 1991; Kim and DeMars, 2001; He *et al.,* 2017; Hepler *et al.,* 2018). The MOMP protein also served as the basis of CT serotyping, allowing for species-specific differentiation prior to molecular techniques (Baehr *et al.,* 1988). Some have reported that MOMP is also involved in attachment and entry into host cells, being a key tissue tropism determinant (Su *et al.,* 1990; Su and Caldwell, 1991).

Because of the importance of MOMP to CT cell entry and its immunodominance, MOMP remains one of the leading targets for CT vaccine development (Olsen *et al.,* 2021; Collar *et al.,* 2022). However, the *ompA* gene, which encodes MOMP, is under significant selective immune pressure (Dean *et al.,* 1995; Nunes *et al.,* 2009; Seth-Smith *et al.,* 2021), with ~90% of nucleotide substitution in the *ompA* gene being associated with amino acid changes (non-synonymous substitutions) as evidence for this immune pressure (Dean *et al.,* 1991; Dean *et al.,* 1992; Brunham *et al.,* 1994; Hayes *et al.,* 1994), occurring in regions encoding both variable and conserved domains (Fitch *et al.,* 1993; Frost *et al.,* 1993; Dean and Millman, 1997; Gomes *et al.,* 2005). In particular, the 3’ end of *ompA* segment has been mapped to contain several T cell epitopes, important for eliciting protective immunity (Su *et al*., 1990). Recombination in this region is thought to allow CT to evade host immunity, allowing the organism to survive within the host cell (Dean, 2013).

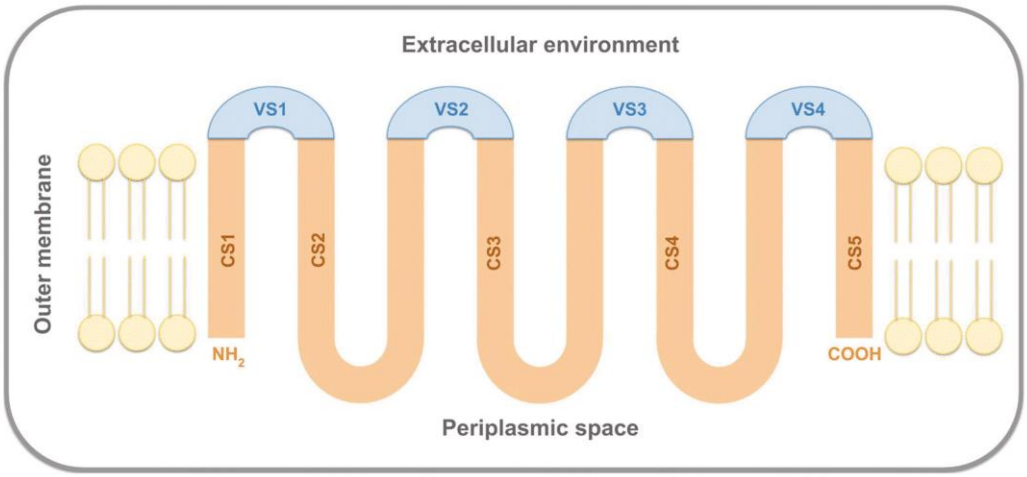

Figure 1.4. **Schematic representation of chlamydial MOMP**. The outer membrane of the chlamydial envelope is a 39 500 Da protein. The periplasmic and extracellular spaces are exposed to five conserved sequence (CS1–CS5) and four variable sequence (VS1–VS4) regions of the protein, respectively. Reproduced with permission from (Gitsels *et al.*, 2020).

1.8.1.2 Other members of the *Pmp* family

Like MOMP, other *Pmp* family members that have been characterized from CT are bacterial adhesins that are necessary for infection of host cells (Moelleken and Hegemann, 2008; Liu *et al.*, 2010; Becker and Hegemann, 2014). *Pmp* genes constitute >7% of the *Chlamydia* genome, with <50% amino acids homology between species (Grimwood and Stephens, 1999), making them among the most variable regions in the CT genomes, with a high rate of mutation among CT strains, evidently from immune pressure (Carlson *et al.*, 2005; Hölzer *et al.*, 2020). There are nine members of the *Pmp* family (*PmpA*, *PmpB*, *PmpC*, *PmpD*, *PmpE*, *PmpF*, *PmpG*, *PmpI*), which have relatively heterogeneous sequences (Grimwood and Stephens 1999; Henderson and Lam 2001). All CT *Pmp* proteins are auto-transporters (Becker and Hegemann, 2014; Vasilevsky *et al.*, 2016; Favaroni and Hegemann, 2021), with passenger domains being exposed to the outside of the bacterial cell (Henderson and Lam, 2001; Paes *et al.*, 2018; Doyle and Bernstein, 2021). Motif-segments of CT, *C. pneumoniae*, and *C. psittaci* that have been sequenced are putative adhesins, thought to be essential for establishing infection (Mölleken *et al.*, 2010; Becker and Hegemann, 2014; Favaroni *et al.*, 2021). The *Pmp* genes require at least two motifs to mediate infection but they can harbour multiple domains (Mölleken *et al.*, 2010).

1.8.1.3 *PorB*

PorB is a surface exposed outer membrane porin of 38 kDa, with similar characteristics and abundance as MOMP (Kubo and Stephens, 2000; Kubo and Stephens, 2001; Kawa and

Stephens, 2002), that is known to elicit neutralizing antibodies (Kawa and Stephens, 2002). Due to the *porB* sequence being conserved among *Chlamydia* species (Kubo and Stephens, 2000; Kawa and Stephens, 2002), and that it raises neutralizing antibodies, *porB* has also been evaluated as a potential CT vaccine candidate.

1.8.1.4 Translocated actin-recruiting phosphoprotein

EBs secret Tarp outside of the bacterial cell via their type III secretion system, into the host cell phagosome (Clifton *et al.,* 2004; Jamison and Hackstadt, 2008; Ghosh *et al.,* 2020). During host cell invasion by EBs, Tarp have been shown to facilitate actin-binding, nucleation and cytoskeleton rearrangement (Clifton *et al.,* 2004; Jewett *et al.,* 2006). Recent study demonstrated that early effector Tarp is able to form F-actin bundles *in vivo*, which is crucial for chlamydial entry into epithelial cells (Aranjuez *et al.,* 2022).

1.8.1.5 Heat shock proteins

Three chlamydial heat shock proteins (HSPs) that have been identified, including HSP10, HSP60 and HSP70, which are homologous to *Eschericia coli* GroES, GroEL and DnaK, respectively (Danilition *et al.,* 1990; Cerrone *et al.,* 1991; LaVerda *et al.,* 1999). *Chlamydia* HSPs are highly conserved proteins, with >95% amino acid identity, that share ~50% homology to human HSPs (Peeling and Mabey, 1999). CT HSPs are expressed throughout the chlamydial life cycle and their expression is upregulated during stressful conditions (Peeling and Mabey 1999). HSPs are considered to be proteins that assist the conformational folding or unfolding of large bacterial proteins (Zügel and Kaufmann, 1999). In cases of severe chlamydial disease in women, antibodies against HSP10 and HSP60 have been suggested to contribute to immunopathology in the upper genital tract, by cross-reacting with HSP epitopes in the host cells (Witkin *et al.,* 1997), leading to host tissue damage by autoimmune mechanisms (LaVerda *et al.,* 1999; Karinen *et al.,* 2004). However, HSP60 and HSP70 are not components of the chlamydial outer membrane complex (Liu *et al.,* 2010).

1.8.1.6 Chlamydial protease-like activity factor

CPAF is a type II secreted broad-spectrum serine protease, which is highly conserved among *Chlamydia* species, having 99% amino acid identity among CT serovars (Elwell *et al.,* 2016; de la Maza *et al.,* 2017), essential for CT infection (Tan and Sütterlin 2014; Witkin *et al.,* 2017; Prusty *et al.,* 2018). CT secretes CPAF midway during its development cycle into the host

cytosol (Zhong, 2011; Conrad *et al.,* 2013; Yang *et al.,* 2016), where it functions to suppress host cell apoptosis to ensure survival of intracellular CT (Zhong *et al.,* 2001; Pirbhai *et al.,* 2006; Jorgensen *et al.,* 2011; Tang *et al.,* 2015; Yang *et al.,* 2015). CPAF has also been shown to degrade innate immune effectors like host nuclear factor-kappa β and other transcription factors, which trigger production of multiple pro-inflammatory host mediators, in so doing suppressing the release of pro-inflammatory cytokines (Patton *et al.,* 2016; Witkin *et al.,* 2017; Schott *et al.,* 2020).

1.8.1.7 Chlamydial Membrane Attack Complex/Perforin

Chlamydial Membrane Attack Complex/Perforin (MACPF) proteins are also encoded by CT (Keb and Fields 2020), with horizontal gene transfer from a mammalian host proposed to be the origin of this protein (Ponting, 1999; Wolf *et al.,* 1999). The identification of MACPF-containing proteins as components of host interactions has been proposed as proof that *Chlamydia* species most likely acquired this domain via co-evolution with a mammalian host (Keb and Fields, 2020). Genes encoding MACPF have been identified in all WGS generated for CT, being highly conserved, and located in the highly variable plasticity zone (Taylor *et al.,* 2010). Furthermore, animal isolates of *C. pneumoniae* that originate from human sources can be distinguished using the MACPF gene sequence (Mitchell *et al.,* 2010).

1.8.2 CT plasmids

Most members belonging to *Chlamydiaceae* contain one or more virulence-associated plasmid/s that are approximately 6.3 - 8 kbp in size, apart from *C. abortus* strains isolated from livestock, and some human strains of *C. pneumoniae* (Zhong, 2017; Szabo *et al.,* 2020). Nearly all CT clinical isolates that have been described have this highly conserved plasmid (Turman *et al.,* 2023b), which has eight putative coding sequences (CDS), and two small antisense RNA sequences (Ricci *et al.,* 1993). The eight CDS are called plasmid gene proteins (Pgp) 1 to 8. Plasmid maintenance is attributed to Pgp1, Pgp2, Pgp6, and Pgp8 (Song *et al.,* 2013; Jones *et al.,* 2020; Yang *et al.,* 2020). Pgp4 and Pgp5 are putative transcriptional regulators (Gong *et al.,* 2013; Song *et al.,* 2013; Liu *et al.,* 2014a), which have control over other chlamydial genes, while plasmid loci Pgp3, Pgp4, and Pgp5 have been proposed to be related to virulence (Liu *et al.,* 2014b; Turman *et al.,* 2023a; Turman *et al.,* 2023b). In addition, the CT plasmid contains

a 22bp repeat region that serves as the origin of replication (Jones *et al.,* 2020; Szabo *et al.,* 2020; Fields *et al.,* 2022).

The plasmids are proposed to help survival of CT and to confer a selective advantage to CT by carrying of antibiotic resistance or virulence-associated determinants (Williams and Thomas, 1992). It has been suggested that the conserved chlamydial plasmids promote infection by facilitating better host cell entry and exit (Turman *et al.,* 2023b). CT plasmids are also thought to accelerate innate inflammatory responses that cause tissue damage (Turman *et al.,* 2023b). Some have suggested that CT plasmids are virulence determinants in the host. While CT strains containing no plasmids do exist, these are rarely found among clinical samples (Yeow *et al.,* 2016; Hadfield *et al.,* 2017; Jones *et al.,* 2020).

Chlamydial plasmids are vertically inherited, with the phylogeny of plasmids and chromosomes being similar (Hadfield *et al.,* 2018). While there is some evidence of homologous recombination between plasmid sections and exchange of whole plasmids, these events are rare in comparison to CT chromosomal recombinant events (Peterson *et al.,* 1990; Farencena *et al.,* 1997; Harris *et al.,* 2012; Wang *et al.,* 2013). The most impactful variation in CT was reported from Sweden, where the new variant CT was described, which had a deletion in the plasmid, resulting in these isolates being undetectable by some PCR-based diagnostics. In the case of this new variant of CT, this plasmid deletion resulted in a false-negative diagnosis and rapid expansion of the new variant (Unemo *et al.,* 2010). However, the variant had no other biological advantage (Unemo *et al.,* 2010).

1.8.3 Immunopathogenesis and protection from CT infection

Genital tract inflammation, including mucosal infiltration of inflammatory cells, are features of CT infection (Mascellino *et al.,* 2011), with multiple factors contributing to the extent of inflammation and ensuring development of disease, including both host and CT virulence factors. Both innate and acquired immune responses are induced and maintained by CT infection. Growth factors, cytokines, and chemokines are produced, predominantly in response to chlamydial replication or persistence in infected cells (Stephens 2003). Without use of antibiotics, host immunity to CT rarely leads to the infection being eradicated, in part because of CT's capacity to evade host immunity (Horne *et al.,* 2008; Agrawal *et al.,* 2009; Darville and Hiltke 2010). These ongoing host inflammatory processes in persistently infected tissues

lead to severe clinical consequences of ongoing CT infection, such PID, tubal blockage, and tubal infertility.

Several cytokines are involved in inflammatory processes during CT infection, including tumour necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-8, and IL-17 (Agrawal *et al.,* 2007; Mascellino *et al.,* 2011; Faris *et al.,* 2019; Poston *et al.,* 2019; Xiang *et al.,* 2021), which will be evaluated in this dissertation. To balance these pro-inflammatory responses, regulatory T-cells, a T-cell subsets that control or regulate inflammation, migrate to the infection sites mucosa and secrete inhibitory cytokines like IL-10 (Belkaid, 2007). As such, the role of IL-10 will be discussed and evaluated in this thesis.

Antigen-specific $CD4^+$ that secrete interferon gamma (IFN-γ) are one of the most important cellular immune responses in the development of protective immunity against chlamydial infection (Belland *et al.,* 2003; McClarty *et al.,* 2007; Ziklo *et al.,* 2016a; Wang *et al.,* 2022).

1.8.3.1 Interferon gamma

IFN-γ, produced predominantly by activated T-cells, increase following both *in vivo* and *in vitro Chlamydia* infections (Williams *et al.,* 1988; Tau and Rothman 1999; Cohen *et al.,* 2000; Morrison *et al.,* 2000), involving both innate and adaptive immunity (Xiang *et al.,* 2021). The important of IFN-γ in protection against CT has been demonstrated in the mouse model of *Chlamydia*, using *C. muridarum*. Mice deficient in IFN-γ (IFN-$\gamma^{-/-}$) and its receptor (IFN- $\gamma R^{-/-}$) and mice treated with anti-IFN-γ antibody experience more severe disease following chlamydial infection than wild-type controls or untreated mice (Williams *et al.,* 1988; Cotter *et al.,* 1997; Ito and Lyons 1999). In patients with CT infections, IFN-γ has been associated with more rapid resolution of *Chlamydia* infections (Xiang *et al.,* 2021). However, excessive production of IFN-γ has also been associated with exacerbated sequelae following infection, suggesting that too much of this cytokine can drive pathology (Ishihara *et al.,* 2005; Mascellino *et al.,* 2011). Others have suggested that the immunological microenvironment and the stage of infection influence the impact of IFN-γ on *Chlamydia* infection-induced immune response (Morrison, 2000; Zhang and Starnbach, 2015). While high levels of IFN-γ are been shown to be important in CT eradication, some studies have suggested that lower levels facilitate

persistence and produce aberrant, non-infectious CT bodies *in vitro* (Figure 1.2) (Ziklo *et al.*, 2018; Ziklo *et al.*, 2019).

Studies have also shown that IFN-γ alters the availability of vital nutrients for *Chlamydia* development which it acquires from the host, preventing it from metabolising and replicating normally. IFN-γ significantly reduces CT metabolic development through host cell tryptophan depletion (needed by CT) and glucose deprivation (Brunham and Rey-Ladino, 2005). Byrne et al. showed that IFN-γ was able to restrict intracellular *Chlamydia* growth *in vitro* due to increased catabolism of the essential amino acid tryptophan within the host cell, by increasing the activity of indoleamine-2,3-dioxygenase , a catabolic enzyme that degrades tryptophan into N-formylkynurenine and kynurenine (Byrne *et al.*, 1986). Others have shown *ex vivo* that CT bacterial burdens were higher in women with higher indole cervicovaginal concentrations, associated with lower tryptophan concentrations (Wang *et al.*, 2022). However, exogenously added indoles were reported to protect CT from the effects of IFN-γ *in vitro*, enabling them to produce infective progeny (Ziklo *et al.*, 2016b).

IFN-γ also interferes with host iron metabolism (Jerchel *et al.*, 2014), and all prokaryotes (including CT) have an absolute requirement for iron or related metals for survival (Brunham and Rey-Ladino, 2005). *In vitro* studies have shown that *Chlamydiae* enter a different growth state, described as viable but non-cultivatable, when deprived of iron by treatment with a hydrophilic chelator like deferoxamine mesylate (Beatty *et al.*, 1994) or 2,2-bipyridyl (Thompson and Carabeo, 2011). *Chlamydia* species also lack key enzymes needed for glucose metabolism and rely on host cells for this critical source of carbon (Stephens *et al.*, 1998; Elwell and Engel 2012; Cox *et al.*, 2016). Axenic medium (single species media) experiments revealed that glucose-6 phosphate, not ATP, was necessary for protein synthesis in CT EBs (Omsland *et al.*, 2012).

While IFN-γ is considered a correlate of protection against CT infection, high concentrations of IFN-γ in the female genital tract has been linked with an excessive inflammatory reaction and infectious sequelae (Morrison, 2000; Ishihara *et al.*, 2005; Mascellino *et al.*, 2011; Zhang and Starnbach, 2015), suggesting that this key cytokine can also be a cause of pathology.

1.8.3.2 Tumour necrosis factor

TNF an important inflammatory factor, produced by activated monocytes, macrophages and activated T cells (Idriss and Naismith 2000; Horiuchi *et al.,* 2010; Jang *et al.,* 2021). TNF-α, released in response to inflammatory stimuli, is recognised for its ability to destroy intracellular pathogens (Kamalakaran *et al.,* 2013). *Chlamydia* and its antigens, such as LPS and pORF5 (a plasmid protein), have been shown to stimulate TNF-α production *ex vivo* in cervicovaginal secretions, and *in vitro* in cultured cell supernatants (Kaukoranta-Tolvanen *et al.,* 1996; El-Asrar *et al.,* 1998; Kragsbjerg *et al.,* 1998). TNF-α expression is regulated by host Toll-like receptor (TLRs). Studies have shown that deletion of TLR2 (which binds diacyl and triacylglycerol moieties, proteins like CT MOMP, chlamydial plasmid-regulated ligands and peptidoglycans) or TLR4 (which binds CT LPS and HSPs) on macrophages during chlamydial infections result in significant reduction in TNF-α levels (Jiang *et al.,* 2008; Nagarajan *et al.,* 2011; Massari *et al.,* 2013; Elwell *et al.,* 2016). During early stages of chlamydial infection, in contrast, deficiency of TLR3 by epithelial cells was associated with increased TNF-α levels (Carrasco *et al.,* 2018).

TNF-α, in partnership with IFN-γ, has been shown to suppress host cell metabolism by raising the activity of IDO, that limits CT development (Brunham and Rey-Ladino 2005; Njau *et al.,* 2009). Furthermore, TNF-α may induce apoptosis of CT-infected host cells that are providing suitable intracellular conditions for the growth of CT (Darville *et al.,* 2000; Perfettini *et al.,* 2000; Radomski *et al.,* 2019). Adoptive transfer of TNF-α in the mouse model has been shown to lower CT bacterial burden in the lungs of newborn mice from *Chlamydia*-infected mothers (Xiang *et al.,* 2021). TNF-α deficiency or inhibiting TNF-α, on the other hand, had no effect on *Chlamydia* clearance *in vivo,* indicating that TNF-α may not be necessary for *Chlamydia* clearance (Kamalakaran *et al.,* 2013).

TNF-α has been linked to *Chlamydia*-induced pathology (Xiang *et al.,* 2021). TNF-α increases recruitment of immune cells to the site of inflammation, in addition to promoting the release of inflammatory cytokines such as IL-6 and IL-8, which are associated with tissue fibrosis and scar formation (Shen *et al.,* 2019). TNF-α has also been shown to promote collagenase release by increasing tissue fibroblast proliferation, which may exacerbate histopathological damage in women infected with CT (Igietseme *et al.,* 2003). Mice deficient in TNF-α receptors were shown to experience significantly less severe fallopian tube scaring following chlamydial

infection than wild-type mice, further confirming the critical role TNF-α plays in immunological damage caused by *Chlamydia* (Manam *et al.,* 2015; Zafiratos *et al.,* 2019). TNF-α may therefore mediate immunopathological damage associated with CT infection, even though it is not required for *Chlamydia* clearance.

1.8.3.3 Interleukin-1

IL-1 is a central mediator of innate immunity and inflammation (Dinarello 2018), with its two isoforms produced by macrophages (IL-1β) and epithelial cells (IL-1α), each exerting a variety of local (and systemic) responses to bacterial infections in addition to being involved in the pathogenesis of several chronic inflammatory diseases (Dinarello, 1996; Dinarello, 2018), including in chlamydial infections (Rothermel *et al.,* 1989; Gervassi *et al.,* 2004). High concentrations of both IL-1α (an alarmin) and IL-1 β can be detected in cervicovaginal secretions from women infected with CT infection (Marconi *et al.,* 2014). Similarly, during *C. pneumoniae* infections, alveolar macrophages and peripheral blood mononuclear cells obtained from chronic obstructive pulmonary disease (COPD) patients infected with *C. pneumoniae* produces significantly higher amounts of IL-1β and lower amounts of IL-1R-antagonist (which regulates IL-1 activity) than COPD patients negative for *C. pneumoniae* (Rupp *et al.,* 2003).

A wide range of inflammatory cytokines and chemokines, including IL-6 and IL-8, are induced by IL-1α and IL-1β to aid host defence against chlamydial infection (Hvid *et al.,* 2007; Shimada *et al.,* 2011). However, IL-1β has been shown to contribute to the worsening of upper genital tract disease during infection (Prantner *et al.,* 2009).

1.8.3.4 Interleukin-6

IL-6 is produced by a wide range of immune cells, including macrophages, dendritic cells, T-cells, B-cells, as well as fibroblasts (Tanaka *et al.,* 2014), and it is involved in a wide range of immune cell function-related activities. IL-6 most noticeable role is in the defence against infection (Heinrich *et al.,* 2003; Rose-John 2018), it promotes terminal differentiation of B cells (Heinrich *et al.,* 2003), T cell survival (Tanaka *et al.,* 2014), and helps T cell to overcome suppression by Tregs (Weissenbach *et al.,* 2004). High levels of IL-6 are detected in mice with *Chlamydia*-related tubal factor infertility and in humans with *Chlamydia*-related disease (Kishimoto 2010; Sun *et al.,* 2017). Given that IL-6 defective mice were substantially more vulnerable to being infected with *Chlamydia* than wildtype mice, elevated IL-6 levels were

suggested to prevent *Chlamydia* infection (Williams *et al.,* 1998; Zhang *et al.,* 2010). Anti-IL-6 treatment was found to increase the risk of CT infection in patients who received it (Edwards 2012; Refaat *et al.,* 2016).

IL-6 is thought to modulate inflammation to prevent infection by encouraging the recruitment of leukocytes (neutrophils) to the site of infection, as well as promoting neutrophil apoptosis (Cunningham *et al.,* 2013; Rose-John 2018). IL-6 also activates $CD4^+$ Th1-like cells to better clear infection, by encouraging their production of IFN-γ (Sun *et al.,* 2017). Additionally, IL-6 inhibits TNF-α in a dose-dependent manner, which is important to limit *Chlamydia*-induced fallopian tube blockage and maintenance of the ongoing infection (Ilumets *et al.,* 2007; Zhao *et al.,* 2015). Although IL-6 is thought to be essential for controlling *Chlamydia* infections, its effects may not always be positive. For instance, IL-6 functions differently in *Chlamydia* infection at various inoculating doses in mouse models (Sun *et al.,* 2017). Irrespective of *C. muridarum* dose, IL-6 is necessary to limit pathogen replication. However, IL-6 also exacerbates infection-induced fallopian tube blockage at low doses, which is not necessary for high doses of the pathogen (Sun *et al*., 2017). Sun *et al*. (2017) proposed from these data that IL-6 might increase inflammatory infiltration into tissues, as well as the specific $CD4^+$ and $CD8^+$ T-cells that produce TNF-α in response to low-dose chlamydial inoculation, as previously suggested (Ilumets *et al.,* 2007; Refaat *et al.,* 2016). These findings also suggest that inflammatory responses involving IL-6 may not always correspond to the severity of infection.

1.8.3.5 Interleukin-17

IL-17 is secreted by activated $CD4^+$ Th17 cells, performing a proinflammatory function, particularly at mucosal sites, providing defence in many mucosal infectious diseases (Khader and Gopal 2010; Amatya *et al.,* 2017). IL-17 has been detected in cervicovaginal secretions and tissues from CT-infected patients (Rizzo *et al.,* 2011; Hakimi *et al.,* 2014a). IL-17 deficient mice or mice treated with IL-17 inhibitors experience more severe sequalae following *Chlamydia* infection than wild-type mice (Andrew *et al.,* 2013; Bai *et al.,* 2017), suggesting that IL-17 protects against *Chlamydia* infection. IL-17 responses during early stages of infection appear critical in amplifying inflammation and initiating host defence against *Chlamydia* through its interactions with other cytokines (like IL-6) (Zhang *et al.,* 2010).

While IL-17 has been shown to protect against chlamydial infections via its pro-inflammatory function, it has also been shown to promote inflammatory pathology and participate in the pathogenesis of chlamydial diseases (Xiang *et al.,* 2021). During the early stages of *Chlamydia* infection, IL-17 increases local neutrophil infiltration by regulating the expression of chemokines and adhesion molecules (Arkatkar *et al.,* 2015; Qiao *et al.,* 2019). Additionally, IL-17 can trigger the release of a number of other cytokines that greatly increase tissue damage and repair fibrosis (Zheng *et al.,* 2007; Bagri *et al.,* 2022; Mills, 2023).

1.8.3.6 Interleukin-10

IL-10 is an anti-inflammatory cytokine which serves to dampen inflammatory responses, produced predominantly by regulatory T cells (Ouyang *et al.,* 2011). However, the role of IL-10 in chlamydial infection is complicated. Studies have shown that CT HSP60 and LPS interact with host TLRs, which increases expression of IL-10 in host cells (Jha *et al.,* 2011; Hakimi *et al.,* 2014b). Elevated IL-10 levels have been found in *Chlamydia*-infected patient serum, scar tissue homogenates, bronchoalveolar lavage fluid and cervical secretions (Faal *et al.,* 2006; Agrawal *et al.,* 2007; Kaiko *et al.,* 2008) as well as the supernatant of *Chlamydia*-infected HeLa cells, dendritic cells, and PBMCs (Holland *et al.,* 1996; Yu *et al.,* 2003; Han *et al.,* 2006).

IL-10, although anti-inflammatory, often correlates positively with inflammatory cytokines, produced proportionately to counter the potentially harmful effects of unregulated inflammation (Masson *et al.,* 2015b). Studies have shown that the absence of IL-10 is often initially beneficial to the host, associated with heightened inflammatory responses, but that prolonged IL-10 deficiency can be detrimental in the long term, with enhanced and prolonged production of inflammatory cytokines being detrimental (Iyer and Cheng 2012). Complications caused by *Chlamydia* infections are caused by inflammatory processes, and IL-10 is important to controlling these (Xiang *et al.,* 2021). In mice, IL-10 attenuates pathological damage like tubal swelling associated with *Chlamydia* infections, by reducing local inflammatory infiltration (Hvid *et al.,* 2007). The activity of $CD8^+$ T-cells is suppressed by IL-10, therefore reducing tissue damage (Caspar-Bauguil *et al.,* 2000).

1.9 Host genetic risk factors for CT

Host genetic factors are likely to influence susceptibility to, resistance against, and infection severity to CT, although these have not been well characterized. In several other bacterial infections, the extent of inflammation to have been connected to functional polymorphisms in

cytokine genes (El-Omar *et al.,* 2000; Laine *et al.,* 2000; Witkin *et al.,* 2002). Therefore, development of severe inflammation following CT infection in some women may be a consequence of a similar genotypic predisposition.

A range of cytokine gene polymorphisms have been identified in women that experience tubal infertility following CT infection, including genetic polymorphisms in genes encoding IL-10 (Öhman *et al.,* 2009), TNF-α (Öhman *et al.,* 2009), and IL-12 (Öhman *et al.,* 2012). In addition, a single nucleotide polymorphism in the TLR6 promoter, associated with increased expression of TLR1, TLR6, and TLR10 and higher secreted levels of ten CT-induced cytokines, was more prevalent in European than African populations, suggesting that CT pathology may be more severe in Europeans than Africans (Barnes *et al.,* 2022). No polymorphisms in IL-1β and IL-1 receptor genes have been associated with CT-related tubal pathology (Murillo *et al.,* 2003; Poston *et al.,* 2017)

1.10 CT vaccines

Changes in antigen dominance during the different phases of CTs unique life cycle has posed significant challenges in vaccine design. Initial experiments showed that live and attenuated *Chlamydia* vaccines protected mice against infection following genital challenges (Karunakaran *et al.,* 2016; Poston and Darville 2016; Yu *et al.,* 2016; de la Maza *et al.,* 2017; Liang *et al.,* 2017; Phillips *et al.,* 2019). However, other studies showed that mice were more susceptible to *Chlamydia* infection when killed organisms were inoculated directly into their uterus's, which was thought to be due to vaccine-induced tolerance, from *Chlamydia*-specific regulatory T cells producing IL-10 (Stary *et al.,* 2015). During early trachoma vaccine trials in humans with whole-cell CTvaccines (and in non-human primate pre-clinical testing), increased risk of delayed hypersensitivity and susceptibility to reinfection were noted, (Taylor 2008), which made resulted in this approach not proceeding into larger human trials. Furthermore, these vaccine trials indicated that protection was serovar specific (Taylor 2008), which was likely due to MOMP (Fitch *et al.,* 1993; Stephens *et al.,* 1998). Because of this, understanding CT *ompA* genovars circulating regionally, particularly the diversity in MOMP, is crucial in vaccine design.

Several sub-unit vaccines for CT have been considered, predominantly including those containing CT MOMP, but also CT Pmps and CPAF (de la Maza *et al.,* 2021). Preclinical

studies employing MOMP-based vaccines have demonstrated that protective immune responses can be elicited from animal models (Kari *et al.,* 2009; Verma *et al.,* 2018; Pal *et al.,* 2020; Russi *et al.,* 2023). A multi-subunit vaccine, incorporating MOMP and other chlamydial antigens, has also demonstrated efficacy in preclinical studies by inducing robust T-cell immune responses (Yu *et al.,* 2016).

The results of the first-in-human phase 1 clinical trial of a recombinant CT vaccine was published in 2019 (Abraham *et al.,* 2019), which showed that the recombinant antigen called CTH522 was safe and well tolerated. CTH522 contains a version of MOMP, which showed both strong humoral and cell-mediated immune responses in preclinical testing. In this clinical study, CTH522 was administered by intramuscular injections in three doses to women, followed by intranasal administrations. CTH522 vaccination in this phase 1 trial was associated with CT-specific IgG seroconversion (Thoma, 2019). The primary outcome of the trial was safety, with no treatment-related serious adverse events were noted although some women experienced mild injection site reactions.

In addition, some Pmps have also been shown to be good immunogens in pre-clinical testing (Inic-Kanada *et al.,* 2015; Paes *et al.,* 2016; Vasilevsky *et al.,* 2016; Müller *et al.,* 2017), although these have been most comprehensively tested in mouse respiratory models of *C. pneumonia* (Pal *et al.,* 2017). Similarly, vaccination of mice with CPAF shortened the duration of the *Chlamydia* infection, protected them against oviduct pathology and offered cross-species protection (Li *et al.,* 2007). However, these pre-clinical studies demonstrated that CPAF-based vaccines did not protect against infertility, suggesting incomplete immunity (Murthy *et al.,* 2011).

1.11 Relationship between CT and HPV infections

A causal link between CT and HPV infections has been suggested, with CT infection increasing the risk of HPV-associated cervical cancer progression (Zhu *et al.,* 2016). HPV is one of the most common viral STIs, which is highly prevalent in adolescents, with infections starting soon after sexual debut and decreasing with age (Schiffman and Castle, 2005; Mbulawa *et al.,* 2021; Dos Santos *et al.,* 2022). In South Africa, AGYW (<25 years of age) have high HPV prevalence, with HPV prevalence in cohort studies (including those from our own lab) from Western Cape, Gauteng and KwaZulu-Natal Provinces ranging from 44-85% (Richter *et al.,*

2013; Adler *et al.,* 2014; Giuliano *et al.,* 2015; Mbulawa *et al.,* 2015; Ebrahim *et al.,* 2016; Mbulawa *et al.,* 2018). Infection with other STIs (including CT), early sexual debut, increased number of lifetime sexual partners and increased numbers of current sexual partners are some of the factors that influence HPV natural history (Munoz *et al.,* 2006; Insinga *et al.,* 2010; Gravitt, 2012; Yamaguchi *et al.,* 2021).

HPV is a small double stranded DNA virus that belongs to the family *Papillomaviridae,* having a distinct tropism for mucosal or cutaneous squamous epithelia. The ~60 nm HPV icosahedral capsid is composed of the HPV late proteins 1 and 2 (L1 and L2), which contain a single small genome of ~8,000bp (Knipe *et al.,* 2013). The *Papillomaviridae* family has been divided into 39 genera, based on a L1 sequence identity of >60%, with each genus designated by Greek letter (de Villiers *et al.,* 2004; Bernard *et al.,* 2010; Harden and Munger, 2017). HPVs in the alpha genus are of the greatest medical significance given that they are linked to anogenital tract, mucosal, and oral cancers (Harden and Munger, 2017).

To date, more than 200 HPV types have been isolated and characterized (de Villiers *et al.,* 2004; Bernard *et al.,* 2010; Bruni *et al.,* 2023). Based on epidemiologic association with cervical cancer, mucosal HPV genotypes are divided into high-risk (HR-HPV) and low-risk (LR-HPV) types (Akarolo-Anthony *et al.,* 2014; Menon *et al.,* 2016b; Mudini *et al.,* 2018; Yakub *et al.,* 2019). HR-HPVs are confirmed etiological agents of cervical cancer (and a subset of other human cancers; (Ghittoni *et al.,* 2010; Harden and Munger, 2017; Pešut *et al.,* 2021), and include HPV-16, 18, 26,31, 33, 35, 39, 45, 51, 52,53, 56, 58, 59, 68, 73, and 82 (de Sanjose *et al.,* 2010; Haedicke *et al.,* 2013; Szymonowicz *et al.,* 2020; Pešut *et al.,* 2021). Their association with cervical cancer is particularly strong in women who have persistent infections with HR-HPV types (Burd 2003; Trottier *et al.,* 2006; Okunade and Gynaecology, 2020). LR-HPV types include HPV-6, 11, 40, 42, 43, 44, 54, 55, 61, 62,67,69,70,71,72,81 and 84, which are associated with benign warts and even recurrent respiratory papillomatosis.

In 2020, there were 604 127 cervical cancer cases and 341 831 deaths recorded (Singh *et al.,* 2023). The most carcinogenic HR-HPV types are considered to be HPV-16 and HPV-18, being the most commonly associated with development of cervical cancer, accounting for ~70% of invasive cervical cancers (Muñoz *et al.,* 2003; Smith *et al.,* 2007; Kaliff *et al.,* 2018). In South Africa, HPV-16 and HPV-18 are highly prevalent and they are noted to be the major cause of over 65% cervical cancer cases in the region (Bruni *et al.,* 2010; Okoye *et al.,* 2021; Seyoum

et al., 2022). A recent 20-year systematic review revealed that the most prevalent HR-HPV types in HIV positive and negative women diagnosed with invasive cervical cancer in Sub-Saharan Africa were HPV-16, HPV-18 and HPV- 45 (Okoye *et al.,* 2021). In addition, a significant portion of vaginal, penile, vulvar, and oropharyngeal cancers are also believed to be caused by HR-HPV infection (CDC, 2012; De Martel *et al.,* 2017). There are several safe and effective prophylactic HPV vaccines available, but they do not protect against all HPV types or those who are already infected with HPV (Harden and Munger, 2017). The most commonly used HPV vaccine in LMICs currently, including South Africa, is Cervarix® (from Glaxo-SmithKline) which contains HR-HPV types HPV-16 and HPV-18 virus-like particles (just the capsid protein L1 which self-assembles), where it is administered to a subset of 10-year old girls through a National school-based vaccination program (free to schools in the bottom two quintiles economically) (Delany-Moretlwe *et al.,* 2018; Ledibane *et al.,* 2023). However, other cost-effective vaccines have now been developed by the Serum Institute in India (Sharma *et al.,* 2023) and from China (Li *et al.,* 2023).

While HPV infection in AGYW is highly prevalent in many LMICs, including South Africa, this potentially oncogenic viral infection commonly co-occurs with CT (Bellaminutti *et al.,* 2014; Escarcega-Tame *et al.,* 2020), and other inflammation-causing STIs like MG (Harrison *et al.,* 2019; Borgogna *et al.,* 2020) and NG (Lim *et al.,* 2015; Seo *et al.,* 2021). CT is considered a co-factor for cervical cancer progression (Bellaminutti *et al.,* 2014; Di Pietro *et al.,* 2018), infecting the same endocervical environment with similar risk factors and modes of transmission (Chen *et al.,* 2020). According to several studies, CT infection promotes HPV persistence, which increases risk for cervical cancer progression (Bhatla *et al.,* 2013). Inflammation due to CT infection increases reactive oxygen species and free radical production which may damage the host mucosal barrier and compromise cell-mediated immunity, enhancing the oncogenic potential of HPV (Conde-Ferraez *et al.,* 2017; Chen *et al.,* 2019). CT infection may also facilitate HPV transmission (Chen *et al.,* 2020). For effective prevention of both HPV and CT infections, it is therefore essential to understand the prevalence and genotype distribution of CT and HPV infection, particularly in AGYW who are most at risk.

1.12 Conclusion

STIs, including CT and HPV, increase the risk of HIV infections. In order to reduce the burden of CT in regions with high burden of HIV infection, routine screening and molecular typing of CT are crucial. Appropriate diagnostic services are required to identify CT infection, particularly in women, where these are frequently asymptomatic. Although continuous efforts are being made to develop a safe and effective chlamydial vaccine, these are not currently available. Understanding bacterial factors, the host inflammatory environment (including cytokines and T cell activation) involved in CT pathogenicity will aid in the development of optimal vaccines that prevent chlamydial infections progression to infertility. Because of the overlap in risk factors for CT and HPV, considering common co-infections are critical, particularly in AGYW, as CT-associated inflammation may enhance HPV persistence and cervical disease progression. As many LMICs are adopting population wide administration of the HPV vaccine to protect women from cervical cancer, these networks can be harnessed to roll-out an effective CT vaccine, once this is approved and available. Understanding regional diversity in CT MOMP will be critical to facilitate these efforts.

1.13 Rationale for this study

HIV is a major health issue among young women in South Africa, and genital inflammation increases HIV risk. Although most women are clinically asymptomatic, CT infection causes genital inflammation, although factors that influence CT pathogenesis and symptoms are poorly understood. There is little molecular epidemiology on urogenital CT infections in South Africa, and most genomic research has concentrated on ocular CT strains (Last *et al.,* 2017; Pickering *et al.,* 2017; Alkhidir *et al.,* 2019; Last *et al.,* 2020; Pickering *et al.,* 2022).

Buckner *et al*. (2016) suggested that the endocervix is the most common site of CT infection, with CT thought to primarily infect columnar epithelial cells. Conditions like cervical ectopy (were the columnar epithelium of the endocervix extends onto the proximal portion of the ectocervix immediately adjacent to the multilayer squamous epithelium, forming a visible squamocolumnar junction) are common in AGYW and put them at high risk for infections with CT infection (Lee *et al.,* 2006). AGYW with immature epithelium had higher genital concentrations of inflammatory cytokines than older women with mature epithelium, and younger age has been independently associated with increased cervicovaginal immune function (Hwang *et al.,* 2011; Dabee *et al.,* 2019).

High resolution typing of CT using MLST has been shown to be a useful tool in epidemiological studies and investigation of CT infection, transmission or recurrence, sexual network analysis, and surveillance of emerging strains (Ripa and Nilsson 2006; Borges *et al.*, 2019; Escobedo-Guerra *et al.*, 2019; Feodorova *et al.*, 2019; Hokynar *et al.*, 2019; Borges *et al.*, 2021; Puolakkainen *et al.*, 2023). HPV and CT share the same transmission pathway and risk factors, and CT-associated inflammation may increase risk for cervical cancer progression.

1.14 Aims and Objectives

1.14.1 Aim 1

To investigate the impact of CT infection on host correlates of protection from CT and pathogenesis following infection. This will be addressed in the following two objectives:

Objective 1. To investigate impact of CT bacterial burden and plasmid copy number on genital tract inflammation (cytokines: IL-1β, IL-6, IL-17A, IFN-γ and TNF-α.) and cellular activation (CD4:CCR5 and $CD38^{+}$) in AGYW.

Hypothesis: Higher CT bacterial load and plasmid number will increase genital inflammation, indicated by elevated cytokines, $CD4^{+}CCR5$ and $CD4^{+}CD38^{+}$).
The results of Objective 1 are presented in Chapter 2.

Objective 2. To investigate variation in CT genotype (hr-MLST) and associated genital pathology (cytokines).

Hypothesis: High diversity of genetically distinct CT sequence types circulating in Cape Town young women are associated with genital pathology, indicated by elevated cytokines.
The results of Objective 2 are presented in Chapter 3.

1.14.2 Aim 2

To investigate the impact of CT infection on risk for HPV infection in AGYW.
This will be addressed in the following objective:

Objective 3. To evaluate the impact of CT infections, bacterial load and plasmid copy number on prevalent and persistent infections with HPV in AGYW.

Hypothesis: CT infection will increase HPV infection in AGYW.
The results of Objective 3 are presented in Chapter 4.

CHAPTER 2: Determination of CT bacterial load, plasmid load, proinflammatory cytokines and T cell activation in adolescent women

2.1 Introduction:

Various social and biomedical factors have been linked with higher HIV risk in young women, including gender inequality and biological susceptibility (Sia *et al.,* 2016). A recent study in our laboratory of HIV-seronegative adolescent females (16–22 years) from two socioeconomically disadvantaged South African communities (Johannesburg and Cape Town) compared the prevalence of symptoms associated with laboratory-diagnosed STIs and BV and revealed a high STI burden in both communities (Barnabas *et al.*, 2018).

Inflammation of the genital mucosa, which is associated with STIs like CT, increase HIV acquisition and transmission risk in women, most likely because of an increase in HIV target cells in mucosal tissues (Li *et al.,* 2009; Masson *et al.,* 2015a). High rates of CT infections may be due to AGYW being socially, immunologically and physiologically naïve (Batteiger *et al.,* 2010b). This raises the question as to whether the high STI rate among adolescents is partially due to them being immunologically naïve to CT infections. Furthermore, does acquired immunity to STIs like CT become more prevalent as women age?

The endocervix is the most frequently infected site in women, where columnar epithelial cells of the genital mucosae are the primary target of CT (Lanjouw *et al.,* 2015). Some studies have shown that AGYW with an immature epithelium (predominantly columnar and early/mid squamous metaplasia cells) had higher genital concentrations of IL-1, IL-6, IL-8, MIP-1, RANTES (regulated upon activation, normal T-cell expressed and secreted), TNF-α, IL-10, IL-12, and IFN-γ than those with a mature epithelium (Hwang *et al.*, 2011), and younger age was independently associated with increased cervicovaginal inflammation (Ghanem *et al.,* 2005). Inflammation is an important biological process in the initiation of an effective host immune response and HIV is able to take advantage of both inflammation and immune activation processes (Douek *et al.,* 2009; Deeks *et al.,* 2013; Lv *et al.,* 2021). Additionally, increased cervicovaginal cytokines were linked to significant changes in the genital epithelial barrier function and protease activity, as well as an increase in the recruitment of activated $CD4^+$ T-cells (Arnold *et al.*, 2016).

The mechanism by which *Chlamydia* invades host cells is poorly understood but CT infection compromises and interferes with the function of infected host cells (Pais *et al.,* 2019; Bishop and Derré, 2022; Cheong *et al.,* 2023; Luís *et al.,* 2023). It is important for the host to mount an immune response against CT that includes production of cytokines that activate or recruit immune cells to trigger or amplify inflammation against *Chlamydia* (Mpiga *et al.,* 2006; Faris *et al.,* 2019) *In vitro* and *in vivo* studies on *Chlamydia* infection show that a variety of cytokines, including IL-1α, IL-β, IL-6, IL-8, IL-17A, IFN-γ and TNF-α are involved in the inflammatory response, whilst IL-4 and IL-10 are involved in the anti-inflammatory response (Xiang *et al.,* 2021).

During chlamydial infection, $CD4^+$ T-cells are essential (Morrison *et al.,* 1995; Su *et al.,* 1995; Gondek *et al.,* 2012). Murine models using MHC II deficient mice infected with *Chlamydia* have shown the significance of $CD4^+$ T-cells in clearing the disease (Morrison *et al.,* 1995). Study of murine upper genital *Chlamydia* infection models have suggested that $CD4^+$ T-cells are necessary and sufficient for clearance of infection and protection against reinfection (Gondek *et al.,* 2012). Examining of the immune response against *C. pneumoniae* also involved proliferation and activation of both $CD4^+$ and $CD8^+$ T-cells during primary infection, although activation of only $CD4^+$ T-cells was observed in the later stages of the infection (Halme *et al.,* 2000). CCR5 has been described as a major co-receptor of HIV-1 entry expressed on various immune cell types (macrophages, dendritic cells, and natural killer cells) and in cells of other tissues in addition to $CD4^+$ T-cells, which are the primary host cell targets of HIV-1 (Sankaranantham 2019). CT infection also enhances HIV replication in peripheral mononuclear cells and a CCR5 T cell line, suggesting that it could promote HIV infection or replication in underlying target cells (Buckner *et al.,* 2016).

In other bacterial STIs, including MG (le Roux and Hoosen, 2017) and NG (Bissessor *et al.,* 2011), higher bacterial load has been associated with increased disease severity and risk of the infected person transmitting to their sexual partners. The relationship between CT bacterial load and pathogenicity is however sparse (Pickett *et al.,* 2005; Seth-Smith *et al.,* 2009; Ferreira *et al.,* 2013; Last *et al.,* 2014; Last *et al.,* 2017; Last *et al.,* 2018; Last *et al.,* 2020). It has been suggested that efficacy of CT infection in the urogenital tract is related to the bacterial load (Frost *et al.,* 1993; Eckert *et al.,* 2000) and that the bacterial load might influence CT transmission within a population (Last *et al.,* 2017). There are inconsistent findings about

higher CT loads being associated with symptoms (Gomes *et al.,* 2006; Jalal *et al.,* 2011; Walker *et al.,* 2012; Vodstrcil *et al.,* 2015). Murine models of *Chlamydia* infection and some trachoma clinical studies have shown that higher *Chlamydia* bacterial loads increase the transmission potential (Pal *et al.,* 1998; West *et al.,* 2005), and the risk of sequelae (Pal *et al.,* 1998; Pal *et al.,* 2001; West *et al.,* 2005). The susceptibility for prolonged *Chlamydia* infection, which generates harmful inflammatory disease, is a crucial but poorly understood pathophysiological aspect of chlamydial infection.

Highly conserved small cryptic 7.5-kb plasmids that most CT strains harbour is an extra-chromosomal, self-replicating genetic element that improve survival, although mechanism not clear (Song *et al.,* 2013; Zhang *et al.,* 2019; Szabo *et al.,* 2020; Turman *et al.,* 2023b). The chlamydial plasmid is thought to affect both the regulation of chromosomal gene expression and virulence (Carlson *et al.,* 2008; Rockey 2011; Song *et al.,* 2013; Turman *et al.,* 2023b). Plasmid-less and wildtype CT strains have shown phenotypic differences with respect to infectivity glycogen accumulation (plasmid-less isolates did not accumulate glycogen in inclusions), induction of host inflammation (plasmid-less isolates elicit reduced levels of inflammation and pathology), and activation of Toll-like-receptor pathways (O'Connell *et al.,* 2011; Lehr *et al.,* 2018). In animal models, it has been shown that the CT cryptic plasmid serves as a virulence factor (Kari *et al.,* 2011; O'Connell *et al.,* 2011; Russell *et al.,* 2011; Frazer *et al.,* 2012). Studies employing CT plasmid deletion mutagenesis demonstrated that removing the plasmid *pgp4* gene causes an *in vitro* phenotype that is the same as a plasmidless strain (Song *et al.,* 2013). This is consistent with bacterial transcriptome analysis, which revealed a decrease in transcript levels of a subset of chromosomal genes in a naturally occurring plasmid-free strains of CT, demonstrating that the plasmid is a transcriptional regulator of genes linked to virulence (Carlson *et al.,* 2008). In addition, plasmidless *Chlamydia* strains do not accumulate glycogen in their inclusions and they elicit reduced levels of inflammation and pathology (Turman *et al.,* 2023b). A study from Malaysia also suggested that women infected with plasmid-bearing CT strains were more likely to be diagnosed with infertility, inflammation in the reproductive tract, irregular menses, and polycystic ovarian syndrome than those infected with plasmidless variants of CT, suggesting plasmids were associated with virulence (Yeow *et al.,* 2016).

This study included samples from AGYW enrolled in the uCHOOSE study, which was an open-label, randomized crossover study of healthy, sexually active, HIV-negative female

adolescents aged 15 to 19 years, that investigated the safety and acceptability of different forms of hormonal contraceptive (Gill *et al.*, 2020). With CT prevalence in the uCHOOSE cohort being 33% (43/130), the aim of this Chapter was to investigate the influence of CT pathogenic determinants (including bacterial load and plasmid copy number) on genital inflammation in young women (focusing on cervicovaginal cytokines and $CD4^+$ T-cell activation). The hypothesis that was being tested was that higher CT bacterial load and plasmid number will increase genital inflammation.

2.2 Methods

2.2.1 Cohort and sample collection

As part of the uCHOOSE study, lateral vaginal wall swabs were collected and used for this study. The uCHOOSE study recruited AGYW through the Desmond Tutu Health Foundation clinical research site in Cape Town, South Africa, located in a peri-urban low-income community with a high HIV prevalence. For the first 16-weeks, eligible participants were randomly assigned to one of three study arms: (A) monthly vaginal Nuvaring (etonogestrel 11.7 mg/ethinyl estradiol 2.7 mg), (B) bi-monthly injectable contraceptive (Norethisterone enantate 0.2 g/mL), or (C) daily COC (Combined oral contraceptive pill) (Levonorgestrel 0.15 mg/Ethinyl estradiol (Balle *et al*., 2020; Figure 2.1). After 16 weeks, participants were "crossed over," and those in arms B and C continued to receive Nuvaring for another 16 weeks, while those in arm A were given the option of injectable or COC. Participants had follow-up visits every eight weeks. The study was approved by the University of Cape Town Health Science Research Ethics Committee and registered on ClinicalTrials.gov (NCT02404038) Before undergoing any trial-related procedures, all participants, and their parents/legal guardians (if a participant was 18 years old), provided written informed consent and assent (if a participant was <18 years old). To be eligible for the study, participants had to agree to use a randomly assigned contraceptive method for the duration of the study (32 weeks). Participants were ineligible if they were pregnant, HIV-positive, or had medical contra-indications to using the contraceptive study products.

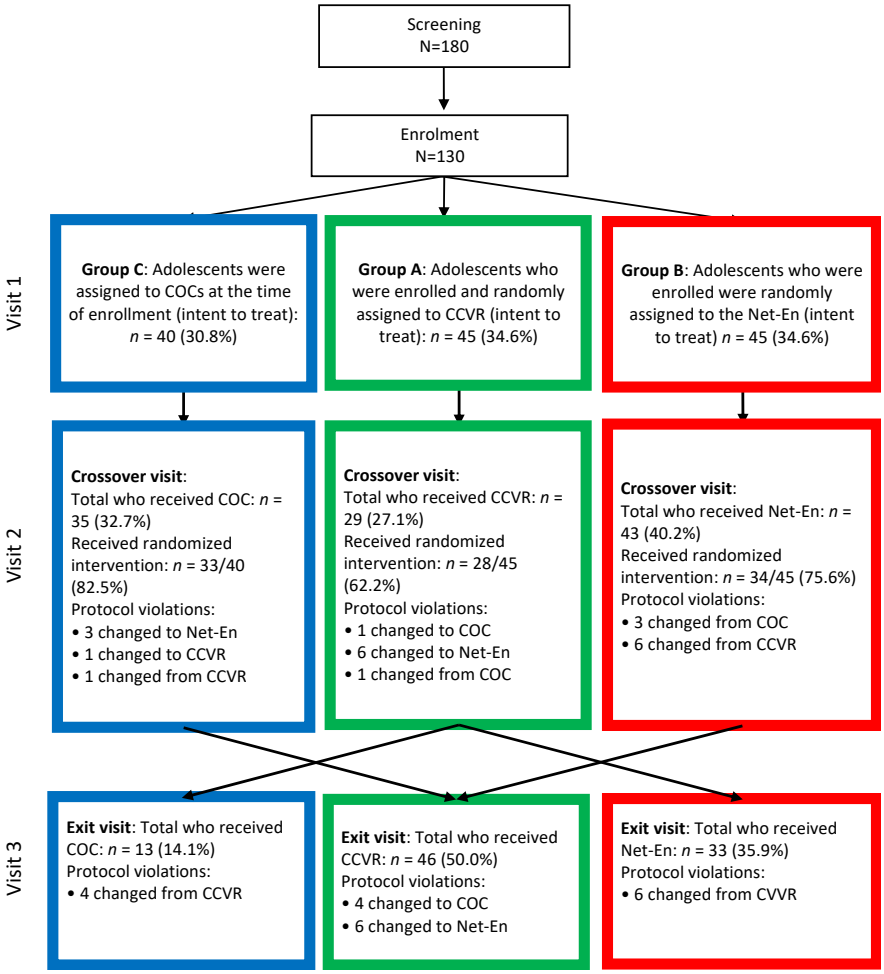

Figure 2.1. **Overview of the uCHOOSE study design and randomization**. At enrollment, participants were divided into three groups: Nuvaring (group A), injectable (group B), and combined oral contraceptives (COC, group C). The figure depicts the number of participants who switched to a different study arm before the crossover visit, when all participants from groups B and C were switched to Nuvaring and those who had previously been in group A were free to select their next method of contraception.
Pregnancy (urine) and HIV testing (finger prick; blood) were done at screening, enrolment, and weeks 8, 16, 24, and 32. For confirmation, HIV rapid testing was performed in series using a third generation Determine^{TM} HIV-1/2 Combo and Uni-GoldTM Recombigen HIV-1/2. STI testing was performed at the screening, week 16, and exit visits. Taken from (Gill *et al.*, 2020)

2.3.2 STI testing

Vulvo-vaginal swabs was used to test for CT, NG, TV, and MG, using a Multiplex PCR (at the NICD STI surveillance lab, Sandringham, Johannesburg). A vulvo-vaginal swab was used to screen for BV by Nugent scoring on a wet-prep slide, where Nugent 0-3 was considered BV negative, Nugent 4-6 was considered intermediate; and Nugent 7-10 was considered positive. The wet-prep slide was also used to screen for Candidiasis, based on the presence of *Candida* hyphae and/or spores. Color-fixed indicator strips (Macherey-Nagel, Duren, Germany) were

used to measure vaginal pH. When the results were available, all STIs were treated by study clinicians according to South African National Guidelines.

2.2.3 Quantification of CT bacterial load and plasmid copy number

For both CT+ and CT– cases, Microbial DNA was extracted using Quick-DNA™ Fungal/Bacterial Miniprep kit (Zymo Research) following the manufacturer's protocol from vaginal lateral wall swabs as described by (Balle *et al.,* 2020) and used for CT qPCR. A multiplex qPCR assay was set up for this study, using two sets of published primers and probes to detect the plasmid-encoded ORF2 open reading frame of CT and the chromosomal-encoded outer membrane complex B (*omcB*) gene, as described by (Pickett *et al.,* 2005) (Table 2.1). Recombinant standards were prepared by cloning and expressing the CT *omcB* gene and a segment within ORF2 of the CT plasmid (described in Appendix 2.1).

Table 2.1. **Primers and probes used for PCR reactions**

Target	Oligonucleotide	Name	Sequence (5'–3')
pORF2	Forward primer	Ctrachplas11F	CAGCTTGTAGTCCTGCTTGAGAGA
	Reverse primer	Ctrachplas119R	CAAGAGTACATCGGTCAACGAAGA
	Probe	Ctrachplas57T	HEX-CCCCACCATTTTTCCGGAGCGA-BHQ-1
omcB	Forward primer	Ctrach60k127F	GACACCAAAGCGAAAGACAACAC
	Reverse primer	Ctrach60k232R	ACTCATGAACCGGAGCAACCT
	Probe	Ctrach60k159T	FAM-AAGCAAAAAAGCAAGAAAAAACCACAGCAAAGAG-BHQ-1

Because only one copy of the *omcB* gene is found on the chlamydial chromosome, the ratio of the plasmid to *omcB* was considered to equal to the average number of plasmid copies per bacterium. All samples were run in triplicate, and the estimated *omcB* and plasmid quantities are expressed as copies/µl of DNA.

The following conditions were used to process DNA from CT positive samples, using multiplex real-time PCR test to amplify the *omcB* gene and the CT pORF2 gene: A 20µl reaction mixture including forward and reverse primers (300 nM each), probe (100 nM each), and GoTaq Probe Master Mix (Promega, US) was added to two microlitres of the sample (Promega). PCR cycles were performed in a Biorad CFX96 Real-Time System C1000 (USA) Thermal cycler according to the manufacturer's instructions. The *omcB* gene and pORF2 were

diluted 10-fold (range, 10^0-10^9) to create two standard curves. The threshold cycle values for the *omcB* gene and pORF2 were used to calculate the average CT and plasmid loads for each sample, as described by Pickett *et al*., 2005. The geometric median was used as the bacterial load and plasmid load. Estimated quantities of CT bacterial load and plasmid load are expressed as copies/1µl of extracted DNA. The limit of detection was 30 copies/µl of *omcB* gene. Samples determined to have <30 copies/ul of *omcB* gene were assigned a bacterial load that was half the lowest detection limit, as described by (Hornung *et al.,* 1990).

2.2.4 Measurement of cervicovaginal cytokines

A Softcup® menstrual cup was provided to each uCHOOSE participant, who inserted it for 30 mins, to collect undiluted cervicovaginal secretions and returned them to the study investigators. Menstrual cup secretions were weighed using the menstrual cup-mean weight of ten 50 mL tubes containing an unused menstrual cup according to a previously developed protocol for processing menstrual cup, and then stored at -80°C (Jaumdally *et al.,* 2017; Jaumdally *et al.,* 2018). Previously, cervicovaginal cytokine concentrations were determined using the Bio-Plex Human Th17 cytokine Luminex panel (Bio-Rad Laboratories, Hercules, CA) by Konstantinus *et al*., (2020), including IL-1β, IL-6, TNF-α, IL-23, IL-33, IL-17A, IL-17F, IL-21, IL-22, IL-25, IL-31, IFN-γ, and soluble CD40 ligand. For the purposes of this study, cytokines, which have previously been associated with CT infection were selected, including IL-1β, IL-6, IL-17A, TNF-α, IFN-γ and IL-10. A Bio-plex Suspension Array reader was used to collect the data (Dabee *et al.,* 2019). The instructions provided by the manufacturer were followed during the experiments. Lower detection limits ranged from 1–307pg/ml for cytokines measured. 5PL regression lines were used to determine cytokines concentration. For each cytokine, half of the lowest measured concentration for each cytokine was recorded for all values below the detection limit. Ten samples were included in all assay plates to measure interplate variability, with samples being distributed at random on the various assay plates. For each cytokine across all the plates, Spearman correlations between these ten samples were performed in order to ensure that the data were comparable.

2.2.5 Measurement of cervical T cell activation by Flow Cytometry

A Digene cervical cytobrush was used to collect cervical cells from each AGYW, which were processed with in four hours and stained immediately, as previously described and performed

by Kontantinus *et al*. (2020). A violet amine-reactive Live/Dead stain was used to determine cell viability (ViViD; Invitrogen, Carlsbad, CA) and the cells stained with the following antibodies: Pacific Blue- CD14, Pacific Blue-CD19 (both Invitrogen, Carlsbad, CA) APC-H7-CD3, BV605-CCR6, and APC-CCR5 (all from BD Biosciences, San Jose, CA); BV711-CD8,PE-CCR10, and Alexa-700-human leukocyte antigen (HLA) DR (Biolegend, San Diego, CA); PE-Cy5.5-CD4 (Invitrogen, Carlsbad, CA); and PE-Cy7-CD38 (eBioscience, San Diego, CA). Dead cells, CD14+ monocytes and CD19+ B cells were excluded. (Konstantinus *et al.,* 2020). Cells were acquired on a BD Fortessa flow cytometer using BD FACS Diva. For the purposes of this study, data were analysed using FlowJo v9.9.3 (FlowJo, LLC, Ashland, OR). The gating strategy was previously published by Konstantinus *et al*. (2020).

2.2.6 Statistical analysis

For STI prevalence calculation, the proportion of participants with STIs at the screening, crossover (week 16), and exit (week 32) visits was used. The number of new infections per person per year was used to calculate STI incidence rates. Any infection that was present for the first time or had been treated at a previous study visit was defined as a new infection. GraphPad Prism 8 (GraphPad software) was used for analyses. Non-parametric statistical tests were used because CT bacterial load was not normally distributed. Where indicated, CT bacterial load was stratified into low CT load (≤ 454 copies/µl; $<50^{th}$ percentile) and high CT load (> 454 copies/µl; $>50^{th}$ percentile). Statistical significance was calculated using Mann–Whitney U test, with multiple comparison adjustment using the false discovery rate step-down procedure where indicated. Regression analysis was done for CT load and cytokine. Where indicated, regression analysis for plasmid load and cytokine levels was adjusted for CT chromosomal load.

2.3 Results

Samples from 130 AGYW were available for this study, from the randomized cross-over uCHOOSE study that took place between 2015 and 2017 at one of the DTHF Clinical Research Sites in Cape Town (Balle *et al.,* 2020; Gill *et al.,* 2020). Table 2.2 summarizes the socio-behavioral demographics of the cohort. Overall, the median age of the AGYW was 17 years (IQR 16-18). Their median reported age of sexual debut was 15 years (IQR 14-16), 21% (27/130) reported having relationships with partners >5-years older than them, and 9.2% (12/130) reported having had several relationships since sexual debut. Almost half of the young women (64/130) reported that they had not used a condom during their most recent sex act. Alcohol use was reported by 61.5% (80/130) of young women.

Prior to enrollment, most of the AGYW used injectable hormonal contraceptives (88/130; 67.7%) (Table 2.2). Very few of the young women had never used hormonal contraception prior to the study (5/130), while 20% (26/130) had not used hormonal contraceptives recently (for three months before enrollment). Thirteen percent (17/130) of the young women had been pregnant.

BV was common in the cohort, being observed in 42.3% of women (55/130; Nugent 7-10) (Table 2.2). Similarly, the overall STI prevalence of the cohort at baseline was 43.1% (56/130), with CT being the most prevalent (33.1%; 43/130), followed by NG and TV (both 10%; 13/130). Of the 130 women enrolled, 107/130 (82.3%) returned for the second study (4 months later) and 92/130 (70.8%) completed the third study visit at 8 months. The prevalence of CT remained high throughout the study: 15% (16/107) of AGYW were CT+ at visit 2 and 19% (17/92) were CT+ at their third study visit. All CT infections were asymptomatic. All women that were diagnosed with an STI or BV Nugent 7-10 were offered appropriate antibiotic treatment. Partner testing and treatment were offered to women diagnosed with CT or NG, but uptake was poor (Balle *et al.,* 2020).

Balle *et al*. (2020) previously evaluated STI or BV prevalence by study arm, showing that the injectable contraceptive Net-En may be associated with increased risk of MG compared with oestrogen-containing contraceptives but not with overall STI risk. Furthermore, they reported that COC use decreased STI risk relative to the vaginal ring. CT prevalence was similar across HC arms, being 4/35 (11.4%) in those randomized to Net-En, 4/37 (10.8%) in those

randomized to COC, and 8/35 (22.9%) in those randomized to CCVR (chi (χ) square test, P= 0.278).

Table 2.2 **uCHOOSE cohort baseline characteristics**

Characteristic	
Number	**Overall (n=130)**
Age (years; median; IQR)	17 (16-18)
Age of sexual debut (years; median; IQR)	15 (14-16)
Multiple sexual partners (n/N; %)	12/130 (9.2%)
Relationships with partners >5 years older (n/N; %)	27/130 (21%)
Ever been pregnant (n/N; %)	17/130 (13%)
Alcohol use (n/N; %)	80/130 (61.6%)
Condom use at last sex act (n/N; %)	66/130 (51%)
Condom use always (n/N; %)	65/130 (50%)
Condom use sometimes (n/N; %)	64/130 (49%)
Hormonal contraceptive use prior to study:	
None (n/N; %)	5/130 (3.8%)
Injectable (NET-EN) (n/N; %)	88/130 (67.7%)
COC (n/N; %)	6/130 (4.6%)
Implant (n/N; %)	3/130 (2.3%)
Vaginal pH (median; IQR)	4.8 (4.5–5.2)
STI prevalence (n/N; %):	56/130 (43.1%)
CT (n/N; %)	43/130 (33.1%)
NG (n/N; %)	13/130 (10.0%)
MG (n/N; %)	3/130 (2.3%)
TV (n/N; %)	13/130 (10.0%)
BV status (Nugent score)	
Nugent 0–3 (n/N; %)	61/130 (46.9%)
Nugent 4–6 (n/N; %)	14/130 (10.8%)
Nugent 7–10 (n/N; %)	55/130 (42.3%)

2.3.1 CT bacterial load and plasmid copy number in AGYW

Next, CT bacterial loads in CT+ AGYW were determined by quantifying *omcB* gene copy in 130 women with 220 swabs taken from three visits. Estimated quantities of CT bacterial load and plasmid load are expressed as copies per 1µl of DNA (Figure 2.2). We confirmed 51 CT+ cases in 43 different women based on presence of the plasmid (> 30 DNA copies). This included 28 cases from visit 1, 9 cases from visit 2 and 14 cases from visit 3. From the 51 CT+ cases, the *omcB* gene could be amplified from 41/51 samples, with 10/51 samples having <30 copies detected (Figure 2.2A). The median CT bacterial load (*omcB* copy number) was 454 copies/µl DNA (IQR 51,5-1349). All samples above the median were classified as having high bacterial load and those below or equal to the median were classified as having low bacterial

load. *OmcB* copy numbers were similar in CT cases from the first, second, and third visits (Figure 2.2A).

In mice, lower *Chlamydia* bacterial loads were observed in animals previously infected with *C. muridarium*, suggesting that mice developed partial immunity to subsequent infections (Carey *et al.*, 2009). In humans, CT prevalence is higher in younger than older women (Simons *et al.*, 2021). Based on these data, it was hypothesized that older adolescents would have lower CT bacterial loads than young women, because of partial immunity to CT. CT bacterial loads were similar in younger and older adolescents (median 541 [IQR 61-2134] copies versus 406 copies [45-454], respectively; P=0.5), although a higher number of younger than older adolescents were infected (30/83 in younger cohort versus 13/47 cases in the older cohort, respectively; Figure 2.2B).

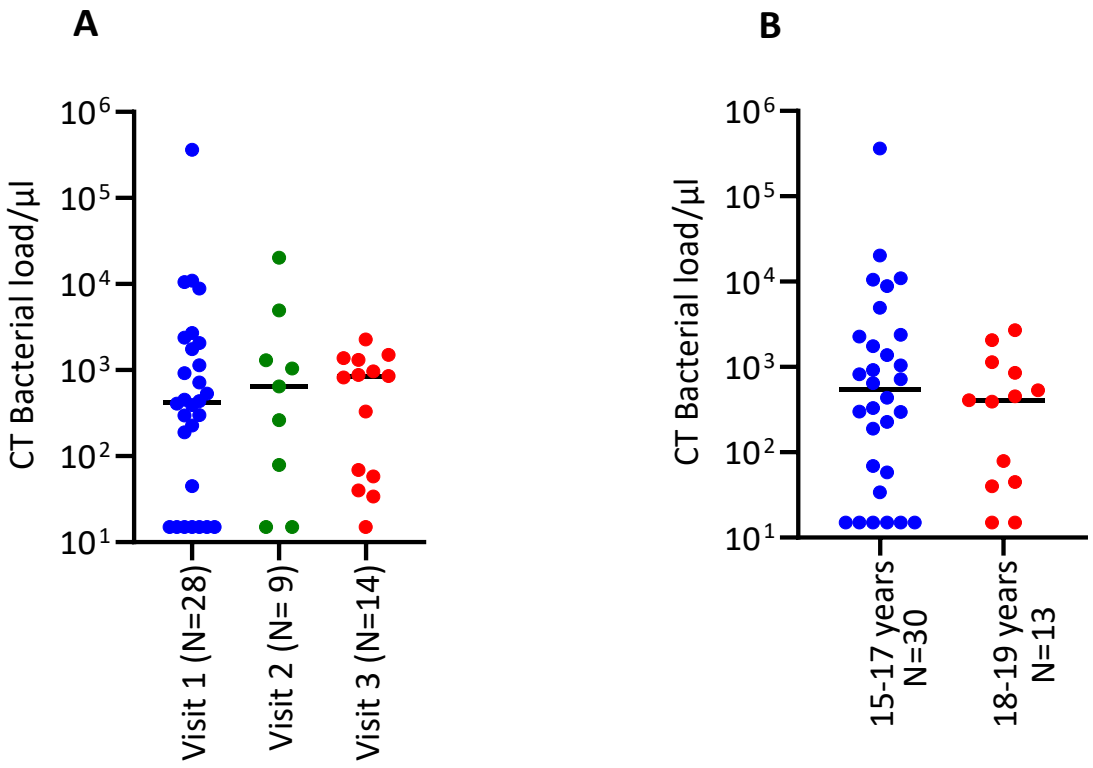

Figure 2.2. **Temporal changes (A) and impact of age (B) in CT bacterial load (inferred from *omcB* copy numbers) in CT+ AGYW.** (A) Blue dots indicate samples positive for CT at visit 1, green dots indicated samples positive for CT at visit 2, and red dots indicated samples positive for CT at visit 3. The horizontal black lines indicate the median. (B) Younger adolescents (15-17 years) are shown by blue dots and older adolescents (18-19 years) are shown by red dots. Differences between groups were evaluated using the Mann-Whitney U test. P-values <0.05 were considered significant. Horizontal black lines indicate the median.

Seven of the 43 young women from which CT *omcB* could be amplified at baseline also had CT+ samples that could be amplified at other study visits (Figure 2.3). Of these, four young women had similar CT bacterial loads at the first and second time points (UC045, UC087, UC155, UC167) while three women had lower bacterial loads at the subsequent visit (UC008, UC019, UC042).

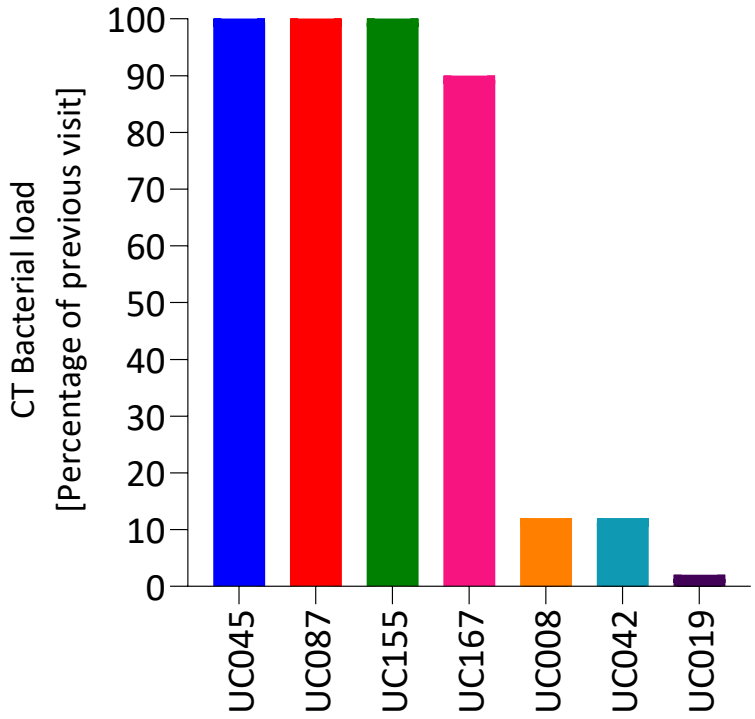

Figure 2.3. **CT bacterial load in young women who were infected at multiple time points**. Data is shown as a ratio of CT bacterial load at [subsequent visit]/[first visit].

2.3.2 Plasmid copy number in CT

CT plasmid copy numbers per cell were calculated by adjusting plasmid DNA for CT bacterial DNA load. Forty-three AGYW infected with CT (CT+ cases) were included in this analysis. The median plasmid copy number for this cohort was 9 plasmids per CT (IQR, 7-13) (Figure 2.4A). Plasmid copy numbers per *Chlamydia* cell were similar in women with high (>50th percentile) and low CT bacterial loads (<50th percentile; Figure 2.4B).

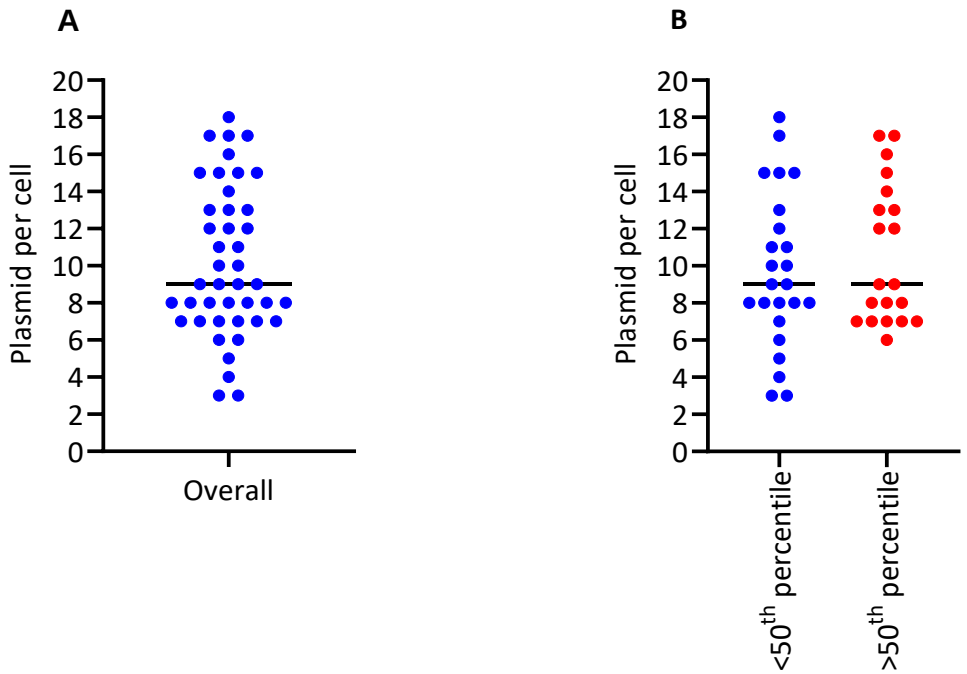

Figure 2.4. **Plasmid copy number in CT cell**. (A) Number of CT plasmid found per CT cell and (B) CT plasmid copy number in CT cases with low CT bacterial load (<50th percentile) versus high CT bacterial load (>50th percentile). Blue dots indicate cases with low bacterial load and red indicates cases with high bacterial load. Statistical significance was calculated using Mann-Whitney U test. The horizontal black lines indicate median. P<0.05 were considered significant.

2.3.3 Impact of CT Infection and bacterial load on cervicovaginal cytokine responses

It was hypothesised that CT infection triggers inflammatory cytokine production, particularly focusing on IL-1β, IL-6, IL-17A, IFN-γ and TNF-α. Cervicovaginal concentrations of these cytokines were compared in women infected with CT (cases) compared to those not infected (controls; Table 2.3). Adolescents infected with CT had significantly higher concentrations of IL-17A and IFN-γ than those with no CT infection. This analysis included IL-10 as a marker of an anti-inflammatory or regulatory host response (Ouyang *et al.*, 2011; Xiang *et al.*, 2021), moderating inflammation at mucosal sites. In this study, cervicovaginal IL-10 concentrations did not differ in CT– and CT+ women. Concentrations of IL-17A (adjusted P=0.0258; Mann-Whitney U test) and IFN-γ (adjusted P=0.0399; Mann-Whitney U test) there significantly higher CT+ cases compared to controls.

Table 2.3. **Comparison of cervicovaginal cytokine concentrations in AGYW with and without CT infections**

Cytokine	**CT cases (pos)** Median pg/ml (IQR)	**CT controls (neg)** Median pg/ml (IQR)	**P-value[a]** (adjusted)
IL-1β	14.2 (IQR,1.6-145.6)	8.2 (IQR,1.0-39.7)	0.2532
IL-6	1.3 (IQR,0.4-7.5)	0.9 (IQR,0.3-4.2)	0.3575
IL-10	0.01 (IQR,0.01-1.4)	0.1 (IQR,0.01-0.8)	0.3179
IL-17A	2.3 (IQR,1.1-5.3)	1.2 (IQR,0.7-2.4)	0.0258*
IFN-γ	2.7 (IQR,1.4-7.1)	1.9 (IQR,0.9-3.5)	0.0399*
TNF-α	1.0 (IQR,0.1-6.6)	0.5 (IQR,0.1-2.5)	0.2962

[a] Statistical significance was calculated using a Mann-Whitney U test. *p<0.05 significance.

Since bacterial load may indicate degree of CT pathogenesis, cytokine concentrations were compared against CT bacterial loads in cases (Figure 2.5). IL-17A (R^2=0.39; P=0.004), I IFN-γ (R^2=0.35; P=0.01), L-1β (R^2=0.34; P=0.01), and IL-6 concentrations (R^2=0.34; P=0.02), positively correlating significantly with CT bacterial loads, with TNF-α tending to correlate although not significantly (R^2=0.24; P=0.09). For IL-10, however, no association was found between bacterial load versus IL-10 concentrations, although cervicovaginal IL-10 concentrations were generally low in this cohort.

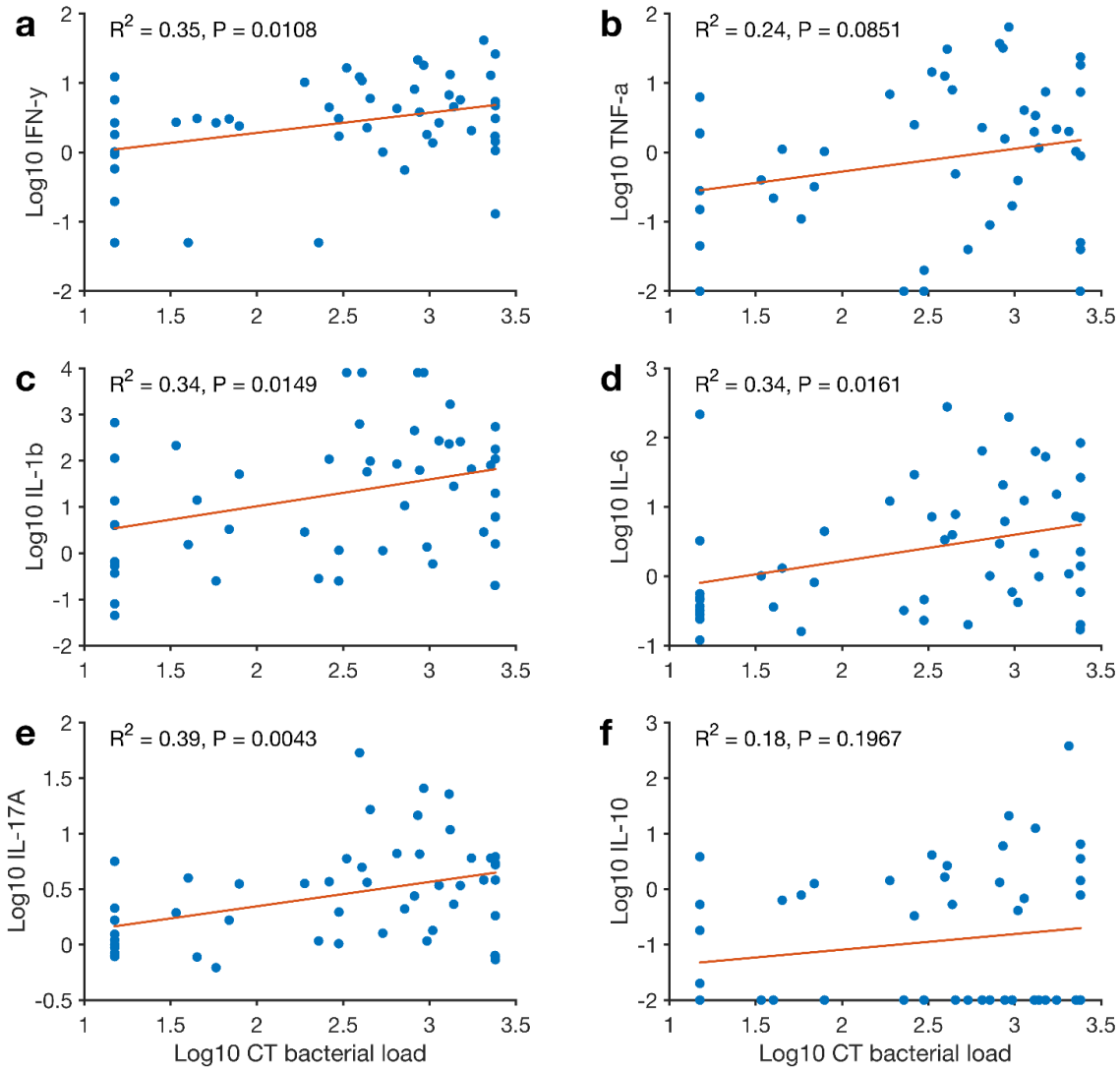

Figure 2.5. **Correlation of proinflammatory cytokines** (A). IFN-γ and CT bacterial load, (B). TNF-α and CT bacterial load, (C). IL-1β and CT bacterial load (D). IL-6 and CT bacterial load, (E). IL-17A and CT bacterial load (F) IL-10 and CT bacterial load. Blue dots represent CT positive samples.

Analyzing this data slightly differently, all inflammatory cytokine concentrations tended to be higher in CT cases with high bacterial loads than in those with lower bacterial loads (Figure 2.6). IL-1β concentrations were significantly elevated in the high CT bacterial load group than healthy controls, with median concentrations being 52-fold higher than controls (adjusted P=0.0003; Mann-Whitney U test) (Figure 2.6A). IL-1β concentrations in the low CT bacterial load group were also higher than in the controls, although this increase was only 5-fold (adjusted P=0.0462; Mann-Whitney U test). Similarly, AGYW in the high CT bacterial load group had significantly elevated IL-6 compared to controls, with concentrations being 5.2-fold higher than controls (adjusted P=0.0006; Mann-Whitney U test) (Figure 2.6B). The low CT

bacterial load group was only moderately elevated IL-6 compared to controls, although this was not significant.

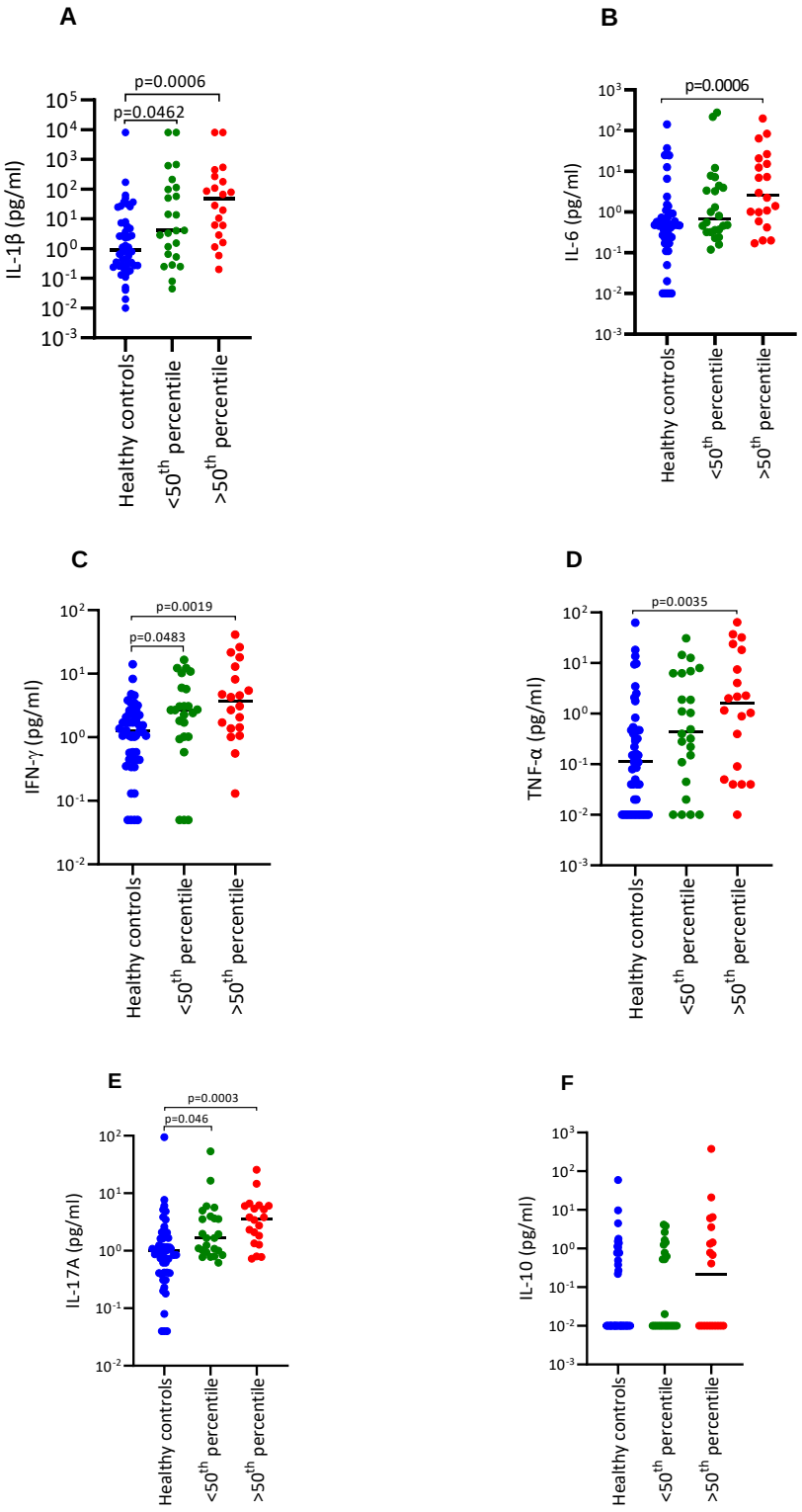

Figure 2.6. **Impact of CT bacterial load on cervicovaginal cytokine responses**: (A) IL-1β, (B) IL-6, (C) IFN-γ, (D) TNF-α, (E) IL-17A, and (F) IL-10. All CT+ categories were compared with CT– controls (blue dots). CT bacterial load was stratified into low (<50th percentile; green dots) or high (>50th percentile; red dots). Statistical significance was calculated using Mann-Whitney U test. Horizontal black line indicates median. P-values <0.05 were considered significant, and only significant p-values are shown.

Compared to CT– controls, IFN-γ concentrations were significantly elevated in CT+ cases with both high bacterial loads (Figure 2.6C; adjusted P=0.0019; Mann-Whitney U test) and low bacterial loads (P=0.0462; Mann-Whitney U test). Similarly, TNF-α was significantly elevated in CT+ cases with high bacterial loads compared to CT– controls (adjusted P=0.0035; Mann-Whitney U test) and tended to be increased in AGYW with low CT bacterial load group compared to the CT– controls (adjusted P=0.0668; Mann-Whitney U test), although this was not significant. Compared to controls, IL-17A was significantly increased in cervicovaginal secretions from CT+ cases with both high (adjusted P=0.0003; Mann-Whitney U test] and low bacterial loads [adjusted P=0.0046; Mann-Whitney U test) (Figure 2.6E). Cervicovaginal IL-10 concentrations were similar in CT cases and controls, irrespective of bacterial load (Figure 2.6F).

As mentioned previously, CT bacterial burden was measured in 7/43 CT+ women at more than one time point. To investigate whether repeat or persistent CT infections were associated with higher levels of inflammation, we compared cytokine concentrations in matched samples from AGYW over time (Figure 2.7). Of the seven CT cases with persistent or repeat infections, four had similar CT bacterial loads at two time points (UC045, UC087, UC155, UC167) and three had a lower bacterial load at subsequent visit (UC008, UC019, UC042) (previously shown in Figure 2.3). Of these, two (UC155 and UC167) generally had lower cytokine concentrations at the second visit compared to first visit. The other two AGYW (UC045 and UC087). IL-1β concentrations were notably decreased from first time point to second time point for UC155 and UC167. However, we had only seven women which is not enough to conclude the association of repeat or persistent CT infections with higher levels of inflammation.

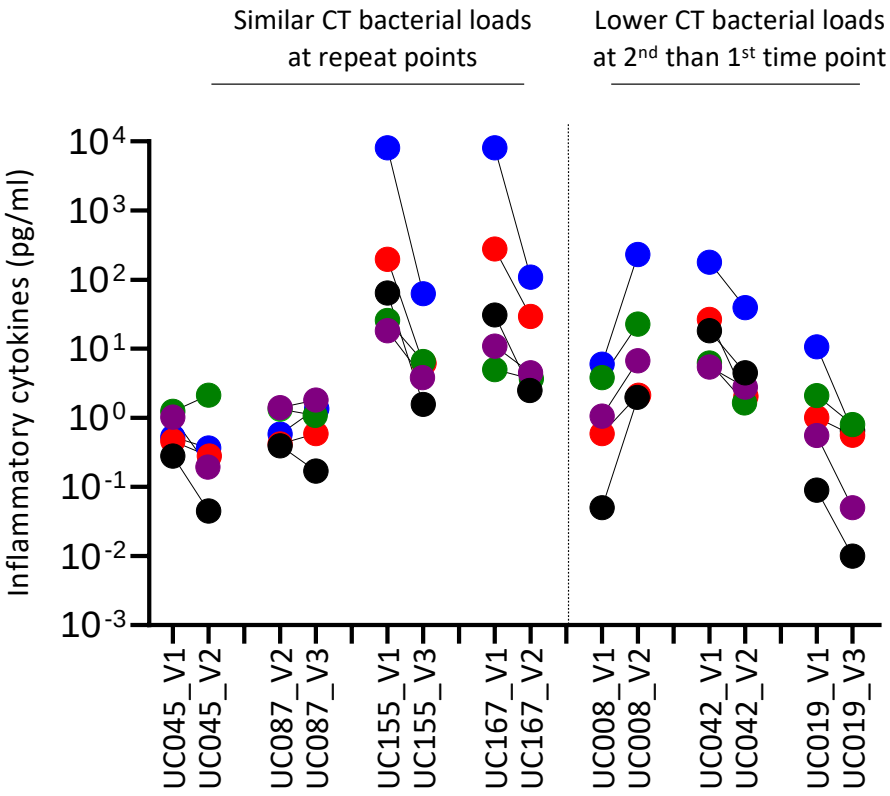

Figure 2.7. **Inflammatory cytokines in young women who were CT-infected at multiple time points**. Blue dots indicate IL-1β, red dot indicate IL-6, green dot indicate IL-17A, purple dot indicate IFN-γ and black dot indicate TNF-α.

2.3.4 Relationship between CT plasmid number and cervicovaginal cytokine concentrations

It was hypothesised that increased plasmid copy number per CT would be associated with more genital inflammatory cytokine production, because the chlamydial plasmids have been suggested in the mouse model to be pathogenesis determinant. To test this, cytokine concentrations were compared in CT+ cases with high or low plasmid copy numbers (Table 2.4). Interestingly, cytokine concentrations were generally lower in women infected with CT with high plasmid copy numbers than those with low copy numbers, although not significantly.

Table 2.4. **Relationship between cervicovaginal cytokines and plasmid copy number**

Cytokine	Cytokine concentrations (median; IQR; pg/ml in CVS)		P-value[a] (adjusted)
	High plasmid copy number (>50th percentile*)	Low plasmid copy number (<50th percentile*)	
IL-1β	5.0 (IQR,1.0-64,5)	66.1 (IQR,3.5-582.6)	0.1292
IL-6	0.7 (IQR,0.4-1.6)	4.4 (IQR,0.9-18.0)	0.1116
IL-10	0.01 (IQR,0.01-0,6)	0.6 (IQR,0.01-3.1)	0.1281
IL-17A	2.0 (IQR,1.1-3,7)	3.6 (IQR,1.1-6.0)	0.3246
IFN-γ	2.7 (IQR,1.3-3.4)	4.7 (IQR,1.4-12.2)	0.1072
TNF-α	0.4 (IQR,0.04-2,0)	2.2 (IQR,0.9-13.6)	0.0597

[a] Mann-Whitney U test.
*Median CT plasmid copy number was 9 plasmids per bacterial cell.

2.3.5 Impact of CT bacterial load on genital tract CD4^{+} T-cell activation

T-cell activation (CD38) and HIV co-receptor expression (CCR5) were measured in cervical cytobrush-derived T-cells collected from a subset of the adolescent women enrolled in this study, as described by Konstantinus *et al*., (2020). It was hypothesised here that higher CT bacterial loads would be associated with more cervical T-cell activation, and higher expression of the HIV co-receptor CCR5. CT infection or CT bacterial load did not appear to influence the proportion of T-cells expressing the HIV co-receptor on CD4 T-cells (Figure 2.8A). Similarly, there were no significant difference in expression of CD4 T-cells expressing the activation marker CD38 (Figure 2.8B).

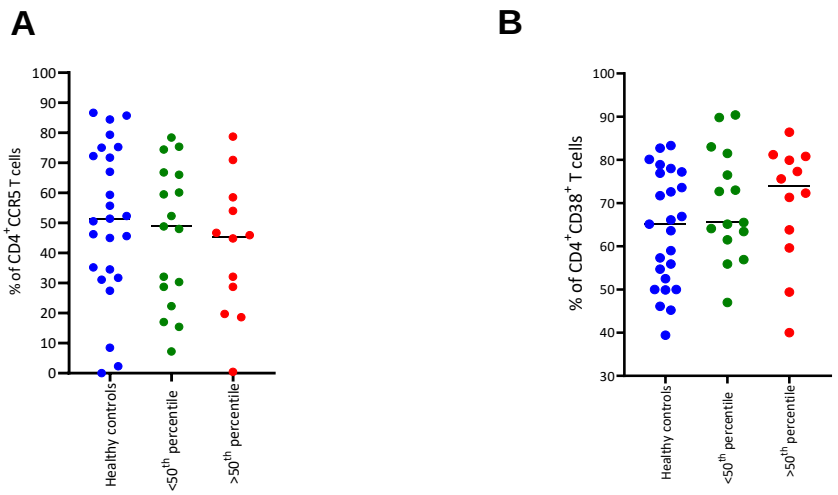

Figure 2.8. **(A) The frequency of CD4$^+$CCR5 T-cells and (B) CD4$^+$CD38$^+$ T-cells stratified by CT bacterial load.** Blue dots represent healthy controls, green dots represent low CT load group and red dots indicates high CT load group. Statistical significance was calculated using a Mann-Whitney U test. The horizontal black lines indicate medians.

2.3.6 CT plasmid copy number and CD4$^+$ T-cell activation or CCR5 expression

There was no significant difference in CD4$^+$ T-cell expression of CCR5 or CD38 in CT cases with either low or high plasmid copy numbers (Figure 2.9A and B).

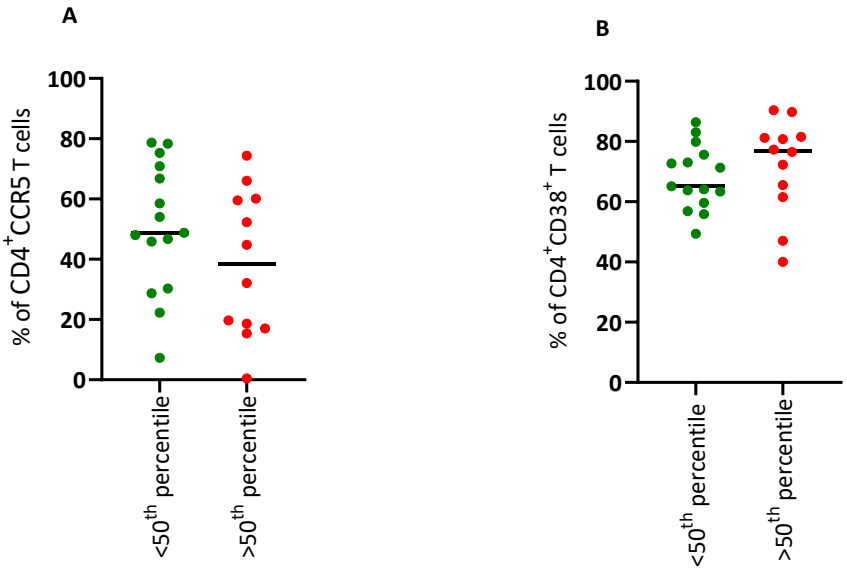

Figure 2.9. (A) **The frequency of CD4$^+$CCR5 and CT plasmid load**, (B) CD4$^+$CD38$^+$ and CT plasmid load**.** Green dots represent low plasmid load group and red dots indicates high plasmid load group. Statistical significance was calculated using Mann-Whitney U test. Horizontal black line indicates median.

2.4 Discussion

In this study, CT prevalence was confirmed to be high overall in AGYW, with many young women being infected at multiple time points, despite treatment. CT bacterial burden was stable over time in these adolescents. Cervicovaginal concentrations of IL-1β, IL-6, TNF-α, IFN-γ, and IL-17A were found to be higher in CT cases than uninfected controls, which was higher in cases with higher CT bacterial loads. In those who were infected with CT for >1 study visit, four cases had similar bacterial loads at follow-up visits while the other three cases had lower *omcB* copy numbers, possibly providing evidence of subsequent immune control, if not protection from CT infection. However, inflammatory cytokine profiles did not differ significantly in those who had similar bacterial loads at subsequent visits versus those that had lower bacterial loads. Plasmid copy number per CT cell varied considerably, ranging from 3 to 18 plasmids per cell. No cases of CT were noted that did not have a plasmid present. Despite mouse studies suggesting that plasmids may contain pathogenesis determinants, no significant difference in cytokine concentrations was noted in CT cases with high versus low plasmid copy numbers. Neither CT bacterial load nor plasmid copy number appeared to influence cervical CD4 T-cell activation or expression of CCR5.

All the AGYW infected with CT in this study were clinically asymptomatic, despite huge variation in CT bacterial loads. It seems plausible to argue therefore that these clinically asymptomatic CT infections in these young women may allude to difficulties in clinically detecting CT infections in women, without more sensitive NAATs. Others have confirmed that clinical symptoms of CT infection are more difficult to detect in women than men (Vodstrcil *et al.,* 2015), possibly because of the target site of CT infection in women is the upper rather than lower genital tract (Russell *et al.,* 2016a; Brunham *et al.,* 2020). Alternatively, absence of clinically evident symptoms may suggest that these AGYW tolerate persistently or intermittently high levels of bacteria without the development of significant disease. Previous studies in South Africa estimated that ~50% of CT infections in women are not clinically evident and therefore not treated, despite being associated with significantly increased cervicovaginal inflammatory cytokine concentrations that may increase their risk for other infections, including HIV (Mlisana *et al.,* 2012; Masson *et al.,* 2014; Barnabas *et al.,* 2018). While this study did not have a group of women with symptoms of CT as a comparator group, Jalal *et al*. (2011) suggested that higher CT bacterial loads [All women with cervicitis had a CT bacterial load of >1950 copies/µl, Jalal *et al*.(2011) used 10µl DNA sample in 25µl PCR

reaction and 1µl DNA sample in 25 µl PCR reaction was used in this study and seven women with cervicitis in Jalal *et al*. (2011) study had a CT load >1.0x 10^5 copies/µl] significantly increased the likelihood of clinical signs and symptoms of CT being evident (which included vaginal discharge, dysuria, pelvic discomfort/pain, post-coital or intermenstrual bleeding). In the study by (Jalal *et al.,* 2011), the median CT bacterial load determined was 316 copies/µl, which was slightly below the median bacterial load in this study. A multivariable analysis of these samples revealed a significant correlation between CT bacterial load and clinical features (P value=0.02) (Jalal *et al.,* 2011).

In adolescents who were infected with CT for more than one visit, some had bacterial loads of similar magnitude at their second CT positive visit, while others had lower bacterial loads. Low CT bacterial loads may reduce risk of transmission to sexual partners (Wiggins *et al.,* 2009; Dirks *et al.,* 2015; van Liere *et al.,* 2019) and adverse outcomes (Wiggins *et al.,* 2009; van Liere *et al.,* 2019). While lower CT bacterial loads at subsequent visits could demonstrate acquired immunity to CT, young women were not tested for cure following antibiotic treatment so unresolved infections cannot be excluded. Two other studies have reported that CT bacterial burden tended to be lower for recurring infections, although the duration of follow up was longer in these studies as was the number of participants higher (Gomes *et al.,* 2006; Walker *et al.,* 2012). Gomes *et al*. (2006) included 91 Portuguese women (aged 16–67) attending STD, family planning and general practice clinics in over a three-year period, while Walker *et al*. (2012) included 55 Australian women infected with CT followed for a 12-months period. Adaptive immune responses in humans appear to develop slowly, with robust immunity against CT only being noted after multiple exposures to CT, over several months (Katz *et al.,* 1987; Molano *et al.,* 2005). Gupta *et al*. (2018) investigated factors influencing CT bacterial loads in women with repeat infections for 6 months, including 37 women (>16 years) who were predominantly African American. They found a significant decrease in the CT bacterial loads over time in women with repeat infections. Another study that included young women from the USA (aged 14 to 19 years) found that those who had with a history of CT infection had lower bacterial loads than those with no history of infection, suggesting protective effects of acquired immunity (Batteiger *et al.,* 2010a). Previous histories of CT infections were not available for this cohort, so it is not possible to determine whether CT infections within this study were primary or repeat exposures. In this subset of AGYW in this study, it is unknown whether the chronic CT infections may affect the course of the disease and pathologies (like

PID, tubal infertility, and risk for ectopic pregnancies (Stephens, 2003), because inflammatory cytokine responses were evident in those infected with CT that persisted.

The balance between protective versus disease-enhancing immune responses to CT infection in the female genital tract has not been fully characterized, partly due to the difficulties associated with accessing sites of infection in women (like the upper reproductive tract). However, evidence from animal models of *Chlamydia* infection have been useful for dissecting the specific types of immune responses important for chlamydial protective immunity, with CD4 T-cells and IFN-γ appearing to be correlates of protection (Yu *et al.,* 2012). Innate immune responses following *Chlamydia* infection have been shown to activate or recruit adaptive immune cells to the site of infection, that are important in clearing infection and eliminating the pathogen and often depend on the production of pro-inflammatory cytokines (Mpiga *et al.,* 2006; Faris *et al.,* 2019). In this study, genital CT infection in young women was associated with higher cervicovaginal concentrations of IL-1β, IL-6, IL-17A, IFN-γ and TNF-α compared to the healthy controls, particularly in the CT cases with the highest CT bacterial loads.

IL-1β has been shown to be produced in response to different stimuli (including microbes and cytokines) predominantly by monocytes and macrophages, but also by other non-immune cells [eg. endothelial cells and epidermal cells; (Stylianou and Saklatvala, 1998)]. IL-1β induces a wide spectrum of inflammatory cytokines and chemokines, such as IL-6 and IL-8 to assist host defence (Hvid *et al.,* 2007; Shimada *et al.,* 2011). IL-1β has also been suggested to be an important contributing factor of inflammation at other mucosal sites, leading to mucosal barrier damage, for example increasing permeability of intestinal tight junctions in the gut (Kaminsky *et al.,* 2021) which increased intestinal penetration of luminal antigens (Rawat *et al.,* 2020). In the context of *Chlamydia* infections, previous studies have reported significantly higher amounts of IL-1β being produced by alveolar macrophages and PBMCs from COPD patients with *C. pneumoniae* infection than from COPD patients without *C. pneumoniae* infection (Rupp *et al.,* 2003). Similar to this study, during clinical CT infection, studies have reported that CT+ women have elevated cervicovaginal concentrations of IL-1β (Marconi *et al.,* 2014). An interpretation of the elevated IL-1β concentrations in AGYW with CT infections in this study could be that this potent inflammatory cytokine may disrupt the epithelial barrier integrity in the cervicovaginal area, allowing easy access to macrophages and dendritic cells residing

below the mucosal barrier, which would further cause amplified immune activation and disease progression.

IFN-γ, a Type II interferon, is known to regulate the immune system, being recognized to have anti-bacterial, anti-parasitic, and anti-tumour properties, particularly for intracellular organisms like CT (Schroder *et al.,* 2004; Kak *et al.,* 2018). It is produced by macrophages, natural killer cells and neutrophils in response to infections (Schroder *et al.,* 2004; Kak *et al.,* 2018; Zhu 2018). In fact, IFN-γ produced by $CD4^+$ T-cells has been described as a single most important factor in host defence against *Chlamydia infection* (Rank *et al.,* 1992). *In vitro* evidence showed that IFN-γ concentrations were elevated in cell culture, and in cervical secretions in women with CT infection, as has been demonstrated in the AGYW in this study (Williams *et al.,* 1988a; Cohen *et al.,* 2000; Morrison, 2000). Female sex workers from Kenya, who exhibited increased resistance to CT infection, had blood-derived $CD4^+$ T-cells that secreted IFN-γ (Cohen *et al.,* 2005). Beatty *et al*. (1994) showed that women with higher CT bacterial loads produced IFN-γ, and that this was able to inhibit CT growth by activating the enzyme indolamine-2,3-dioxygenase, which was shown to catabolize tryptophan to kynurenine, in so doing depleting cytosolic tryptophan which CT needs as an essential growth factor. To counteract this, tryptophan synthase, which converts indole into tryptophan, was shown to be upregulated by urogenital CT strains in response to tryptophan depletion, to facilitate *de novo* synthesis of tryptophan (Caldwell *et al.,* 2003). While IFN-γ has been shown to accelerate *Chlamydia* clearance and reduce severity of CT-associated lesions, higher concentrations of IFN-γ paradoxically can cause excessive inflammatory reactions that contribute to infectious sequelae (Ishihara *et al.,* 2005; Mascellino *et al.,* 2011). Studies have suggested that the CT bacterial load, possibly associated with stage of infection, are responsible for these contrasting roles of IFN-γ in CT-induced immunity versus pathogenesis (Rottenberg *et al.,* 2002; Zhang and Starnbach, 2015). The finding in this study that a stronger IFN-γ response in adolescents in those with highest CT load may result in excessive inflammatory response and lowest CT bacterial loads suggest that this potent Type II interferon may be inhibiting normal metabolism and replication of CT. In future, it would be interesting to evaluate how cervicovaginal IFN-γ affected the availability of essential nutrients for CT growth.

In this study, cervicovaginal IL-17A concentrations in AGYW were shown to correlate in a dose dependent manner with CT bacterial loads, suggesting a proportionate role for this cytokine in response to increasing bacterial burden. Others have also confirmed that IL-17 is detected in inflammatory tissues of patients infected with *Chlamydia* (Rizzo *et al.,* 2011; Hakimi *et al.,* 2014a). IL-17A is an important mucosal cytokine, that activates macrophages to produce cytokines as well as activating epithelial cell innate immune responses (Veldhoen, 2017). It is produced by Th17 $CD4^+$ T-cells (Cicala *et al.,* 2009; Chege *et al.,* 2012), which express high concentrations the HIV co-receptors CCR5, are abundant in reproductive mucosal tissues, and highly susceptible to HIV infection (Rodriguez-Garcia *et al.,* 2014), which may have implications for the HIV risk of AGYW in this study. During primary HIV infection, previous studies have also shown that Th17 CD4 T-cells were depleted confirming their role in initial HIV infection (Zhang *et al.,* 2004; Chege *et al.,* 2012; Boily-Larouche *et al.,* 2017).

Interestingly, IL-17A is known to synergise with IFN-γ to inhibit intracellular CT by enhancing intracellular inducible nitric oxide synthase expression, which has cytotoxic effects on CT infection (Zhang *et al.,* 2012). Previous research has demonstrated that IL-17A is protective against extracellular pathogens but the role of IL-17A against intracellular pathogens, including *Chlamydia*, has not yet been determined (Bagri *et al.,* 2022). Knockout mouse studies have confirmed that *C. muridarum* bacterial loads in the lungs of infected mice were higher in the absence of IL-17A, and that their survival decreased (Zhang *et al.,* 2009). IL-17 has also been shown to synergize with other cytokines such as IL-6 and macrophage inflammatory protein-2 (MIP-2) in the early stages of infection, amplifying inflammation and initiating host defence against *Chlamydia* (Zhang *et al.,* 2010). It has also been reported that excessive production of IL-17 contributes to other human inflammatory and autoimmune diseases, including psoriasis, arthritis, Sjogren's disease, and inflammatory bowel disease (Moschen *et al.,* 2019; Zwicky *et al.,* 2020; Hwang *et al.,* 2021; Schmitt *et al.,* 2021; Schnell *et al.,* 2021), all of which are diseases associated with compromised epithelial barrier function (Zhou *et al.,* 2022; Kotla and Rochev 2023).

In this study, cervicovaginal TNF-α was elevated in adolescents with high CT bacterial loads compared to uninfected controls, which reflects potent host inflammatory responses ongoing in the lower reproductive tract to this pathogen. TNF-α is the major cytokine produced by macrophages in response to inflammatory stimuli and is well known for its killing effect against

intracellular microbes (Kamalakaran *et al.,* 2013; Xiang *et al.,* 2021). Previously, secretion of TNF-α by CT infected women has been associated with severe inflammation and tissue damage in the female genital tract (Zhao *et al.,* 2015; Shen *et al.,* 2019), with TNF-α being causally linked to histopathological damage caused by CT (Igietseme *et al.,* 2015). Previous studies have also demonstrated that *Chlamydia* and its inflammatory antigens (like LPS), induce TNF-α that can be detected in various host fluids, including serum and bronchial lavage fluid in patients infected with *C. pneumoniae*, and in vaginal secretions of patients infected with CT, (Kaukoranta-Tolvanen *et al.,* 1996; El-Asrar *et al.,* 1998; Kragsbjerg *et al.,* 1998). In women with CT lesions, TNF-α has been implicated in the formation of the lesions (Xiang *et al.,* 2021). Furthermore, TNF-α induces expression of IL-6 and IL-8, which have also been implicated in CT-associated tissue fibrosis and scar tissue formation (Zhao *et al.,* 2015; Shen *et al.,* 2019). TNF-α is also known to accelerate the release of collagenase, by stimulating the proliferation of tissue fibroblasts, which further contributes to tissue damage (Igietseme *et al.,* 2015).

In this study, activation and CCR5 expression of cervical cytobrush-derived CD4 T-cells was evaluated, as previously described by (Konstantinus *et al.,* 2020). However, frequencies of cervical $CD4^+$ T-cells expressing either CD38 or CCR5 did not differ in CT cases compared to controls. $CD4^+$ T-cells have been shown to be necessary for *Chlamydia* clearance in mice, as well as providing protection against reinfection (Su *et al.,* 1995; Gondek *et al.,* 2012; Russell *et al.,* 2016b; Bakshi *et al.,* 2018).

CT infection has previously been associated with increased expression of several chemokines that bind to the CCR5 receptor (eg. regulated on activation, normal T-cell expressed and secreted [RANTES], interferon-inducible protein 10 [IP-10], macrophage inflammatory protein 1α [MIP-1α], MIP-2α, and GRO1 oncogene [GROα]) (Belay *et al.,* 2002; Ficarra *et al.,* 2008; Olive *et al.,* 2011; Menon *et al.,* 2016a; Poston *et al.,* 2019). Previous studies have also shown that higher concentrations of these CCR5-binding chemokines lead to CCR5 receptor internalization, which lowers cell surface expression of the cognate receptor (Lederman *et al.,* 2006; Barmania and Pepper, 2013). Although CCR5 binding chemokines were not evaluated in this study, this may have confounded the analysis of CCR5 receptor expression in this study, as a proxy for HIV susceptibility of cervical CD4 T-cells. Alternatively, it is also possible that CT, being an intracellular pathogen, might be avoiding immune detection by hiding from or interfering with HLA class II antigen presentation processes, to avoid CD4 T-cell immune detection (Wong *et al.,* 2019).

This study was embedded in a randomized cross-over study that evaluated the preference for different hormonal contraceptives in AGYW (uCHOOSE), and the impact of these on mucosal inflammation and vaginal microbiota (Balle *et al.,* 2018; Balle *et al.,* 2020; Gill *et al.,* 2020; Konstantinus *et al.,* 2020). However, the parent study was not powered to detect differences in STI or BV incidence, and the CT cases included in this study did not differ in terms of prevalence or bacterial load between uCHOOSE study arms (injectable Net-EN, COC, and vaginal ring).

In the CT+ cases in this cohort, CT plasmid numbers per cell ranged from 3 to 18, with a median of 9 plasmids per cell, which was broader than previously reported: Dirks *et al*. (2021) reported plasmid copy number ranging from 1-11 plasmids per cell (in a study of 42 vaginal swabs from 42 women), with a median of 6; Last *et al*. (2014) reported 1-18 plasmids per bacterium in clinical trachoma samples; CT lab-adapted strains had plasmid numbers ranged from 1-10 per cell, which were stable over time in sub-culture (Palmer and Falkow 1986; Pickett *et al.,* 2005; Ferreira *et al.,* 2013). However, CT plasmid copy number in this study did not appear to influence CT bacterial loads, suggesting that plasmid copy number did not predict CT virulence. Studies in mice infected with *C. muridarum* first suggested that plasmids may enhance the pathogenesis of infection, with plasmid containing strains being more efficient at infecting mice, which experienced more potent inflammatory responses in reproductive tract tissues (O'Connell *et al.,* 2007; Kari *et al.,* 2008; Olivares-Zavaleta *et al.,* 2010; Kari *et al.,* 2011; O'Connell *et al.*, 2011; Zhong 2018; Zou *et al.,* 2019). As with other plasmid-containing microbes, genes found on bacterial plasmids are thought to confer a selective advantage for survival in specialised environments, including virulence and resistance to antibiotics and toxic heavy metals, enabling specialized ecological interactions and nutrient utilization (Williams and Thomas, 1992; Bennett, 2008; Petersen, 2011). However, this relationship between chlamydial plasmids and virulence has been less clear from human studies of CT infection (Borges *et al.,* 2010; Last *et al.,* 2014). In some studies, women infected with plasmid-containing CT strains were found to be more likely to experience infertility, inflammation, irregular menstruation, and polycystic ovarian syndrome than those infected with plasmid-free variants of CT (Yeow *et al.,* 2016). In other studies, including individuals with ocular CT infections, plasmid copy number was not associated with disease severity (Last *et al.,* 2014).

There were several limitations to the results presented in this Chapter that should be acknowledged. As discussed, the study was not powered to evaluate the effect of hormonal

contraceptives on CT infection risk or pathogenesis. Lateral wall vaginal swabs might not be optimal for CT molecular characterization but unfortunately the available DNA from high vaginal swabs was not stored optimally and may have degraded. Therefore, DNA extracted from lateral wall vaginal swabs was used instead. CT is an intracellular infection so the relatively non-invasive vulvovaginal swabs used to test for the presence of CT DNA may have underestimated CT bacterial loads. It was not possible to determine if the CT infections determined in this study were primary or repeat infections, as all AGYW were enrolled after sexual debut and had been sexually active for ~2 years prior to joining this study. It was also not possible to differentiate live versus dead CT in this study as NAAT was used, rather than culture. AGYW who were treated for their CT infections were not screened shortly after treatment completion for cure and sexual partners were difficult to treat so it was difficult to differentiate between repeat, recurrent or persistent infections. Another limitation is that CT-infected women who had elevated cytokines were also infected with other STIs like HPV.

In conclusion, results presented in this chapter suggested that CT infections in AGYW were largely asymptomatic, although several inflammatory cytokines associated with pathology were elevated, including IL-1β, IL-17A and IFN-γ. Anti-inflammatory treatment in CT infected patients may provide a means of reducing the inflammation and subsequent fibrosis that leads to tubal damage associated with PID and its complications. This chapter supports previous evidence suggesting that the majority of STIs are asymptomatic among sexually active adolescents and encourages testing for asymptomatic adolescent women for highly prevalent STIs, like CT.

CHAPTER 3: High-resolution MLST of urogenital CT strains infecting young South African women

3.1 Introduction

CT genital infections are a major cause of morbidity and mortality across the globe, particularly in women (Chesson *et al.,* 2017; Perslev *et al.,* 2019; Armstrong-Mensah *et al.,* 2021). CT infections are asymptomatic, if left untreated, late complications raise the risk of ectopic pregnancy, PID and female infertility (Elwell *et al.,* 2016; Brunham *et al.,* 2020). In addition to facilitating HIV transmission, CT infections are associated with cervical cancer (Buckner *et al.,* 2016; Arcia Franchini *et al.,* 2022; Dzakah *et al.,* 2022).

The ~1Mbp CT genome is largely conserved as it is an obligate intracellular pathogen, although it does contain areas with a great deal of nucleotide variability and recombination activity (Hadfield *et al.,* 2017; Hadfield *et al.,* 2018; Borges *et al.,* 2019; Borges *et al.,* 2021). Classification of CT MOMP, encoded by *ompA,* has been shown to correlate with CT tissue tropism and disease outcome (Joseph and Read, 2012; Patiño *et al.,* 2018), and serotyping MOMP was the most common method used to type CT infections until development of NAATs. Because CT MOMP is under host immune pressure and therefore prone to variability, these serological typing approaches have largely been abandoned because they lack sensitivity to distinguish between the high degree of polymorphism in the *ompA* (Dean and Millman, 1997; Brunelle and Sensabaugh, 2006; Joseph and Read, 2012; Nunes and Gomes, 2014). As such, MOMP serotyping has been replaced with more sensitive typing approaches that can detect recombination events, which are useful to distinguish between new or persistent infections (Götz *et al.,* 2014), and understand transmission patterns (Smelov *et al.,* 2017).

Most modern epidemiological studies on circulating *Chlamydia* strains now use sequence analysis of the *ompA* gene, although some have argued that *ompA* variability does not offer enough discriminating power to understand CT diversity and transmission (Pedersen *et al.,* 2009; Herrmann *et al.,* 2015). Indeed, *ompA* genotyping alone is not recommended for molecular typing, due to its recombinogenic nature (Gomes *et al.,* 2007; Harris *et al.,* 2012; Hadfield *et al.,* 2017), with MLST being more widely adopted for many global epidemiological studies, as this approach allows for CT intraspecies discrimination (Klint *et al.,* 2007; Ikryannikova *et al.,* 2010; Bom *et al.,* 2011; Christerson *et al.,* 2011a; Isaksson *et al.,* 2016; Pilo *et al.,* 2021; Ulianova *et al.,* 2021). Importantly, phylogenetic trees generated from CT

MLST approaches have compared well to whole-genome-based trees, with comparable levels of congruence in the phylogeny for CT strains (Herrmann *et al.,* 2015; Gupta *et al.,* 2017; Floridia-Yapur *et al.,* 2021). MLST has been argued to be cheaper and faster than whole genome-based sequencing (Gupta *et al.*, 2017; Patino *et al.*, 2018). There have been a number of MLST schemes developed for genotyping CT, each with a different goal in mind. This Chapter used the Uppsala MLST scheme, developed by Klint *et al*. (2007), as it offers high discrimination power compared to the other MLST schemes. This MLST scheme simultaneously detects three hypothetical CT genes (CT058, CT144, CT172) and two known genes (*hctB* and *pbpB*), all situated among the five most highly variable regions of CT genome (Klint *et al.*, 2007), putatively under selection, and supported by the PubMLST database (Jolley *et al.,* 2018).

While approaches like MLST provide important information to track CT transmission patterns that are epidemiologically important, there is still very much a need to monitor CT MOMP characteristics within a population, to inform on effective CT vaccine design (He *et al.,* 2017; Phillips *et al.,* 2019; Murray and McKay 2021; Olsen *et al.,* 2021). As there is so much variability in MOMP globally, an effective CT vaccine should ideally cover a broad spectrum of MOMP variants to ensure protection against different strains, through identifying and targeting conserved regions or key epitopes within the MOMP protein or considering a multivalent vaccine approach that includes multiple MOMP variants (Badamchi-Zadeh *et al.,* 2016; Abraham *et al.,* 2019; Olsen *et al.,* 2021).

The aim of this Chapter was, therefore, to examine molecular diversity in CT strains causing infections among AGYW in South Africa using MLST, including providing critical molecular *ompA* genotyping to guide CT vaccine needs in high-risk communities. In addition, the impact of CT strain diversity on genital inflammatory cytokine and T-cell activation was explored to identify strain characteristics associated with high levels of pathology.

3.2 Methods

3.2.1 Description of cohort and collection of CT samples

The cohort and samples collected were described in Chapter 2 sections **2.3.1.** CT cases were diagnosed using 51 clinical samples from 43 women as described in section **2.4.1.** All CT cases identified in Chapter 2 section **2.4.1** (n=43) were included in this chapter.

3.2.2 High resolution-MLST

High resolution-MLST (Hr-MLST) was used to type clinical samples from this cohort that were positive for CT by NAAT. For the hr-MLST scheme used in this study, nested PCR primers were used, described by Pilo *et al*. (2021). Internal fragments of the highly variable CT046 (*hctB*), CT058, CT144, CT172, and CT682 (*pbpB*) and the *ompA* gene were amplified. Figure 3.1 shows the position of these genes in the CT genome. The resulting primer-to-primer sequences were compared to the CT hr-MLST database (Jolley *et al.*, 2018).

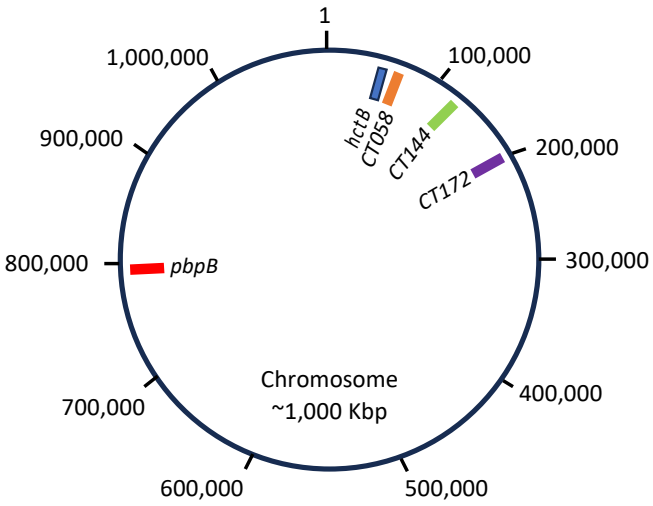

Figure 3.1. **CT genome map showing positions of the five regions used as the basis for hr-MLST typing**

As described by Bom *et al*. (2011), two consecutive PCR reactions were performed. In the first PCR, external primers (blue) were used to amplify the regions containing the target genes (Table 3.1). This was followed by a second round of PCR with internally located primers (yellow) using the product from the first reaction as template. Products from this second reaction were sanger sequenced in both directions using a third set of primers (green) that targeted the final sequence used in the MLST scheme.

Table 3.1 Details of the hr-MLST primer list used for PCRs

Primer Number	Gene	Gene position	Primer name	Sequence 5'-3'	5' Chrom-position (1)	Tm
1	**CT172**	External	CT172_CONF	CAGTGACTTGATTGGATAAC	195282	60
2		External	CT172_CONR	TGATCAAGCCATCTTAGAC	195799	60
3		Internal	CT172_new-F	TAAAGGTCGCCCAAATTCC	195373	60
4		Internal	CT172_new-R	GCTCCGGCTATTTTGTTTAGG	195778	60
5		Seq_F	CT172-seqF	AAATTCCATGTTATAATC	195385	61
6		Seq_R	CT172-seqR	TATTTCCCTCAGTTATTC	195756	45
7	***pbpB* (CT682)**	External	CT682_CONF	TGGGATTTATTATTGCTTTG	780726	58
8		External	CT682_CONR	TACACCTAATTTGCCGATCA	781453	58
9		Internal	CT682_new_F	TCATCACTTTGCGTATATGG	780750	58
10		Internal	CT682_new_R	ATAGGCATGCATCTCTAAC	781420	58
11		Seq_F	CT682-seqF	TGGCATGTTGCTGTTGTG	780767	60
12		Seq_R	CT682-seqR	CGACGCTCGATATTCCTG	781376	59
13	***ompA***	External	*ompA*_CONF	AAGATAGCGAGCACAAAGAG	780107	60
14		External	*ompA*_CONR	ACTGCGTATTTGTCTGCATC	778943	60
15		Internal	*ompA*_new-F	CTCTTGAAATCGGTATTAG	780051	60
16		Internal	*ompA*_new_R2	CTTGCTCGAGACCATTTAAC	779174	60
17		Seq_F	*ompA*-seq_F2	TTGCCGCTTTGAGTTCTG	780029	60
18		Seq_R	*ompA*-seq_R2	GAGAGCTAAACTTGCTTG	779230	55
19	***hctB* (CT046)**	External	CT046_CONF	ACAACGTTTCCTCGAAGAC	51209	62
20		External	CT046_CONR	GAAGCGCAAACCTTTACAC	52030	62
21		Internal	CT046_new-F2	ATGGGCACATTTGGACATTG	51239	62
22		Internal	CT046_new-R2	TACACCAGAAGCAGCTACAC	52016	62
23		Seq_F	CT046_seq_F2	AAGAAGAGTTGCCTTACC	51280	55
24		Seq_R	CT046_seq_R2	CAAGCTGCAGTATGTTTATG	51991	56
25	**CT144**	External	CT144_CONF	AAGGAGGCAGATTAATGAC	160680	60
26		External	CT144_CONR	TCCTGAAGAAGTTGCATAG	161467	60
27		Internal	CT144_new_F	AACAGCAACAGCCAATTAC	160810	60
28		Internal	CT144_new_R	TATACCATGTGGACCCTAAAC	161414	60
29		Seq_F	CT144_seqF	TCGTGACTAATCCGAAATC	160908	56
30		Seq_R	CT144_seqR	CTAAACATACGGCTATTCC	161399	54
31	**CT058**	External	CT058_CONF	TAAGCACAGCAGGGAATG	67871	62
32		External	CT058_CONR	TGCGTGATTGAGGTCTTG	68754	62
33		Internal	CT058_new_F2	AATCCTCCTTGGCCTCTC	67903	62
34		Internal	CT058_new_R2	TTGGAAGGCGCGGATAAC	68739	62
35		Seq_F	CT058_seqF	TTGGCCTGAAGTAGAGAC	67995	57
36		Seq_R	CT058_seqR	AAAGGTGGCTGCGTTAAG	68637	59

Colours signify different groups of primers: light blue, external gene position (Nested PCR 1). Yellow, internal gene position (Nested PCR 2). Green, primers used for sequencing.

For all targets, the first round PCR amplification was performed in a 25µl reaction volume containing: 2µl extracted DNA, 0.5µM of each primer and 2x KAPA HiFi Hot Start Ready-mix (Roche, USA) (which contained 0.3 mM of each dNTP [1x], and 0.5U of KAPA HiFi Hot Start DNA Polymerase; per 25µl reaction). Cycling conditions were as follows: 5 min of polymerase activation at 95°C, followed by 30 cycles of denaturation at 95°C for 30s, annealing at 58-62°C for 30s and elongation at 72°C for 60s. A final 'polishing' step was carried out at 72°C for 7 mins. In the second round of PCR reactions, 2µl of the first PCR reaction was added

to a reaction tube containing 0.5 μM of each primer and 2x KAPA HiFi Hot Start Ready-mix. The second round PCR cycling conditions were as follows: 5 min of polymerase activation at 95°C, followed by 30 cycles of denaturation at 95°C for 30s, annealing at 58-62°C for 30s and elongation at 72°C for 60s, followed by a final elongation at 72°C for 7 mins. The final PCR products (15 μl) were confirmed by gel electrophoresis in 2% agarose gels stained using CondaSafe (CondsaLab, Madrid) and Sanger sequenced from both directions (Inqaba Biotech, South Africa).

Chromas (version 2.6.6) was used to QC and edit Sanger reads. Mega X was used to assemble sequences (Kumar *et al.,* 2018), which were saved in FASTA format. Locus allele numbers and subtypes were assigned by matching to the PubMLST database (Jolley *et al.*, 2018) using the hr-MLST scheme (Herrmann *et al.*, 2015). Each sequence was then aligned with published *ompA* sequences to determine the *ompA* genovar**.**

3.3.3 Measurement of cervicovaginal cytokines

Refer to Chapter 2.3.5, with enough detail.

3.3.4 Measurement of cervical cytobrush T-cell activation by FACS

Refer to Chapter 2.3.6, with enough detail.

3.3.5 Statistical analysis

One-way ANOVA was conducted to compare cytokine expression levels (IL-1β, IL-6, IL-17A, IFN-γ, and TNF-α) and T-cell activation markers ($CD4^+CCR5$, and $CD4^+CD38^+$) among the remaining groups. Post-hoc analyses were then performed to determine the specific group differences.

3.3 Results

A total of 43 CT+ samples were included here, obtained from the open-label randomized uCHOOSE trial (Gill *et al*., 2020; described in Chapter 2). This included 25 CT+ samples from the baseline visit, 10 from the cross-over visit, and 8 from the exit visit. This also included samples from 7 AGYW who were infected with CT at 2 visits and one who was infected with CT at all three study visits.

3.3.1 CT *ompA* genovars in the uCHOOSE cohort

Circulating CT *ompA* genotyping has important implications for selection of CT vaccine candidates. Six different *ompA* genovars were detected in the 43 CT samples sequenced (Figure 3.2), including *ompA* genovars D, E, F, G, Ia and Ja. We could amplify ompA gene in 8 samples which were CT+. Genovar E was most common in this cohort, with 39.5% (17/43) of AGYW being infected with this genovar, followed by D (27.9% [12/43]), F (14.0% [6/43]), G (7.0% [3/43]), and Ja (7.0% [3/43]). CT genovar Ia was the least common with only two women being infected with this type.

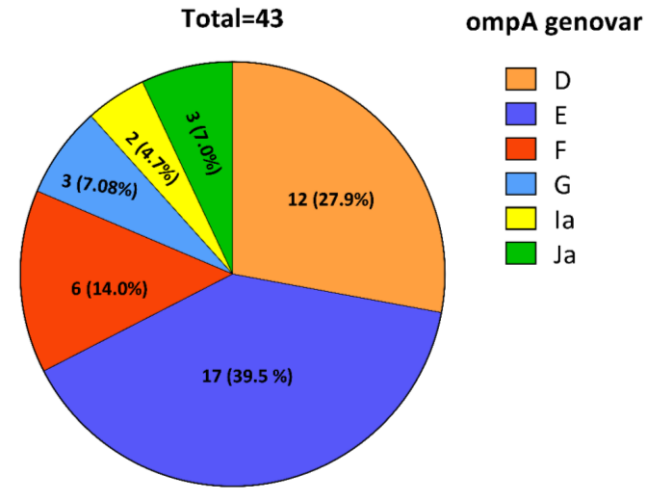

Figure 3.2 **Distribution of CT *ompA* genovars found in AGYW in Cape Town**. *ompA* D and E were the most common genovars circulating in young women. *OmpA* genovars key are indicated by the following colours in pie chart: D (Tan), E (purple), F (orange), G (blue), Ia (yellow) and Ja (green).

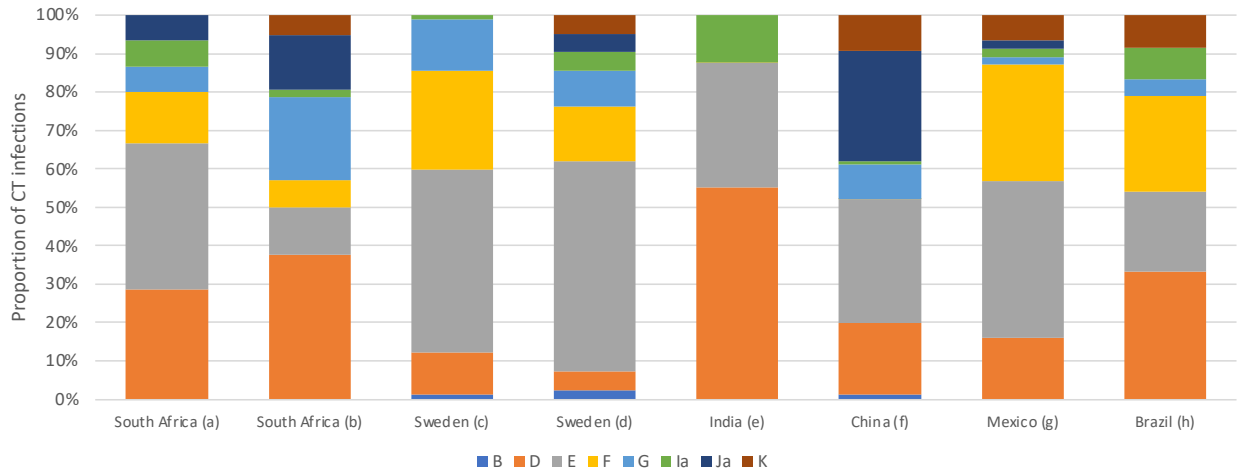

Figure 3.3. **Comparison of CT *ompA* genovars distribution detected in the uCHOOSE cohort in South Africa (a), compared with historical South African and international data.** Here, data was included from studies in (b) South Africa (Limpopo Province) by Versteeg *et al*. (2015a); (c and d) Sweden by Lysén *et al*. (2004) and Lagergard *et al*. (2010); (e) India by *Rawre et al*. (2019); (f) China by Lui *et al*. (2022); (g) Mexico by Casillas-Vega *et al*. (2017); and (h) Brazil by dos Santos *et al*. (2022). *OmpA* genovars are indicated by the following coloured bars: B (medium blue), D (orange), E (grey), F (yellow), G (light blue), Ia (green), Ja (navy blue), and K (brown).

3.3.2 CT *ompA* genovars and host cytokine responses

It was hypothesised that CT *ompA* genovars would differ in the amount and type of host inflammation induced. To test this, cervicovaginal cytokine concentrations were stratified according to CT *ompA* genovars (Figure 3.4). Genovar G was excluded from analysis because there was no cytokine data. Consistently, AGYW infected with genovar Ja tended to have higher concentrations of all cytokines measured, although this was not significant. IFN-γ concentrations appeared to be significantly different according to *ompA* genotype (one-way ANOVA F = 4.2832, P = 0.0079). None of the other cytokines measured appeared to differ according to *ompA* genovar. Furthermore, significant differences were observed in frequencies of activated CD4 T-cells (expressing CD38), but not in those expressing CCR5, by genovar (Figure 3.5).

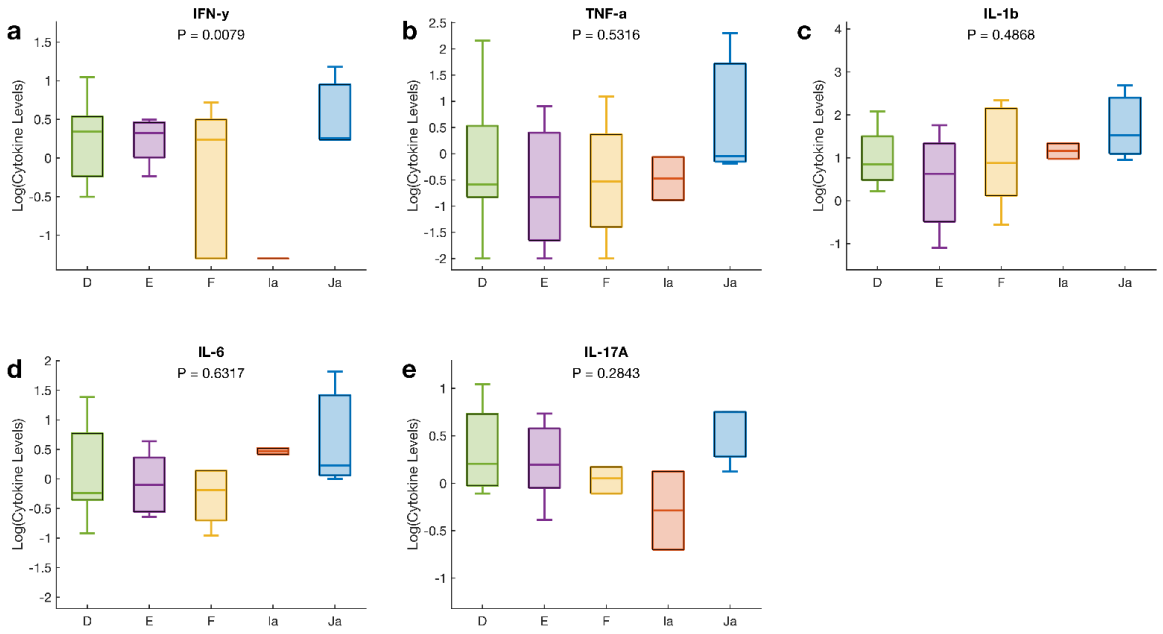

Figure 3.4. **Comparing cervicovaginal cytokine concentrations in CT+ cases by CT *ompA* genovar**. (a) IFN-γ (b)TNF-α (c)IL-1β (d) IL-6 (e) IL-17A. Green boxes represent genovar D (n=11 cases), purple boxes genovar E (n=17 cases), Tan boxes genovar F (n=6 cases), orange boxes genovar Ia (n=2 cases) and blue genovar Ja (n=3 cases). One-way ANOVA was used to compare groups, with p-values <0.05 considered significant.

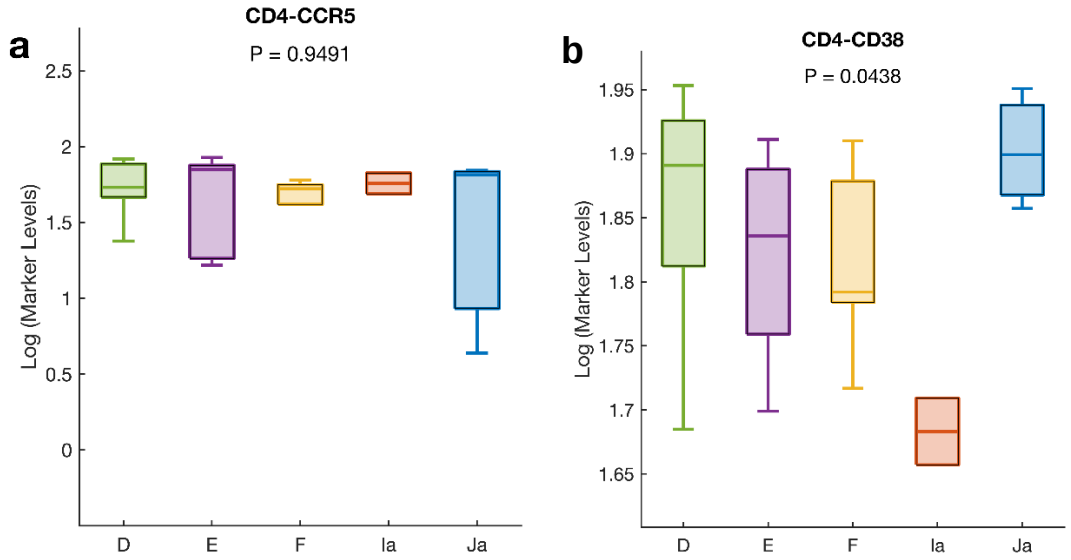

Figure 3.5**. Impact of different CT genotypes on cellular markers of genital tract inflammation, including** (A) $CD4^+CD38^+$ T-cell expression of CCR5 (co-receptor for HIV infection), and (B) $CD4^+$ T-cell expression of CD38 (T-cell activation marker). Green boxes represent genovar D (n=10 cases), purple boxes genovar E (n=12 cases), Tan boxes genovar F (n=6 cases), orange boxes genovar Ia (n=2 cases) and blue genovar Ja (n=3 cases). One-way ANOVA was used to compare groups, with p-values <0.05 considered significant.

3.3.3 MLST of circulating CT in Cape Town

Of the 51 CT+ cases, 7/51 samples had some alleles that could not be amplified by PCR. From the 44 CT+ samples that could be amplified, a total of 27 unique CT subtypes (STs) were identified, including 18 STs that had not been detected before, and were not present in the PubMLST database (Figure 3.6 and Table 3.2). ST3 was the most common MLST identified in the cohort (8/44; 18.1%), followed by ST12 (6/44; 13.6%). One of the CT+ cases (UC042_V2, UC042_V3), at two of the visits that she was infected with CT, had multiple chromatograms in allele CT172.

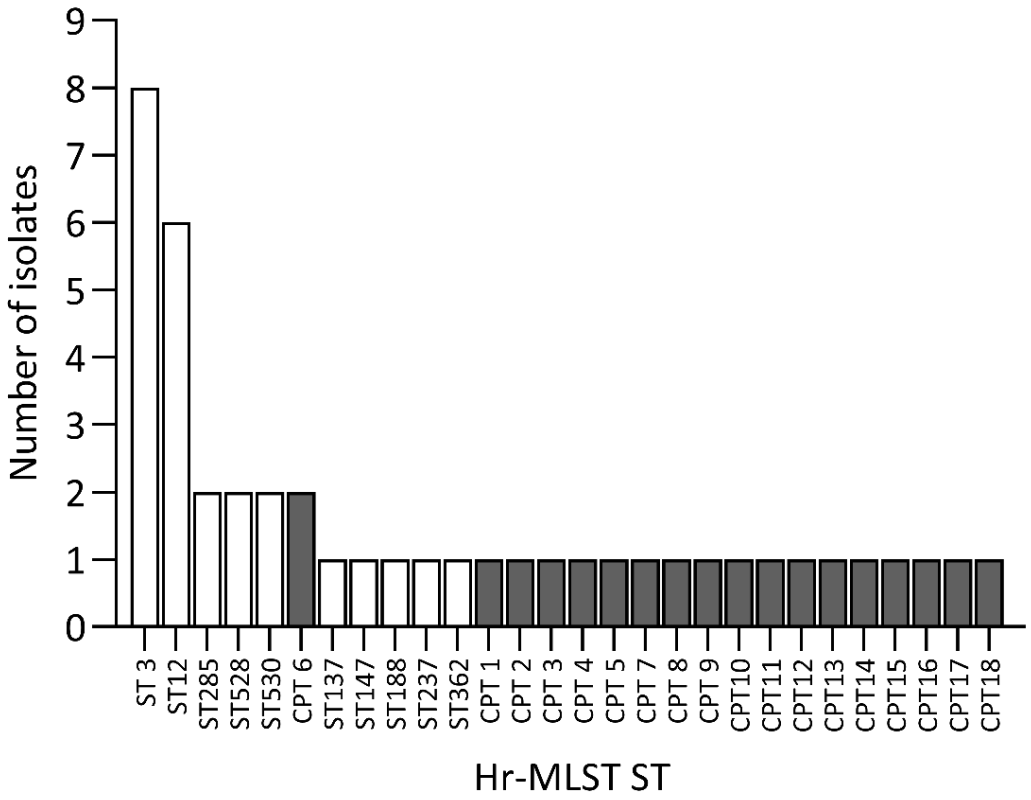

Figure 3.6. **Frequency of CT hr-MLST STs detected in women in Cape Town**. Clear bars represent STs that have previously been detected internationally; filled bars represent STs that are not presently listed in the pubMLST database, being unique to this cohort.

Table 3.2. *Chlamydia* STs identified in South African AGYW by hr-MLST

PIDs	CT046 (*hctB*)	CT058	CT144	CT172	CT682 (*pbpB*)	ST
UC008_V1, UC008_V2 UC009_V1, UC040_V3 UC057_V1, UC132_V1 UC138_V2, UC155_V1	1	2	6	2	2	3
UC013_V1, UC056_V1 UC087_V2, UC110_V1 UC138_V1	5	19	7	1	4	12
UC106_V2	10	8	22	4	6	137
UC072_V2	1	19	7	2	2	147
UC087_V3	5	19	7	2	2	237
UC167_V1, UC167_V2	10	8	22	3	6	285
UC019_V1	5	2	5	2	4	188
UC082_V2	57	2	6	2	2	326
UC056_V3	5	2	5	40	4	520
UC094_V1, UC123_V1	45	2	5	2	4	528
UC155_V3, UC007_V1	93	19	7	1	4	530
UC001_V1	95	19	7	2	4	CPT1
UC020_V1	5	2	5	**40**[a]	4	CPT2
UC021_V2	10	5	12	**7***	18	CPT3
UC025_V1	1	2	7	2	**4**[a]	CPT4
UC033_V3	5	2	7	1	4	CPT5
UC042_V2, UC042_V3	10	8	22	**4***	6	CPT6
UC048_V1	5	8	22	2	4	CPT7
UC051_V3	1	2	6	2[a]	2	CPT8
UC059_V1	5	2	6	1	2	CPT9
UC070_V1	95	19	6	2	2	CPT10
UC071_V1	10	5	12	**26***	18	CPT11
UC083_V2	57	2	6	2	4	CPT12
UC101_V1	95	19	7	2	4	CPT13
UC102_V1	1	2	6	2	4	CPT14
UC106_V3	5	2	6	2	34	CPT15
UC111_V1	1	2	5	2	2	CPT16
UC118_V1	95	19	7	2	4	CPT17

*Mixed sequence; a-closest matching allele

Of the five CT MLST regions evaluated in this cohort (Figure 3.7), CT046 (*hctB*) and CT172 showed the most variations with each having seven unique variants evident, followed by CT144 and CT682 (*pbpB*) with five, and CT058 with four. In most regions, one or two variants were most dominant, with the rest being detected in only 1 or 2 isolates.

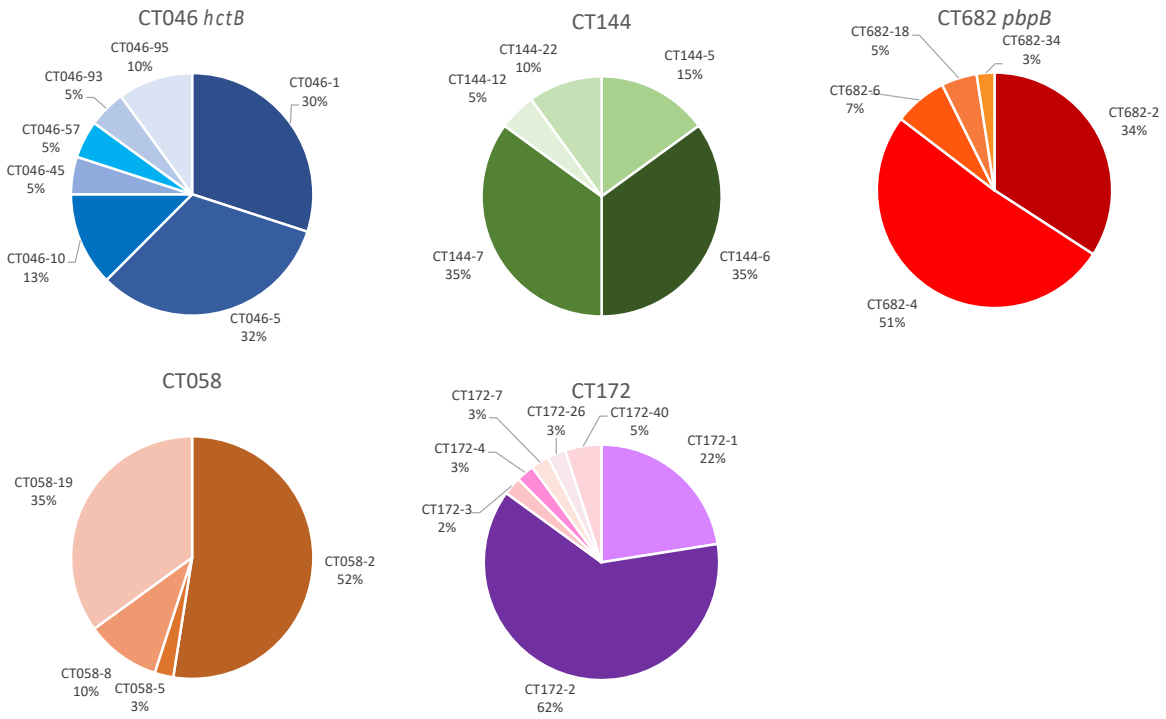

Figure 3.7. **Distribution of CT MLST regions identified in the uCHOOSE cohort,** including CT046 (*hctB*) variants in shades of blue, CT144 variants in shades of green, CT058 variants in shades of tan/peach, CT172 variants in shades of purple/pink, and CT682 (*pbpB*) variants in shades of red/orange.

3.3.4 Longitudinal MLST profiles of AGYW who were CT positive at multiple time points

Eight of the CT+ cases in this study had CT infections at multiple visits. Since all women were treated for CT infection, these likely represent repeat infections, although the possibility of strain persistence cannot be ruled out. Of these, four were infected with the same ST at multiple visits [Figure 3.8; participants 8, 42,87 (strain has evolved slightly over time, but is still basically the same strain) and 167] and four were infected with new CT variants at follow up visits (participants 56, 106, 138, and 155).

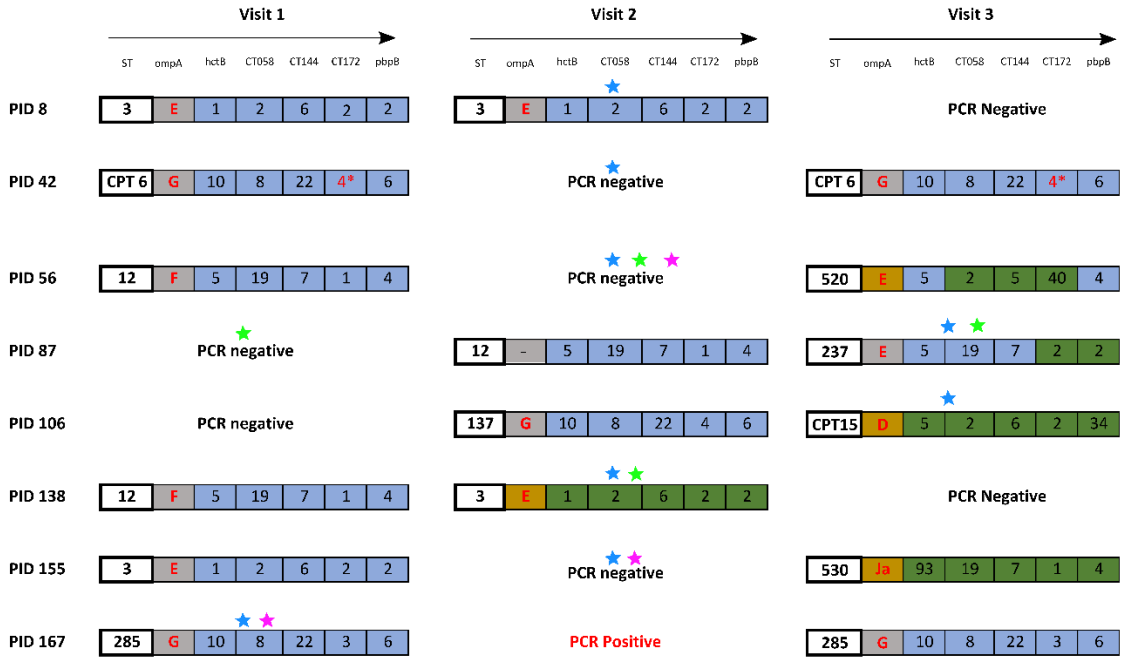

Figure 3.8. **Temporal changes in CT STs in women infected with CT at multiple visits**. *ompA* genovars highlighted in red front and ST in white bold box. Green colour shows change in allele. When participants were recorded to have received Azithromycin, Metronidazole or Ceftriaxone, this is indicated by a blue, green or pink star, respectively. *Indicates were allele CT172 had multiple chromatograms, 'evidence of mixed sequence in the chromatograms.

3.4 Discussion

Overall, the results presented in this Chapter revealed genetic diversity of CT strains among adolescent women found in Cape Town. Unique STs were detected that were not present in the pubMLST database. *OmpA* genovars D and E were the most common genovars in this population. The longitudinal study showed that some women were persistently infected with CT and some were reinfected with unique strains. There was some evidence that suggested mixed infection in a small number of samples. Immunologically, significant differences in IFN-γ secretion across the groups (*ompA* DEF) were noted, after excluding *ompA* G (one sample available for analysis) and there was a significant difference in the expression of the $CD4^{+}CD38^{+}$ activation marker.

All CT+ AGYW in this study were asymptomatic, which is clinically interpreted to mean they do not have evident pathology associated with infection. The relationship between CT genovars and clinical manifestations, however, is still a subject of debate among researchers. Six genovars were identified in the CT+ cases, based on sequencing the *ompA gene.* Genovars D and E are also the most prevalent genovars among participants who had CT infection in previous studies (Morré *et al.,* 2000; Lysén *et al.,* 2004; Bom *et al.,* 2013; Versteeg *et al.,* 2015a; Versteeg *et al.,* 2015b; Tang *et al.,* 2022). In China, Tang *et al*. (2022) and Bom *et al*. (2013) identified the major circulating genovars in women as being E, D, F, and J, suggesting that genovar prevalence was stable over time in China. Similarly, in India, the most found *ompA* genovars were E, D, and F in various studies (Singh *et al.,* 2003; Rawre *et al.,* 2016; Rawre *et al.,* 2019). In Japan, the predominant genovars were E, D, F in one study and F in another that was carried out 14 years later (Ikehata *et al.,* 2000; Satoh *et al.,* 2014). The reason for the global dominance of genovars D, E, and F is unknown, but previous research has shown that *ompA* genovars E and F mutate at a significantly slower rate than the other genovars (Nunes et al. 2009). A *Chlamydia* vaccine must prevent pathology while also providing protection against the vast majority of genovars found worldwide.

Distribution of *ompA* genovars from the uCHOOSE cohort were compared to previous *ompA* data collected from earlier cohort studies conducted by our UCT laboratory (Women's Initiative in Sexual Health Study cohort; Barnabas *et al.,* 2018) and from other global published *ompA* genovar studies (Brazil, China, Mexico, South Africa, Sweden and India) (Figure 3.3). Genovar D and E were the most common in in the lower genital tract of uCHOOSE AGYW, consistent

with previous studies that included CT strains from India (Rawre *et al.,* 2019) and Brazil (dos Santos *et al.,* 2022). In contrast, genovar E and F were most common in two different cohort studies from Sweden (Lysén *et al.,* 2004; Lagergard *et al.,* 2010). Genovar F was third most prevalent in the uCHOOSE cohort, with 14% of the CT cases genotyped being infected with genovar F.

To investigate whether different *ompA* genovars were associated with different levels of inflammation in this cohort, cervicovaginal cytokine concentrations were stratified by *ompA* genotype. Genovars E, D, F, Ia and Ja were all positively associated with IFN-γ concentrations, although genovar G was not. IFN-γ production is regulated positively by IL-12 and IL-18, and negatively by IL-10, but is also enhanced through a positive feedback mechanism where it can induce IFN-γ production by immune cells (Yang *et al.,* 1996; Netea *et al.,* 2004; Zhang and Starnbach, 2015). Although production of IFN-γ in responses to certain CT genovars suggests that $CD4^+$ T-cells become activated, proliferate, and are recruited to the genital mucosa (Johansson *et al.,* 1997; Roan *et al.,* 2006; Marks *et al.,* 2007; Olive *et al.,* 2011), this was not evident in this study, although the sample size was relatively small. Activated CD4 T-cells are important producers of IFN-γ and TNF-α, although the type and amount of effector cytokines produced by CD4 T-cells are also dependent on the types of cytokines present in the environment while T-cells are differentiating into effector cells (Roan and Starnbach 2008). IFN-γ detected in cervical secretions might also inhibit the normal metabolism and replication of *Chlamydia,* affecting availability of essential nutrients for *Chlamydia* growth by cellular tryptophan depletion and glucose starvation (Brunham and Rey-Ladino, 2005). IFN-γ also plays an important role in increasing cell surface expression of HLA class I molecules, thereby enhancing presentation of CT antigens to CD4 T-cells (Gaczynska *et al.,* 1993; Steimle *et al.,* 1994). However, intracellular CT is also known to downregulate HLA class II expression, by degrading the host transcription factor USF-1 that is involved in induction of HLA class II (Zhong *et al.,* 1999; Zhong *et al.,* 2000). CPAF, a *Chlamydia* protease that is secreted into the host phagosome, mediates degradation (Zhong *et al.,* 2001).

Many of the CT STs from this cohort overlapped with other STs described in other countries, although several novel CT STs were also identified by MLST, which appeared to be unique to Cape Town. No particular CT STs was associated with distinct or exacerbated inflammation,

as others have reported (Morré *et al.*, 2000; Christerson *et al.*, 2011a; Gravningen *et al.*, 2012), although the number of samples for each ST were small, which limited statistical power. The most prevalent MLST STs in this study was ST3, followed by ST12. This is similar to another study that included South African samples, from women (18–49 years) living in the Mopani District in Limpopo Province (Versteeg *et al.*, 2015a). Interestingly, while all infections in this cohort were asymptomatic, 61% of the women in the latter study had symptoms. The predominance of ST3 (found in 8 countries) and ST12 (found in 5 countries) strains has also been reported in studies conducted in other parts of the world (Gravningen *et al.*, 2012; Herrmann *et al.*, 2015). Herrmann and colleagues (2015) suggested that the dominance of ST3 and ST12 globally possibly represents 'founder strains', from which many other STs have subsequently evolved, evidenced by the finding that ST12 and ST3 have the highest number of single locus variants branching from them. While the hr-MLST scheme targets hypervariable CT loci, these are sufficiently stable to persist over long periods of time. For example, ST12 was detected in a reference strain collection from 1960 (Herrmann *et al.*, 2015). Therefore, founder strains that entered a geographic area in the past may persist and predominate ST collections.

Some young women in this study had persistent or repeat CT infections over the study period. Reinfection or persistent CT infection increases the risk of severe reproductive complications (Hosenfeld *et al.*, 2009) and some studies have suggested that reinfected women have a higher chance of ascension of CT into the upper genital tract than for women experiencing their first infection (Russell *et al.*, 2016a). Re-infection from a new or untreated partner, non-adherence to the treatment regimen, insufficient exposure to the antibiotic due to host pharmacokinetics or short duration of treatment, and heterotypic or homotypic antimicrobial resistance are possible reasons for persistent/reinfection (Horner, 2012; Lanjouw *et al.*, 2015; Pitt *et al.*, 2018). To prevent re-infection and break the chain of *Chlamydia* transmission, partner treatment is necessary for heterosexuals with CT infection (Lang *et al.*, 2018). Partner treatment uptake was low in this cohort (Gill *et al.*, 2020), which reflects previous experiences in South Africa where >90% of women disclosed their STI diagnosis to their sex partners, although ~25% reported that their sex partner(s) did not seek medical care, and 10% reported that they were unaware if their sex partner(s) sought medical care (Medina-Marino *et al.*, 2020).

This study had some limitations. The relatively small numbers of non-genovar D and E strains limited the ability to detect relationships between CT genovars and specific inflammatory

cytokines. All five alleles could not be amplified from some samples, due to insufficient chlamydial DNA being available. Finally, since test-of-cure was not carried out for women with CT, it was not possible to distinguish treatment failure/ST persistence from re-infection.

In summary, hr-MLST analysis of urogenital CT isolates provided insights into the CT strain types circulating in the Cape Town study population. However, genital inflammation did not appear to differ by CT STs. Whole genome analysis and tissue culture may help shed more light on the potential connections between genital inflammation and CT infections.

CHAPTER 4: Prevalence of HPV coinfection in AYGW infected with CT

4.1. Introduction

HPVs are a large group of DNA viruses that infect the genital, oral, and cutaneous epithelia, with >200 different HPV types being described (Van Doorslaer *et al.,* 2012; Van Doorslaer *et al.,* 2016). HPV-16 and HPV-18 are the most common HR-HPV genotypes associated with cancers in humans (Bruni *et al.,* 2010), being detected in 69.4% of cervical cancers (de Sanjose *et al.,* 2010). LMICs account for 88% of cervical cancer cases and 91% of deaths globally, with Africa, South Central Asia, and South America being the highest risk regions (Torre *et al.,* 2015; Idehen *et al.,* 2018; Swanson *et al.,* 2018; Ji *et al.,* 2019). In addition, women in Low and middle-income countries are more likely to die from cervical cancer than women residing in high income countries (Herrero *et al.,* 2015).

Infections with HPV and CT commonly co-occur (Bellaminutti *et al.,* 2014; Ji *et al.,* 2019; Ssedyabane *et al.,* 2019), sharing a common sexual transmission route and replicating in the same cell types – cervical epithelial cells (Newman *et al.,* 2015; Vos *et al.,* 2016; Naldini *et al.,* 2019). Both can cause chronic persistent infections, with persistence leading to severe reproductive health outcomes (Shew *et al.,* 2013; Karim *et al.,* 2018). CT infection synergistically facilitates HPV infection by disrupting the epithelial barrier, thereby promoting HPV entrance and persistence, a major risk factor for carcinogenesis (Samoff *et al.,* 2005; Shew *et al.,* 2013; Nonato *et al.,* 2016; Mosmann *et al.,* 2019). Persistent inflammatory CT infections may cause cervical hypertrophy (Markowska *et al.,* 1999; Markowska *et al.,* 2002) and squamous metaplasia (Paavonen, 2012), which also favour infection with HPV. Compelling evidence from large epidemiological studies has also shown that CT infection is a risk factor for HPV-associated disease progression and cervical cancer (Bellaminutti *et al.,* 2014; Di Pietro *et al.,* 2018). CT may increase the risk of cervical neoplasia because it is an intracellular pathogen that interacts with metabolic components in host cells that may disrupt cellular pathways regulating cell proliferation (Chen *et al.,* 2020). Women with cervical cancer are more likely to have had a prior *Chlamydia* infection than the general population (Smith *et al.,* 2002; Zhu *et al.,* 2016). A meta-analysis has shown that women infected with CT were significantly linked to increased cervical cancer risk in both prospective (OR=2.21, 95% CI: 1.88–2.61, P<0.001), and retrospective studies (OR=2.19, 95% CI: 1.74–2.74, P<0.001) (Zhu *et al.,* 2016).

Since interactions between CT and HPV may influence disease outcomes for both pathogens, understanding the respective prevalence, genotype distribution, and risk factors for CT and HPV infection is critical for effective prevention and intervention. Given the high burden of CT infection described in Chapters 2 and 3, this chapter investigated whether CT infected AGYW are more likely to be HPV infected and experience persistent HPV infections than CT uninfected adolescents.

4.2 Methods and Materials

4.2.1 Description of cohort and samples collected

This sub-study used samples collected from the uCHOOSE study, which enrolled 130 healthy, sexually active adolescent and young women between the ages of 15-19 years (Gill *et al*., 2020). Cohort information is described in Chapter 2 section 2.3.1. Of these, 108/130 and 96/107 had samples available for HPV typing at baseline and cross-over, respectively.

4.2.2 Testing for CT infection, bacterial load and CT *ompA* genovars determination

As previously described, infection with CT was determined using specific oligonucleotide primers and probes for CT, CT bacterial load was measured by qPCR for the *omcB* gene, according to the method described in Chapter 2 section 2.3.4. CT genovar was determined from 43/51 samples, according to the method described in Chapter 3 section 3.3.2.

4.2.3 HPV genotyping

The HPV Direct Flow Chip (Master Dianostica, Calle Luis Fuentes Bejarano, Sevilla, Spain) was used to type HPV present in cervical swabs, according to the manufacturer's instructions. Briefly, cervical swabs (previously stored at -80^{o}C) were thawed and 500 µl PBS was added to each cervical swab. Swabs were vortexed and 30µl of the PBS swab eluate was used directly for PCR (without DNA extraction) using PCR reagents provided by the manufacturer, and cycling conditions listed in Appendix 4.1. After this PCR step, DNA was denatured for 10 mins at 95^{o}C, and then immediately placed on ice for 2 min. Prior to hybridization, the hybridization solution provided by the manufacturer was warmed to 41^{o}C in a water bath, before being added to the HPV Chip (270µl) together with the amplified DNA product (5µl). The HPV Chip was incubated at 41^{o}C for 8 min, following which the PCR products were removed and the HPV Chip washed three times with 300µl hybridization solution (kept at 41^{o}C). Adjusting to 29^{o}C, 300µl of the Blocking Solution (provided with the kit) was added to the HPV Chip and incubated for 5 min. Blocking solution was then removed, and 300µl of Streptavidin-Alkaline Phosphatase was added to each Chip for a further 5 min at 29^{o}C. This was then removed, the chip washed thoroughly and 300µl of the developing reagent was added for 10 mins at 36 ^{o}C. Finally, the Chip membranes were washed twice, and images captured with the Hybrisoft Software.

4.2.4 Statistical analysis

The percentage of participants who were infected with CT at the screening, crossover, and exit visits (weeks 16 and 32, respectively) was used to calculate the CT prevalence at each study visit. CT incidences were computed using the number of new infections per person per year. Analyses were performed using GraphPad Prism 8 (GraphPad software). Due to the non-normal distribution of CT bacterial load, non-parametric statistical tests were used. CT bacterial load data was stratified into lower CT loads (<50th percentile) and higher CT loads (>50th percentile), as previously defined (Chapter 2). The Mann-Whitney U test was used to determine significance of differences between unmatched groups. A Fisher's exact test was used for comparison of frequencies among groups.

4.3 Results

4.3.1 HPV prevalence in AGYW

Overall, the prevalence of HPV was high in these young women, with 94.4% (102/108) being infected at baseline, and 90.5% being infected at crossover (86/95) (Table 4.1). HR-HPV infections were also highly prevalent at both baseline [84.3% (86/102)] and crossover [84.9 % (73/85)], with the most common HR-HPV types at baseline being HPV-52 [detected in 26.5% (27/102) of women] followed by HPV-18, HPV-35, and HPV-51 [all 17.6% (19/102)]. HPV-16 was found in 11.8% of this cohort at baseline (12/102) which remained similar at cross-over [14.1 % (12/85)].

LR-HPV types were similarly highly prevalent in this cohort, with 81.5% (88/108) of AGYW being infected with any LR type at baseline (Table 4.1). Prevalence was lower at crossover, however, with 64.2% (61/86) being infected with any LR type (P=0.0116; Fisher's exact test). LR HPV-61 and HPV-67 prevalence was significantly lower at crossover than baseline, and the prevalence of HPV-6 and HPV-11, the most common genital wart associated types, was only slightly lower by crossover, although not significantly. None of the young women infected with HPV-6 and HPV-11 at either study visit had clinically evident genital warts.

Of the 95 AGYW who attended both baseline and crossover visits, 78/95 (82.1%) were infected with any HPV type at both time points (4 months apart), and the majority of these were frequently infected with the same HPV type at both time points (64.1%; 50/78) (Table 4.2).

To investigate whether persistence or repeat infections with any HPV genotype was impacted by infection with multiple HPV strains (Table 4.2), the prevalence of these repeatedly detected HPV genotypes was compared in AGYW with multiple versus single HPV infections. AGYW infected with multiple HPV types were not significantly more likely to have HPV infections that persisted (between enrolment and cross over) than those infected with single HPV types (34/53, 61.1% vs. 29/53, 54.7%, P= 0.4290; data not shown). However, more than 50% of women had persistent infection between enrolment and crossover.

Table 4.1. HPV prevalence in adolescent girls and young women during baseline and crossover

HPV type	Baseline	Cross-over (4 months)	P-value[a]
Any HPV	94.4% (102/108)	89.6% (86/96)	0.2967
Any HR-HPV	84.3% (86/102)	84.9 % (73/86)	>0.9999
HPV-16	11.8% (12/102)	14.1 % (12/85[b])	0.6656
HPV-18	18.6% (19/102)	10.5% (10/85[b])	0.2274
HPV-31	9.8 (10/102)	10.5% (10/85[b])	0.8129
HPV-33	5.9% (6/102)	3.2% (3/85[b])	0.5139
HPV-35	18.6% (19/102)	14.7% (14/85[b])	0.8475
HPV-39	15.7% (16/102)	11.8% (10/85[b])	0.5266
HPV-45	17.6% (18/102)	14.7% (14/85[b])	0.8483
HPV-51	18.6% (19/102)	17.6% (15/85[b])	>0.9999
HPV-52	26.5% (27/102)	16.5% (14/85[b])	0.1125
HPV-53	6.9% (7/102)	3.5% (3/85[b])	0.3513
HPV-56	12.7% (13/102)	14.1% (12/85[b])	0.8313
HPV-58	16.7% (17/102)	10.6% (9/85[b])	0.2903
HPV-59	16.7% (17/102)	9.4% (8/85[b])	0.1957
HPV-66	8.8% (9/102)	5.8% (5/85[b])	0.5800
HPV-68	9.8% (10/102)	11.8% (10/85[b])	0.8129
HPV-73	7.8% (8/102)	8.2% (7/85[b])	>0.9999
Any LR-HPV	86.6% (88/102)	71.8% (61/86)	0.0116*
HPV-6	19.6% (20/102)	16.5% (14/85[b])	0.7039
HPV-11	9.8% (10/102)	8.2% (6/85[b])	0.6042
HPV-40	16.7% (17/102)	7.4% (7/85[b])	0.1233
HPV-42	14.7% (15/102)	12.9% (11/85[b])	0.8330
HPV-43	12.7% (13/102)	7.1% (6/85[b])	0.2316
HPV-44/55	11.7% (12/102)	9.4% (8/85[b])	0.6428
HPV-54	9.8% (10/102)	12.9% (11/85[b])	0.6427
HPV-61	19.6% (20/102)	7.1% (6/85[b])	0.0185*
HPV-62/81	31.3% (32/102)	27.1% (23/85[b])	0.6290
HPV-67	26.5% (27/102)	9.1% (8/85[b])	0.0043**
HPV-69	2.0% (2/102)	0% (0/85[b])	0.5015
HPV-70	5.9% (6/102)	8.2% (7/85[b])	0.5739
HPV-72	5.9% (6/102)	3.5% (3/85[b])	0.5139
HPV-84	10.8% (11/102)	3.5% (3/85[b])	0.0920

a-Fisher's exact test
b-one sample HPV genotype was not determined.
Red text shows the highest prevalent HPV types.

Table 4.2. AGYW with evidence of persistent or repeat infection with HR-HPV types

PID	Enrolment	Cross-over
UC008	HPV39, HPV51, HPV58, HPV68, HPV82	HPV39, HPV51, HPV68
UC009	HPV39, HPV51, HPV59	HPV39, HPV59
UC015	HPV33	HPV33
UC016	HPV18, HPV68	HPV68
UC021	HPV52	HPV16, HPV52
UC022	HPV16, HPV56	HPV16, HPV33, HPV56
UC025	HPV18, HPV35, HPV39, HPV53, HPV82	HPV35, HPV39
UC029	HPV16, HPV31, HPV39, HPV52, HPV58	HPV58
UC037	HPV51	HPV51, HPV58
UC038	HPV33, HPV58, HPV66, HPV68	HPV66
UC039	HPV26, HPV35, HPV51	HPV35, HPV51
UC042	HPV73	HPV73
UC053	HPV16, HPV18, HPV31, HPV35, HPV45, HPV51, HPV52, HPV53, HPV56, HPV59, HPV66	HPV16, HPV31, HPV35, HPV51, HPV52, HPV53, HPV59, HPV66
UC055	HPV45, HPV52, HPV59	HPV45, HPV52, HPV68
UC056	HPV45, HPV52, HPV59	HPV59
UC057	HPV45, HPV52	HPV45
UC062	HPV16, HPV18, HPV35, HPV58, HPV59	HPV18, HPV35, HPV58
UC071	HPV31, HPV52, HPV58, HPV66	HPV26, HPV52, HPV82
UC072	HPV39, HPV52, HPV68	HPV39
UC073	HPV39	HPV39
UC082	HPV35, HPV52	HPV52, HPV58
UC084	HPV18, HPV51	HPV18, HPV39, HPV51, HPV52
UC086	HPV26, HPV31, HPV39, HPV52	HPV31
UC091	HPV56, HPV58	HPV56
UC092	HPV39, HPV45	HPV45
UC094	HPV18	HPV16, HPV18, HPV39, HPV45, HPV68, HPV73
UC095	HPV16, HPV31, HPV35, HPV39, HPV51, HPV56, HPV66, HPV68	HPV31, HPV35, HPV39, HPV68
UC096	HPV39, HPV51, HPV59	HPV26, HPV51
UC099	HPV56, HPV58	HPV56, HPV58
UC106	HPV52, HPV58	HPV45, HPV52
UC113	HPV51	HPV51
UC119	HPV73	HPV73, HPV82
UC120	HPV31, HPV33, HPV35, HPV52, HPV59	HPV31, HPV35, HPV52
UC128	HPV59, HPV66, HPV73, HPV82	HPV51, HPV59, HPV82
UC130	HPV39, HPV45, HPV58	HPV45
UC137	HPV16, HPV53	HPV16, HPV35, HPV53
UC139	HPV35, HPV45, HPV56, HPV59	HPV35, HPV39, HPV45, HPV56, HPV68
UC140	HPV35, HPV45	HPV45
UC143	HPV16, HPV51, HPV52, HPV59, HPV68	HPV16, HPV39, HPV51, HPV52, HPV59, HPV68
UC144	HPV18	HPV18
UC149	HPV18, HPV53, HPV68	HPV18, HPV58
UC156	HPV33, HPV51, HPV52, HPV68	HPV52, HPV82
UC157	HPV52, HPV53, HPV68, HPV73	HPV52, HPV68
UC159	HPV16, HPV56	HPV56
UC160	HPV31, HPV52, HPV58	HPV31, HPV52
UC161	HPV58	HPV58
UC167	HPV56	HPV56
UC170	HPV56	HPV56
UC172	HPV18, HPV35, HPV45, HPV51	HPV18, HPV35, HPV45, HPV51
UC177	HPV16	HPV16

Red text shows identical HR-HPV types that were detected at baseline and cross-over in the same participant

4.3.2 Impact of CT infection on HPV prevalence

At baseline, 108/130 AGYW had samples available for HPV typing, of which 102/108 were infected with HPV (94.4%) and 17/108 were infected with CT (15.7%) (Table 4.3). Of these, all 17 CT+ women were co-infected with HPV, compared to 93.4% (85/91) of those who were CT–, which did not differ (P= 0.5869; Fisher's exact test). Furthermore, the median number of HPV types infecting CT– and CT+ women were similar (Table 4.4). Of those with any HPV infection, more CT+ women were infected with multiple HPVs than CT– women [94.1% (16/17) versus 73.6% (67/91)], although this was not significant (P=0.113; Fisher's exact test). Similarly, the prevalence of HR-HPV types tended to be higher in CT-infected and CT– women, with 94.1% (16/17) of CT+ women having any HR-HPV infection compared to 76.9% (70/91) of CT– women, although this was not significant. The prevalence of any LR-HPV infection was significantly different between CT+ and CT– women [63.7% (58/91) versus 94.1% (16/17); P=0.0114; Fisher's exact test). Interestingly, the prevalence of HPV-16, which is associated with most global cervical cancer cases (de Sanjose *et al.,* 2010; Guan *et al.,* 2012; WHO, 2015; Demarco *et al.,* 2020) and the HPV type included in all HPV vaccines (Bruni *et al.,* 2010; Basu *et al.,* 2021; Markowitz and Schiller 2021), was almost two-fold more prevalent in CT+ women than CT– women but was not significant (P=0.3981; Fisher's exact test). In contrast, HPV-18 was more commonly detected in CT– women than CT+ women, although not significantly.

Table 4.3. Relationship between CT infection and HPV prevalence at baseline

Status	HPV negative	HPV positive	Total
CT negative	6/91 (6.6%)	85/91 (93.4%)	91
CT positive	0/17 (0%)	17/17 (100%)	17
Total	6	102	108

Table 4.4. Comparison of HPV prevalence in AGYW by CT status at baseline

	CT negative	CT positive	P-value
N	91	17	
# HPV types (median; IQR)	3 (2-6)	4 (3-7)	0.2406[c]
Multiple HPVs (>2)	67/91 (73.6%)	16/17 (94.1%)	0.1132[b]
Any HR-HPV	70/91 (76.9%)	16/17 (94.1%)	0.1863 [b]
HPV-16	9/91 (9.9%)	3/17 (17.6%)	0.3981 [b]
HPV-18	17/91 (18.7%)	2/17 (11.8%)	0.7315 [b]
Any LR-HPV	58/91 (63.7%)	16/17 (94.1%)	0.0114[b]

b-Fisher's exact test; c-Mann-Whitney U

4.5.3 CT bacterial burden and HPV prevalence

The impact of CT bacterial load on HPV prevalence was evaluated next (Table 4.5), testing the hypothesis that women with higher CT bacterial loads (indicating more severe infections) had more HPV types causing infections or were more likely to be HPV-infected than women with lower CT bacterial loads. However, there was no significant difference in overall HPV prevalence, multiple HPV infections, HR-HPV or LR-HPV prevalence in women stratified by CT bacterial loads.

Table 4.5. HPV prevalence according to CT bacterial load from baseline visit

	High (>50th percentile)	Low (<50th percentile)	P-value[a]
N	6	11	
Any HPV+ type	6/6 (100%)	11/11 (100%)	>0.9999
Multiple HPV types (2+)	5/6 (90%)	11/11 (100%)	0.3529
HR-HPV	5/6 (90%)	11/11 (100%)	0.3529
LR-HPV	5/6 (90 %)	11/11 (100%)	0.3529

a-Fisher's exact test

4.3.4 CT *ompA* genovars and HPV prevalence

As described in Chapter 3, *ompA* genovars D and E being the most common in this cohort (Table 4.6). The nine CT cases with *ompA* genovars D were infected with 26 HPV types (of which 15 were HR-HPV), giving a median of five HPV types per women (IQR 3-7). Similarly, the 11 CT cases with *ompA* genovars E were infected with 23 HPV types (of which 14 were HR), also giving a median of 5 HPV types per case (IQR 2.5-6.5). Overall, HPV prevalence, number of HPV types detected, and prevalence of multiple HPV infections did not differ significantly by CT *ompA* genovar (P=0.3568; Kruskal-Wallis test) (Table 4.6). Numbers were too small to stratify HPV infections by the type causing infections.

Table 4.6. HPV prevalence according to CT *ompA* genovar

OmpA genovar	CT cases (N)	Any HPV type (n)	Median (IQR) HPV types	Prevalence (%; n/N)	
				HR-HPV	Multiple HPVs
D	5	21	4(IQR,1-0-7.5)	61.9% (13/21)	60% (3/5)
E	5	29	6(IQR,6.0-7.0)	62.1% (18/29)	100% (5/5)
F	2	10	5(IQR,4.5-5.5)	70.0% (7/10)	100% (2/2)
G	2	5	2.5(IQR,2.3-2.8)	40.0% (2/5)	100% (3/3)
Ia	2	9	4.5(IQR,3.6-5.3)	55.6% (5/9)	100% (2/2)
Ja	1	1	1(IQR,1-1)	0 % (0/1)	0% (0/1)

*P-value calculated using Kruskal-Wallis test.

4.3.5 Impact of CT infection on HPV persistence

Of the 95 women who had samples available for CT and HPV testing from two consecutive study visits (baseline and cross-over), 14/95 (14.7%) were infected with CT and 81/95 (85.3%) were not. The prevalence of persistent HPV infections was significantly higher in AGYW with CT infections compared to those not infected with CT (14/14 vs 61/81; P=0.04; Fisher's exact test), particularly HR HPV types (13/14 vs 48/81; P=0.0200; Fisher's exact test) (Table 4.7). CT+ women were not more likely to acquire an HPV infection at a subsequent visit compared to CT– women, nor clear an existing HPV infection.

Table 4.7. Persistence of HPV (over 2 study visits) in CT+ cases versus CT negative controls

HPV status	CT positive (n=14)	CT negative (n=81)	P-value[a]
Persistently negative	0% (0/14)	4.9% (4/81)	1.0
Persistence of any HPV type	100% (14/14)	75.3% (61/81)	0.04
Persistence of any HR-HPV type	92.9% (13/14)	59.3% (48/81)	0.02
Acquired any HPV type	0% (0/14)	1.2% (1/81)	1.0
Acquired any HR-HPV type	0% (0/14)	6.2% (5/81)	1.0
Cleared any HPV type	0% (0/14)	7.4% (6/81)	0.59
Cleared any HR-HPV type	7.1% (1/14)	9.9% (8/81)	1.0

a-Fisher's exact test

4.4 Discussion

This chapter examined the prevalence of HPV in AGYW, and assessed how co-infection with CT may affect the progression of HPV infections. High rates of HPV were noted among the participants: over 90% were infected with any type of HPV, around 80% carried HR-HPV types, and over 80% had persistent infections lasting more than four months. Instances of multiple HPV types were more frequent than single infections within this adolescent cohort. Others have argued that infections with multiple HPV types might synergistically favour persistent infections (Gallegos-Bolaños *et al.,* 2017; Senapati *et al.,* 2017), by HPV manipulation of the host immune system (Stanley, 2006), or by leading to higher overall HPV viral loads which might favour persistence (Oyervides-Muñoz *et al.,* 2020).

Every young woman in this cohort infected with CT also had an HPV infection, and the data indicated that HPV, especially the high-risk HPV-16, was more prevalent in women who tested positive for CT compared to those who did not. Furthermore, women with CT were more likely, but not significantly, to be infected with multiple types than single strains of HPV. Co-infections with CT and HPV have also been commonly reported from several other LMICs, including Uganda (Ssedyabane *et al.,* 2019), Brazil (Lima *et al.,* 2018), Thailand (Sangpichai *et al.,* 2019), Argentina (Deluca *et al.,* 2011), Colombia (Quinónez-Calvache *et al.,* 2016), and Cameroon (Fogue *et al.,* 2018). Co-infections with HPV and CT have also been linked to significantly increased risk of developing cervical abnormalities (Ferrera *et al.,* 2023).

Both behavioural and biomedical reasons have been proposed to explain the high concordance between CT and HPV infections. Both are common, sharing the same sexual risk factors such as having multiple sex partners and unprotected sex; and both infect host epithelial cells (Perry *et al.,* 1999; Bastidas *et al.,* 2013; Egawa *et al.,* 2015; Altamura *et al.,* 2020), particularly at the cervical transformation zone (Ribeiro *et al.,* 2020).

Inflammation, epithelial barrier disruption and micro-abrasions caused by CT infection may increase susceptibility to HPV infection by allowing easier access for the virus to basal epithelial cells (Verteramo *et al.,* 2009; Deluca *et al.,* 2011; Escarcega-Tame *et al.,* 2020). Studies have shown that host cell-cell tight junctions are altered during CT infection (Igietseme *et al.,* 2015; Rajić *et al.,* 2017; Igietseme *et al.,* 2018) due to transcriptional repression of epithelial cadherin (E-Cadherin), prompting the disassembly of adherent junctions (Kalluri and

Weinberg, 2009). Some studies have shown that CT alters the host intracellular environment to favour its replication cycle, by inhibiting apoptosis of the host cell, that may be associated with host cell DNA damage and increased oncogenic risk of HPV disease (Chumduri *et al.,* 2013). CT has also been shown to change host cell gene expression and protein stability at transcriptional, translational, and post-translational levels, which may impact severity of HPV-associated disease (Chumduri *et al.,* 2013; Olive *et al.,* 2014; Elwell *et al.,* 2016). CT infections which persist may also lead to local chronic inflammation which increases oxidative stress and HPV-associated oncogenesis (Wilfert and Gutman 1986).

It was interesting that CT bacterial load did not appear to influence HPV prevalence in this cohort, although data presented in Chapter 2 showed that many of the inflammatory cytokine biomarkers were positively associated with CT bacterial loads. Pro-inflammatory host responses to CT may disrupt cervicovaginal mucosa barrier integrity. However, previously, IFN-γ has been described as a correlate of protection against HPV, with higher levels of genital IFN-γ correlated with more rapid clearance of HPV infections (Scott *et al.,* 1999; Song *et al.,* 2008). HPV infection has also been suggested to downregulate a network of proinflammatory genes that regulate IL-1β, necessary to convert pro-IL-1β to the active form of IL-1β (Pétrilli *et al.,* 2007), which may contribute to lowering secretion of IL-1β by HPV-infected epithelial cells (Karim *et al.,* 2011). An *in vitro* study further suggested that HPV positive cells lines expressed significantly higher levels of IL-6 than HPV negative cells line (Grivennikov *et al.,* 2009; Otter *et al.,* 2019; Bonin-Jacob *et al.,* 2021). A recent study reported that elevated IL-6 concentrations were associated with severity of HPV disease, with concentrations of vaginal IL-6 being higher in women with more severe cervical disease (Li *et al.,* 2019). IL-6 detected in exfoliated cervical cells has been associated with the persistence of HPV infections, which may facilitate the development of neoplastic lesions (Bonin-Jacob *et al.,* 2021). Other studies have suggested that HPV-infected cells may produce a number of cytokines including IL-6 that recruit various other immune cells, which contribute to neoplastic progression (Stone *et al.,* 2014; Prata *et al.,* 2015).

CT genovars D and E were found to be the most prevalent in AGYW, which are also the most prominent globally (Morré *et al.,* 2000; Versteeg *et al.,* 2014; Chen *et al.,* 2017b; Hadfield *et al.,* 2017; Smelov *et al.,* 2017), although CT *ompA* genovar did not appear to impact on HPV prevalence in this study. While data in Chapter 3 show that several inflammatory cytokines

were increased in cervicovaginal fluid from women infected with CT genovars D and E, previous studies have suggested that these genovars are less inflammatory than other genotypes (including F, G, Ja, Ia and K), causing milder infections, and inducing less potent immune responses (Bianchi *et al.,* 2016). CT genovars D and E are thought to have a biological advantage over the other CT genovars because they possess specific virulence factors and are able to escape the host immune response, which facilitate transmission (Mossman *et al.,* 2008; Nunes *et al.,* 2009). Studies have also shown that genovars F, G, and K cause more severe disease (Geisler *et al.,* 2003; Molano *et al.,* 2004; Millman *et al.,* 2006; Gao *et al.,* 2007), and that genovars K and H in particular are more likely to be associated with abnormal vaginal discharge and abnormal cervical cytology, respectively (Liu *et al.,* 2022b). These findings support CT infection as a potential cause of squamous intraepithelial lesions in women infected with HPV (Kun *et al.,* 2013).

The bivalent Cervarix® vaccine against HPV (including HPV-16 and HPV-18) is most widely used vaccine type in most regions of Africa (Muñoz *et al.,* 2003; De Martel *et al.,* 2017; Delany-Moretlwe *et al.,* 2018; LaMontagne *et al.,* 2022). In South Africa, the national school-based vaccination program also uses Cervarix® and began in 2014, only for grade 6 girl learners (~9-10 years old) in schools in the bottom two economic quintiles (Delany-Moretlwe *et al.,* 2018). This program does not include catch-up vaccination for those who were older and had missed vaccinations (Delany-Moretlwe *et al.,* 2018). The AGYW included in this study were 15 years or older when the uCHOOSE study started enrolling in 2015 and were too old to have received the HPV vaccine, although the high prevalence of HPV in these young women provides compelling evidence for the urgent need to vaccinate adolescents.

In this study, Cervarix® vaccine type HPV-18 was the most common HR-HPV types found in AGYW, but other non-vaccine HPV types were also very common (including HPV-35, HPV-51, HPV-52, HPV-62/81 and HPV67). Many of these non-vaccine HPV types were also commonly identified by Mbulawa *et al*. (2021) in unvaccinated female learners in the Eastern Cape. Although HPV-16 and HPV-18 have been strongly and consistently linked to the development of HPV malignant transformation (Araldi *et al.,* 2018), and a high age standardized mortality rate or poor prognosis for women in Africa (Chen *et al.,* 2017a; Global Burden of Disease Cancer 2019; Arbyn *et al.,* 2020), data from this study and others (Mbulawa *et al.,* 2021). 2021) highlight the limited coverage of HPV in many high risk groups of women in South Africa. Together these results suggest that vaccination strategies in the region may

benefit from using vaccines that include more HPV types, such as Gardasil®9, which offers protection to more HPV types associated with cervical cancers including (HPV-6, HPV-11, HPV-16, HPV-18, HPV-31, HPV-33, HPV-45, HPV-52, and HPV-58).

This study has some limitations that should be acknowledged. Although the prevalence of CT and HPV were high in the cohort, small sample size for CT-infected women was an issue that could have limited the statistical power. The assay that was used to type HPV prevalence only identified 18 HR-HPV and 18 LR-HPV types, which represents the most common HPV types although >200 types have been characterized. While inflammatory cytokines were measured in cervicovaginal fluid collected during this study, markers of mucosal barrier function were not included. Since CT may have influenced mucosal barrier integrity, it would be interesting to evaluate markers of mucosal barrier integrity, like MMP-9 and E-cadherin in future studies, which have been associated with epithelial disruption (Frank and Hostetter 2007; Nighot *et al.*, 2015; Cherne *et al.*, 2020; Al-Sadi *et al.*, 2021; Cao *et al.*, 2021; Liu *et al.*, 2023).

In conclusion, the high prevalence of HPV and its co-infection with CT among AGYW is a complex public health challenge that warrants multifaceted interventions. The findings of this study elucidate the synergistic interactions between HPV and CT, which may exacerbate the persistence and oncogenic potential of HPV, particularly the high-risk types. The prevalence of HR HPV types, such as HPV-18, and the association of multiple HPV infections with CT, underscores the need for robust screening and prevention strategies. Despite the introduction of HPV vaccines, the predominance of non-vaccine HPV types in South African AGYW points to the necessity of expanding vaccine coverage with formulations to address a broader spectrum of HPV types. Additionally, the data suggest the potential value of integrating CT management with HPV prevention strategies, given their epidemiological interdependence and the biological interactions that may facilitate HPV persistence and pathogenesis. Implementing comprehensive sexual health education, increasing accessibility to HPV vaccination, and reinforcing STI screening and treatment are imperative to mitigate the burden of these infections and their sequelae. Furthermore, the unique epidemiological patterns observed with CT genovars suggest that tailored interventions considering the local prevalence and virulence of specific genovars may be required. Ultimately, these efforts should be informed by ongoing research and surveillance to adapt to the dynamic nature of HPV and CT infections and their impact on public health. Chlamydia vaccines are in the pipeline, but they have been problems

with them, we need to consider dual vaccination. Pathology is caused by inflammatory cytokines, anti-inflammatory strategies can be used to support CT-infected women.

CHAPTER 5. General Discussion

Sub-Saharan Africa is significantly impacted by STIs, recording some of the highest annual incidences worldwide, including that of HIV (WHO, 2016a; Ginindza *et al.,* 2017; Ngobese and Abbai, 2021; Jarolimova *et al.,* 2022). CT presents a particularly severe threat to women's reproductive well-being, compounding their susceptibility to HIV, especially among younger demographics. This dissertation investigated the biological impact of CT infections among AGYW in Cape Town, South Africa. It examined aspects such as CT bacterial load, plasmid number, and the genetic variability of prevalent CT strains, alongside their effects on inflammation. Furthermore, this study investigated the impact of CT infections on the natural progression of HPV infections.

Adolescence marks a transitional stage from childhood to adulthood, characterized by significant anatomical, physiological, psychological, and social changes. While STIs can impact individuals across all age groups (Newman *et al.,* 2020), AGYW are particularly susceptible due to high-risk sexual behaviours like unprotected sex (Karim *et al.,* 2017), and broader social and behavioural factors that elevate their STI acquisition risk (Dehne and Riedner, 2001; Torrone *et al.,* 2018).

Chapter 2 presents data on the high prevalence and persistence of asymptomatic CT infections among AGYW. This reflects similar findings in young women from South Africa (Mlisana *et al.,* 2012; Barnabas *et al.,* 2018), and other LMICs including Zimbabwe, Peru, China, India, and Russia (Detels *et al.,* 2011). The asymptomatic nature of these CT infections could contribute to increased transmission rates, as those infected remain unaware and untreated. CT infections, if not treated, can persist for years (Molano *et al.,* 2005) and lead to severe complications such as PID and infertility. Infections may silently ascend to the upper reproductive tract, causing fallopian tube damage that often goes undetected, with symptoms like PID commonly overlooked. Additionally, due to stigma and limited reproductive health education, many women do not know what constitutes “normal” vaginal discharge for them, which may hinder them self-identifying discharge associated with CT infection. Barnabas *et al*. (2018) found that reporting a vaginal discharge they considered “normal” was the best predictors of having an STI, highlighting the challenges that adolescents face in recognizing changes in their own discharge status. The frequently silent nature of CT and other STIs

underscores the potential for these infections to contribute to the escalating HIV-1 rates in SSA and other regions where STIs are common (Grosskurth *et al.,* 1995; Masha *et al.,* 2018; Dzakah *et al.,* 2022).

While symptom severity in some infections typically is proportional to pathogen load [eg. *E. coli* (Barletta *et al.,* 2011) and NG (Priest *et al.,* 2017)], this is not always the case for others [*Mycobacterium tuberculosis* [MTB], which is also an intracellular pathogen (Murthy *et al.,* 2018)]. This was not observed in this study, where some AGYW had CT bacterial loads as high as $3.6X10^5$copies/µl DNA without having clinically apparent symptoms. However, qPCR cannot distinguish between viable organisms and non-viable DNA, despite its sensitivity at measuring CT chromosomal and plasmid counts. To address this, other researchers have used a modified PCR protocol, which also assesses cell viability, for a more precise quantification of live CT bacteria (Dirks *et al.,* 2021; Janssen *et al.,* 2021).

Intracellular bacteria like CT and MTB have evolved sophisticated mechanisms to circumvent the host's intracellular defences due to their reliance on host cells for replication. *Chlamydia* spp. employ strategies to interfere with NF-κB signalling (Le Negrate *et al.,* 2008), interferon signalling (Thomas *et al.,* 1993; Elwell *et al.,* 2016), inflammation (Perfettini *et al.,* 2003), apoptosis (Chen *et al.,* 2019), and autophagy (Al-Younes *et al.,* 2004). MTB similarly subverts host defences by disrupting the phagosome (De Jonge *et al.,* 2007; Conrad *et al.,* 2017), inhibiting phagosome maturation, evading autophagy (Watson *et al.,* 2012), manipulating the inflammasome (Briken *et al.,* 2013; Shah *et al.,* 2013), and impairing apoptosis (Velmurugan *et al.,* 2007; Miller *et al.,* 2010). These intracellular pathogens, by residing within host cells, are shielded from other immune system components such as antibodies and complement, which enhances their ability to persist undetected and uninhibited.

The production of cytokines during *Chlamydia* infection is a double-edged sword: inflammation is crucial for controlling the infection, yet it simultaneously acts as the principal contributor to immune-driven pathogenesis. In Chapter 2, AGYW with CT infections had increased inflammation in their genital tracts compared to those who were uninfected, with elevated levels of IL-1β, IL-6, TNF-α, IFN-γ, and IL-17A and increased frequencies of highly activated $CD38^+$ cervical $CD4^+$ T-cells. Although CT bacterial load did not correlate with

symptoms, levels of inflammatory cytokines and $CD4^+$ T-cell activation correlated with bacterial loads, significantly so for cytokines.

The significantly elevated cytokines in women infected with CT—IL-1β, IL-6, TNF-α, IFN-γ, and IL-17A—offer insights into the nature of the immune responses being invoked, as well as their potential to cause pathological damage. For instance, previous studies have shown that TNF-α, which is largely secreted by activated macrophages, collaborates with IFN-γ to impede the metabolism of host cells, which is primarily achieved by upregulating the activity of IDO, thereby restricting the proliferation of *Chlamydia* (Perfettini *et al.,* 2000; Brunham and Rey-Ladino 2005; Njau *et al.,* 2009; Radomski *et al.,* 2019). Additionally, TNF-α is also known to promote apoptosis in host cells, creating unfavourable conditions for *Chlamydia*'s survival *in vivo* (Darville *et al.,* 2000).

However, TNF-α has also been implicated in the formation of pathological lesions in women with CT lesions (Xiang *et al*., 2021), by stimulating the expression of other cytokines, such as IL-6 and IL-8 (Zhao *et al.,* 2015; Shen *et al.,* 2019), which are associated with tissue fibrosis and scarring, as well as the recruitment of leukocytes to sites of inflammation (Xiang *et al*., 2021). In women with endometrial infections with CT, an elevation in tumour necrosis factor-α receptor -induced cytokines has been detected in cervical secretions, which supports their pathological role in human chlamydial infections (Poston *et al*., 2019). The importance of TNF-α in the immune-mediated damage caused by *Chlamydia* is further highlighted by studies showing that the severity of lesions in the fallopian tubes is significantly mitigated in TNF-α knock-out mice (Manam *et al*., 2015; Zafiratos *et al*., 2019).

Similarly, mouse studies have shown that IL-1β is associated with exacerbation of upper genital tract pathology during infection with *C. muridarum* (Prantner *et al.,* 2009). IL-1β evolved to aid host defense against chlamydial infection by inducing a broad range of inflammatory cytokines and chemokines, including IL-6 and IL-8 (Hvid *et al.,* 2007; Shimada *et al.,* 2011). IL-1β overexpression, on the other hand, may promote foam cell formation and accelerate atherosclerosis, notably during *C. pneumoniae* infection in the lung (Tumurkhuu *et al.,* 2018). The link between excessive IL-1β and the intensification of upper genital tract pathology in *C. muridarum* infection (Prantner *et al.,* 2009) underscores the dual nature of this cytokine: it is essential for defence but can also drive pathology when unregulated.

Increased levels of IFN-γ are known to be associated with heightened inflammatory reactions that can lead to complications following infection (Ishihara *et al.,* 2005). The amount of IFN-γ present can significantly shape the immune response to *Chlamydia* infection. At lower concentrations, IFN-γ has been observed to interrupt the developmental cycle of *Chlamydia*, leading to the formation of smaller, atypical inclusions characterized by large RBs and non-replicating aberrant bodies. This disruption results in persistent, asymptomatic infections that can contribute to immunopathogenesis by fostering inflammatory damage and fibrosis (Rottenberg *et al.,* 2002; Mannonen *et al.,* 2004; Agrawal *et al.,* 2009). On the other hand, the secretion of high concentrations of IFN-γ has been linked to overactive inflammatory responses and the resulting infectious complications, emphasizing its critical and complex role in the body's response to infection (Ishihara *et al.,* 2005; Mascellino *et al.,* 2011).

Excessive production of IL-17 has been implicated in various inflammatory and autoimmune conditions (Moschen *et al.,* 2019). Observations from a cross-sectional study of children in a trachoma-endemic area in Tanzania linked high IL-17A expression with signs of active trachoma, highlighting IL-17A's role in inflammatory pathologies that can lead to scarring (Burton *et al.,* 2011). IL-17A is also known to stimulate the release of cytokines that can cause disproportionate tissue damage and fibrotic repair, as seen in trachoma cases (Zhou *et al.,* 2009; Jiang *et al.,* 2010; Burton *et al.,* 2011). Correspondingly, human studies have noted a connection between genital tract disease in female CT infections and heightened neutrophil activity (Wiesenfeld *et al.,* 2002), underscoring the detrimental impact of an overactive IL-17 mediated response.

Among AGYW with recurring CT infections, there was a notable variation in bacterial loads over time. Half of these individuals had consistent bacterial loads at subsequent time points, while the other half exhibited lower bacterial loads later on, hinting at the development of partial immunity. In AGYW who exhibited reduced bacterial loads upon re-infection, reduced CT loads might also lessen their risk of transmitting to their partners. Observational studies have noted that some individuals with CT infections can naturally clear the pathogen from the lower urogenital tract without medical intervention (Geisler 2010; Walker *et al.,* 2012; Gupta *et al.,* 2018; Wijers *et al.,* 2020). Additionally, murine models have demonstrated that

subsequent infections result in lower bacterial loads (Morrison *et al.,* 2011), suggesting the establishment of protective immunity against CT.

In Chapter 2, the CTs causing infections in this cohort all had plasmids, ranging from 3 to 18 plasmids per CT. In mice, the presence or absence of *Chlamydia* plasmids were suggested to account for distinct pathological outcomes and cytokine responses (Chen *et al.,* 2015), although this phenomenon has not been directly observed in human CT infections. Echoing the insights from this study, most clinical CT isolates, particularly those associated with trachoma, are known to harbour a conserved 7.5kb plasmid (Peterson *et al.,* 1990; Farencena *et al.,* 1997; Last *et al.,* 2014; Yeow *et al.,* 2016; Last *et al.,* 2017; Ghasemian *et al.,* 2018; Ghasemian *et al.,* 2021), suggesting its probable significance in bacterial virulence and fitness. Future research could benefit from a more detailed analysis of these plasmids, to deepen our understanding of how the plasmid contributes to chlamydial infectivity and the inflammatory response. The conserved nature of the chlamydial plasmid underscores its role in enhancing bacterial fitness, promoting infection efficiency, facilitating host cell entry and exit, and potentiating innate inflammatory responses that result in tissue damage.

In Chapter 3, CT *ompA* genotyping was conducted to identify the predominant genovars present among adolescents in South Africa. The findings indicated that genovars D and E were the most common among CT-infected individuals, with a total of six unique genovars detected. The *ompA* gene encodes MOMP, which has been the focus of more than 78 vaccine trials due to its potential as a vaccine antigen (Phillips *et al.,* 2019). MOMP is specific to the genovar and consists of multiple T-cell and B-cell epitopes, which are known to elicit T-cell immunity and the production of neutralizing antibodies. Understanding the prevalent CT strains in a specific geographic area is critical as it facilitates the development of vaccines that are closely aligned with the circulating strains, increasing the likelihood of efficacy. A successful vaccine should offer broad protection, covering most of the strains present in the population. This ensures that vaccination efforts are as effective as possible in preventing the spread of diverse *Chlamydia* infections.

In previous research conducted in South Africa, including a study of women from the Limpopo province, genovar D emerged as one of the most commonly circulating types (Versteeg *et al*. 2015a). In contrast, a study from China focusing on high-risk women found CT genovar E to predominate, mostly associated with asymptomatic infections (Gao *et al.,* 2007). Meanwhile,

research from Mexico looking at the prevalence of CT genovars in men whose female partners were infertile suggested that genotypes F and G were frequently linked with lower abdominal pain in women (Lopez-Hurtado *et al.,* 2021). Yet, in the current study, genovars F and G were less common, and no cases of PID were observed. Other investigations have suggested that genovars E and G are often related to lower abdominal pain (Morré *et al.,* 2000; Geisler *et al.,* 2003; Rawre *et al.,* 2019), whereas genovar K has been associated with abnormal vaginal discharge (Gao *et al.,* 2007) and genovar H with abnormal cervical cytology (Liu *et al.,* 2022b).

It appears that the relationship between CT genovars and genital clinical symptoms remains a contentious area, with conflicting results from various studies. There is a growing need to investigate host genetic factors that may influence the manifestation of clinical disease and those that result in asymptomatic infections. Notably, differences in IFN-γ secretion among the *ompA* genovars suggest that CT genotypes may influence host immune responses. IFN-γ is a key cytokine of the Th1-type immune response, and the mucosal genital tissues of the AGYW were secreting IFN-γ to combat chlamydial infection. However, CT has evolved strategies to evade host defences, such as resisting IFN-γ-induced inclusion ubiquitination in human epithelial cells (Haldar *et al.,* 2016), complicating the interaction between pathogen and host immunity.

To date, there have been a limited number of MLST genotyping studies focused on CT strains in South Africa and Sub-Saharan Africa (Versteeg *et al*. 2015a), highlighting the importance and potential impact of this study in understanding the genomic landscape of CT in these regions. This study identified a total of 27 CT STs from 44 samples, which included 18 unique STs and 9 previously known STs (according to the pubMLST database). The discovery of multiple novel STs may reflect the ongoing evolution of CT strains, and genotyping of CT strains from previously uncharted geographical regions often reveals new STs.

The predominant strains circulating among AGYW in this study were ST3, associated with genovar E, and ST12, linked to genovars F. Notably, ST12 has also been identified in European countries (Gravningen *et al.,* 2012; Herrmann *et al.,* 2015). Herrmann *et al*. (2015) analysed CT hr-MLST STs isolated from 16 different countries, identifying four STs that were most common in these regions, including ST3, ST12, ST55, and ST56. These STs accounted for 31% of CT cases in this study. Herrmann *et al*. concluded that ST3 and ST12 were founder

strains, with ST12 detected in a reference strain collected as far back as 1960, indicating that other STs may have evolved from it. The long term prevalence of these STs suggests either genomic stability or that they possess inherent factors that enable successful infection in humans on a global scale. Aligning with this dissertation, ST3 and ST12 have been previously identified in women from the rural Mopani District in Limpopo Province (Versteeg *et al.* 2015a).

Using longitudinal samples from a subset of the cohort infected with CT at multiple visits, some instances of persistent infection or reinfection were observed with the same CT strain, while other AGYW had become infected with new strains. The hr-MLST scheme proved capable of differentiating between persistent infections and reinfections, demonstrating its utility in tracing CT transmission dynamics.

Chapter 4 presented an evaluation of HPV prevalence among AGYW, which revealed high rates of infection. At enrolment, >90% of participants were infected with any HPV, ~80% were infected with HR-HPV types, and >80% experienced persistent HPV infections. Notably, multiple HPV infections were more commonly observed than single infections within the cohort. The data also showed that every young woman infected with CT was co-infected with HPV, which could potentially elevate risk of Cervical neoplasia. Furthermore, HPV prevalence, including that of the oncogenic HPV-16, was generally higher in those who were CT+ compared to CT– individuals. Women with CT infections were more likely to be infected with multiple HPV types. However, CT *ompA* genovar and CT bacterial load did not appear to influence the prevalence of HPV infection, indicating that factors beyond the CT strain and its load may affect HPV co-infection rates.

Globally, the prevalence of HPV and CT co-infections varies widely from 4-59% (Paba *et al.,* 2008; Silva *et al.,* 2014). In women from diverse age ranges in Inner Mongolia and China, high HPV/CT co-infection rates and multiple HPV infections have been linked to an increased risk for abnormal cytology (Ji *et al.,* 2019). Similarly, Colombian women with HPV/CT co-infections were more likely to be infected with multiple HPV types (Quinonez-Calvache *et al.,* 2016).

Both HPV and CT share a common transmission route, have the capacity to invade cells and interact with the innate immune system and metabolic processes, potentially leading to

disruptions in cellular pathways that control cell proliferation (Chen *et al.,* 2020). CT infections can induce chronic cervical inflammation (Beatty *et al.,* 1994) and cervical hypertrophy (Markowska *et al.,* 1999; Markowska *et al.,* 2002), which may facilitate HPV's access to the basal epithelial layer. This localized inflammation caused by *Chlamydia*, regardless of the HPV genotype (Seraceni *et al.,* 2014), might increase women's susceptibility to HPV infections due to the damage inflicted on epithelial tissue.

In this cohort, persistence of HR-HPV infections was observed, which are also more likely to develop into precancerous lesions and potentially progress to cancer (Burd, 2003; Graham 2017; Vintermyr *et al.,* 2018; Della Fera *et al.,* 2021). Interestingly, co-infection with CT seemed to exacerbate the persistence of HPV, particularly HR-HPV types. This persistence could be attributed to the relatively immature immune systems of these young women, as well as the requirement for a robust cell-mediated immune response for established HPV lesions to regress (Schiffman *et al.,* 2016). Additionally, CT may impede the immune response, evidenced by a reduction in effector T-cell populations, dendritic cell activation, and production of proinflammatory cytokines/chemokines, which could enhance viral persistence (Vriend *et al.,* 2015; Di Pietro *et al.,* 2018). The complex interplay of these factors highlights the importance of understanding the dynamics between CT infections, immune response, and HPV persistence to better manage and prevent the potential progression to cervical neoplasia.

Some limitations should be acknowledged. In this cohort, a few CT+ samples were excluded due to an insufficient amount of DNA available for typing. The approach used to quantify CT DNA could not differentiate viable and non-viable bacteria. PCR testing also has limitations as it amplifies the most dominant CT strain, meaning that it has limited ability to detect whether mixed genotypes were causing infections. HPV genotyping detected a total of 36 types most commonly associated with cervical disease or sequalae, although >200 HPV types have been described. As such, HPV types not in the commercial 36-array could not be detected so HPV negative results should be considered tentatively.

There is need to move away from syndromic management because most CT and HPV infections are asymptomatic in AGYW. There is need to increase efforts to raise awareness about STIs, emphasizing the importance of screening, vaccination, and safe sexual practices. We used hr-MLST, the next step is to do whole genome sequencing to study intracellular CT. Whole genome sequencing contributes to the design of better diagnostic tools and enhances

the effectiveness of screening programs. There is also need for further investigations to look into inflammatory cytokines in both women with HPV and women and women without any HPV or other STIs.

This thesis sheds light on the alarmingly high rates of CT and HPV infections, underscoring a significant silent burden of STIs among asymptomatic AGYW. Remarkably, all participants with STIs displayed no symptoms. Recruited for a study evaluating health care options, these women were unaware of their CT and HPV statuses, highlighting a critical gap in sexual health awareness and the potential for unknowing transmission. The clinical implications of these findings are substantial. Undiagnosed and untreated STIs like CT can provoke genital inflammation, which is known to elevate the risk of both HIV transmission and acquisition. There is an urgent need for a shift in policy away from syndromic management of STIs towards a model that includes routine STI screening as a fundamental aspect of health care for young women. Implementing STI screening in this vulnerable demographic is crucial for preventing the spread of HIV.

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Appendix 2.1

Preparation of plasmid standards for qPCR

Two plasmid standards harbouring fragments of the *omcB* gene and the CT plasmid ORF2 gene were constructed for use in the qPCR assay. The P1/P2 and P3/P4 primer pair (qPCR Pickett *et al*., 2005, pORF2[Ctrachplas11F and Ctrachplas119R]; *omcB* [Ctrach60k127F and Ctrach60k232R]) were used to amplify the *omcB* and ORF2 gene products, respectively, from gDNA purified from a sample known to contain CT DNA. Reactions (50µl total volume) consisted of: 25µl Kapa HiFi 2x Ready-mix (Roche, USA), 2.5µl of each primer (10 µM), 1µl DNA template and 19µl molecular grade water. PCR cycling conditions in each case were 95°C for 5min followed by 35 cycles of 95°C for 30sec, 61°C for 30sec and 72°C for 30sec. A final polishing step of 72°C for 5min was included. Products of the correct size were confirmed by gel electrophoresis on 2% agarose gels stained with Condasafe (Condalab, Spain) and visualised using a GelDoc instrument (Bio-Rad, Molecular imager, Gel Doc XR+).

Confirmed PCR products were cloned into the pJet1.2 cloning vector (Thermo Fisher Scientific, USA) using the blunt end cloning protocol provided by the manufacturer. Ligated constructs were cloned into chemically competent *E. coli* DH5α (Thermo Fisher Scientific, USA) using the heat shock method. Briefly, 10µl of each ligation mix was added to 50µl of competent cells and incubated on ice for 30mins. Cells were heat shocked at 42°C for 30secs and then cooled on ice for 2min, after which 250µl of prewarmed recovery medium (SOC, Thermo Fisher Scientific, USA) was added and cells were incubated at 37°C for one hour. Transformants were selected by plating onto Luria broth agar plates containing ampicillin (100µg/ml) and confirmed by colony PCR and Sanger Sequencing using the pJet1.2 sequencing primers (Thermo Fisher, USA).

Appendix 4.1

PCR conditions for HPV amplification

PCR cycles	Temperature	Time
1 cycle	94ºC	3 min
15 cycles	94ºC	30 s
	47ºC	30 s
	72ºC	30 s
35 cycles	94ºC	30 s
	65ºC	30 s
	72ºC	30 s
1 cycle	72ºC	5 min
	8ºC	∞
Post PCR denaturation step - 1 cycle	95ºC	10 min