Effect of progestin-based hormonal contraceptives on genital inflammation and Th17 cell activation in adolescents at high risk for HIV infection

by

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This dissertation is dedicated to my husband **Abisai Konstantinus**, and my dearest parents **Loide Namutenya** and **Jason Mbodo**. Thank you for your continuous prayers and believing in me.

Psalm 23

Plagiarism Declaration

I, **Iyaloo Konstantinus**, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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List of Abbreviations

AGYW	Adolescent girls and young women
AIDS	Acquired Immunodeficiency Syndrome
AMP	Antimicrobial peptide
APC	Allophycocyanin
APC	Antigen presenting cell
ART	Antiretroviral therapy
BARC	Bioanalytical Research Corporation Laboratories
BD	Becton Dickinson
BD	Betadefensin
BMI	Body mass index
BV	Bacterial vaginosis
CCR	Chemokine (C-C motif) receptor
CCVR	Combined contraceptive vaginal ring
CD	Cluster of differentiation
CI	Confidence interval
CMC	Cervical mononuclear cell
COCPs	Combined oral contraceptive pills
CT	Community type
CTL	Cytotoxic T lymphocytes
CXCL	Chemokine (C-X-C motif) ligand
DCs	Dendritic cells
DMPA	Depo-medroxyprogesterone acetate
ENA	Epithelial-derived neutrophil-activating
ENG	Etonogestrel
EE	Ethinylestradiol
E2	Estradiol
FCS	Fetal calf serum
FRT	Female reproductive tract
FSH	Follicle stimulating hormone
FMO	Fluorescent minus one

FSC	Forward scatter
G-CSF	Granulocyte colony stimulating factor
GnRH	Gonadotropin-releasing hormone
GM-CSF	Granulocyte/macrophage colony stimulating factors
GR	Glucocorticoid receptor
HBD	Human beta defensin
HC	Hormonal contraceptive
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
HLA-DR	Human leukocyte antigen-D related
H_2O_2	Hydrogen peroxide
HR	Hazard ratio
HSV	Herpes simplex virus
IFN	Interferon
IL	Interleukin
IQR	Interquartile range
ITT	Intention to treat
IUD	Intrauterine device
LH	Luteinizing hormone
LPS	Lipopolysaccharide
LNG	Levonorgestrel
MC	Menstrual cup
MIP	Macrophage inflammatory protein
MSM	Men who have sex with men
NET-EN	Norethisterone oenanthate
NICD	National Institute of Communicable Diseases
NK	Natural killer
NOD	Nucleotide-binding oligomerization domain
OR	Odds ratio
OTU	Operational taxonomic unit
P4	Progesterone
PCR	Polymerase chain reaction
PE-Cy	Phycoerythrin-cyanine

pH	Potential of hydrogen
PID	Pelvic inflammatory disease
PP	Per protocol
PrEP	Pre-exposure prophylaxis
PRR	Pattern recognition receptor
PR	Progesterone receptor
RPMI	Roswell Park Memorial Institute
SA	South Africa
sCD40L	Soluble CD40-ligand
SDF-1	Stromal cell-derived factor 1
SIV	Simian immunodeficiency virus
SLPI	Secretory leukocyte protease inhibitor
SSA	Sub-saharan Africa
SSC	Side scatter
SST	Serum separating tube
STI	Sexually transmitted infection
TGF	Transforming growth factor
Th	T-helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	T-regulatory
WHO	World Health Organization
UNAIDS	United Nations Programme on HIV/AIDS
VMB	Vaginal microbiome/microbiota

Abstract

Background: Adolescent girls and young women (AGYW) are at high risk for HIV infection, particularly in Southern Africa. In addition, some hormonal contraceptives (HC), such as progestin only-injectable contraceptives DMPA and NET-EN, have been associated with significantly increased risk for HIV infection. These HC together with sexual immaturity may increase activation of CD4+ T cells in the female reproductive tract (FRT), which are target cells for HIV infection. NuvaRing, also a long-acting progestin-containing contraceptive albeit topical, has recently been introduced to South Africa, and may offer an improved safety profile over NET-EN and DMPA in terms of HIV risk for young women. Recently, Th17 cells have been found to be disproportionally susceptible to HIV infection compared to the other T helper subsets although the impact of HC use on Th17 cell frequency and activation has not been investigated. Here, the impact of NuvaRing, NET-EN and combined oral contraceptive pills (COCPs) on the vaginal microenvironment of the FRT in AGYW was investigated as this relates to HIV risk, with particular focus on cervical Th17 cells and their related cytokines (Th17-related cytokines).

Methods: One hundred and thirty HIV-negative adolescent girls between the age of 15 and 19 years were enrolled into a randomized, controlled crossover study comparing NuvaRing (n=45), NET-EN (n=45), and COCPs (n=40) for 16 weeks (~4 menstrual cycles). At crossover (16 weeks), the AGYW changed to another method for the following 16 weeks: 23 of those who used NuvaRing changed to NET-EN while 8 changed to COCPs; 23 of those who used NET-EN and 24 of those who used COCPs changed to NuvaRing. The protocol included three study visits in total (screening, crossover, study completion visits). Of the 130 adolescents enrolled, 107/130 reached the crossover visit and 92/130 reached the study exit visit. All adolescents were screened for STIs (multiplex PCR for *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis* and *Mycoplasma genitalium*), BV (by Nugent scoring), and yeast (visible hyphae on gram stain) at all study visits. Data on relative abundance of vaginal microbial community types (CTs) from ectocervical swabs was available for this study, determined by 16S rRNA sequencing of the V4 region. Several genital samples were

collected, including Digene cervical cytobrushes (for flow cytometry of cervical T cells) and menstrual cups at each study visit (for measurement of genital cytokine concentrations). Multiparameter flow cytometry was performed on cytobrushes to determine the frequency and activation status (CD38 and HLA-DR) of Th17 cells (defined by expression of CCR6+CCR10-), total CD3+CD4+ T cells and CD3+CD8+ including Tc17 cells (CD8+ CCR6+). A panel of fifteen Th17-related cytokines (IL-17A, IL-17F, IL-21, IL-22, IL-6, IL-1β, IL-23, IL-33, TNF-α, IL-4, IL-10, IL-25, IL-31, IFN-γ and sCD40L) were measured in genital secretions by Luminex. Results have been presented as an intention to treat (ITT) and per protocol (PP; which accounted for early switching of HC products prior to the crossover visit). Unless otherwise stated, all statistical testing was non-parametric, and $P \leq 0.05$ were considered significant. The Benjamini-Hochberg method was used to adjust for multiple comparisons.

Results: In the FRT of adolescents at baseline (before randomization), Th17 cells (CCR6+CCR10-) were found to be the major CD4+ T cell subset in cytobrushes (median 54.4%, IQR 43.7% - 64.3%) compared to CCR6-CCR10- (median 42.2%, IQR 33.5% - 52.6%), CCR6+CCR10+ (median 1.2%, IQR 0.4% - 2.8%) and CCR6-CCR10+ (median 0.8%, IQR 0.2% - 1.9%). Higher frequencies of Th17 cells expressed CCR5 compared to CCR6-CCR10- CD4+ T cells (median 68.0% vs 56.2% respectively, $p<0.0001$). However, Th17 cell frequencies did not correlate with genital tract Th17-related cytokines at baseline. The presence of BV or STIs did not appear to influence either the frequencies or activation status of cervical Th17 cells. Although BV (Nugent 7-10) and having a non-*Lactobacillus*-dominated vaginal microbiome (C1) was associated with increased concentrations of all Th17-related cytokines (IL-17A, IL-17F, IL-21, IL-22, IL-6, IL-1β, IL-23, IL-33, TNF-α, IL-4, IL-10, IL-25, IL-31, IFN-γ and sCD40L) compared to those without BV (Nugent 0-3) or C2/3, while adolescents with any STI had increased concentrations of IL-1β and IL-17A compared to those without an STI. After being randomized on to HC for 16 weeks, cervical cytobrush-derived immune cells were analysed within individuals in each arm (intra-individual) and between individuals in the three contraceptive arms, using both ITT and PP approaches. Although the frequency and activation status of cervical Th17 cells was similar across the three HC arms, adolescents using NuvaRing and NET-EN had significantly increased activation (CD38+HLA-DR+) on Th17 cells compared to their respective baselines ($p=0.02$ and

p=0.03, respectively), which was not evident in those using COCPs. Furthermore, adolescents using NuvaRing had reduced frequencies of Th17 cells compared to baseline (p=0.001), which was not evident in those using NET-EN or COCPs. Although it was hypothesized that NuvaRing would offer some safety advantage over NET-EN in terms of mucosal HIV target cell activation, intra-individual analysis showed a significant increase in the frequency of highly activated cervical Th17 cells in those adolescents who started using the ring. A significant increase in genital tract concentrations of several Th17-related cytokine concentrations (including IL-21, IL-1β, IL-33, TNF-α, IL-4, IFN-γ and sCD40L) was noted in adolescents assigned to NuvaRing after 16 weeks of use, suggesting that the presence of the vaginal ring likely increased genital cytokine responses. Although the frequency and activation of CD8+ T cells was similar across HC arm, intra-individual analysis showed changes in the frequency of activation markers on CD8+ T cells in all HC arms. Moreover, frequencies of Tc17 cells were significantly reduced after 4 months of contraceptive use in each HC arm compared to baseline frequencies.

Conclusion: In summary, CCR6+CCR10- Th17 cells were confirmed to be the major CD4+ T cell subset in the FRT of young adolescents. The use of NuvaRing led to decreased frequencies of Th17 cells which were highly activated, and was also associated with an increase in Th17-related cytokines compared to NET-EN and COCPs. All HC altered activation of cervical CD8+ T cells and reduced the frequencies of Tc17 cells. The dramatic alterations observed in cervical immune cells associated with the use of NuvaRing compared to NET-EN and COCPs warrant further investigations.

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1.1 Introduction

Despite some remarkable advances in treatment and more modest advances in prevention, HIV-1 continues to be a global epidemic and AIDS has claimed an estimated 35 million lives. By the end of 2016, UNAIDS estimated that around 40 million people were HIV-infected, and 1.8 million people became newly infected globally (UNAIDS, 2017). While HIV is a global health emergency, African countries bear the highest burden, with approximately 19 million people in this region living with HIV, and approximately 7 million are living in South Africa (SA). Sub-Saharan Africa (SSA) accounts for about 43% of the global total new infections. The scale up of antiretroviral therapy (ART) in eastern and southern Africa, together with improved public health measures such as male circumcision programs, have contributed to a decline of up to 29% of new infections between 2010 and 2016 (UNAIDS, 2017). In lower HIV prevalence settings, the key risk populations continue being men who have sex with men (MSM), sex workers, prisoners and injectable drug users. In high-prevalence settings, adolescent girls and young women (AGYW) between the ages of 15-24 are the key risk population, with 80% of young women who become infected residing in SSA. Even in the era of ARTs, AIDS-related illness remains the second leading cause of death among women of reproductive age in Africa because of poor access to or uptake of HIV screening and/or drugs (UNAIDS, 2017).

This dissertation will focus on HIV-1 risk factors in AGYW living in Southern Africa.

1.2 Sub-Saharan African young women and HIV risk

Women make up the fastest-growing group of people living with HIV-1, especially in SSA (Cowan and Pettifor, 2009). The majority of new HIV infections in SSA are by heterosexual transmission, with younger females carrying a disproportionate burden of HIV infection. AGYW in SSA aged 15-24 years currently represent 25% of new infections, being at approximately eight fold higher risk for HIV infection than males in the same age groups (UNAIDS, 2017). It has also been reported that these younger women acquire HIV 5-7 years earlier than their male peers

(Kharsany and Karim, 2016). Like countries within SSA, the gender discrepancy in HIV prevalence is observed across many African countries. South Africa has the highest HIV prevalence amongst 15-24 year old females (13%) and the largest gender discrepancy (difference of 9%; Dellar et al., 2015). Figure 1.1 summarizes the prevalence of HIV according to gender in southern African countries.

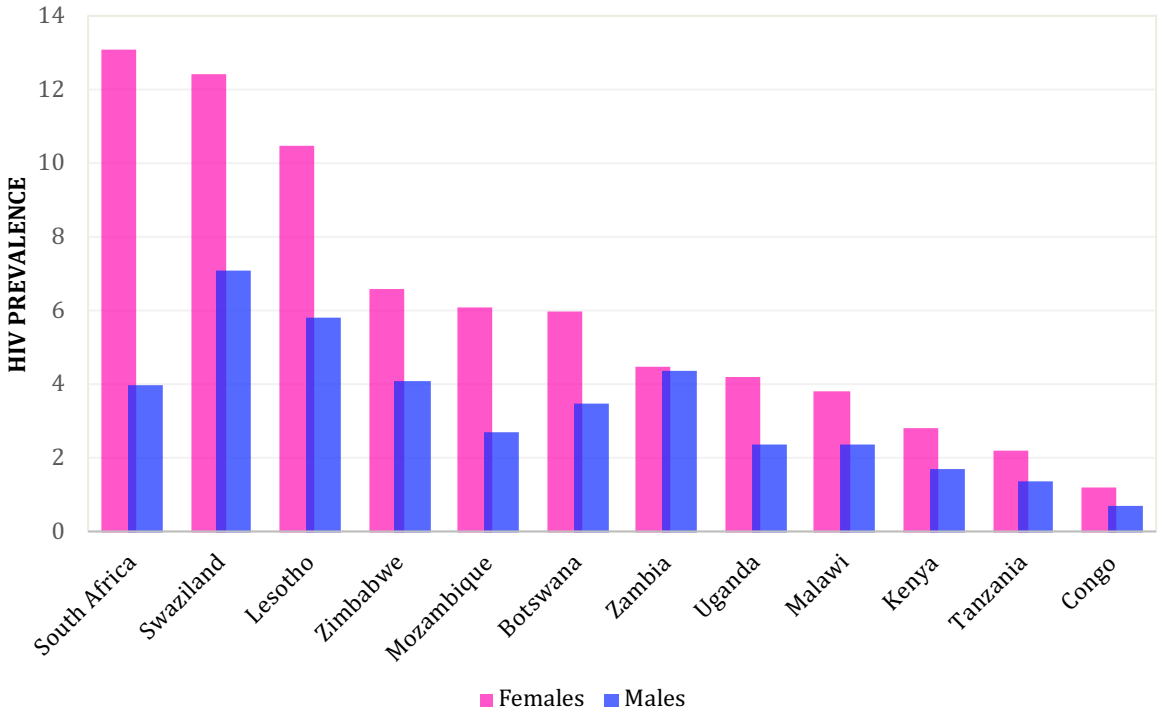

Figure 1.1. HIV prevalence in selected Southern African countries among females (pink bars) and males (blue bars) between 15-24 years old in 2013. Adapted from data reported by Kharsany and Karim, 2016. Graph drawn by Iyaloo Konstantinus.

Although almost certainly multi-factorial in cause, the disproportionate impact of the HIV epidemic on African women has been attributed to various cultural, behavioral, social, economic and biological factors (Ramjee and Daniels, 2013; Mabaso et al., 2018). Women in SSA in some African cultures are not empowered, and do not play a major role in decision-making about sex (Jewkes et al., 2010). Some traditions in Africa still allow marriage for girls as young as 10 years old, which are frequently arranged and sometimes associated with violence within

marriage (Wadesango et al., 2011; Lilian et al., 2015). Moreover, the illegal cultural practice of genital female mutilation that are practiced in some African countries have been associated with increased risk for HIV transmission (Olaniran, 2013). Other cultural practices that may influence HIV acquisition within the African continent include polygamy and the practice of dry sex (Mswela, 2009; Ramjee and Daniels, 2013).

Poverty has also been suggested as an important driver of HIV risk in women, as some resort to transactional sex (Muula, 2008). Young girls from socioeconomically deprived families are sometimes forced or coerced into having sexual relationships with older men, also known as "blessers" in South Africa (Mampane, 2018). As a function of the age and economic disparity between young girls and their older partners, the AGYW cannot negotiate safe sex options. Other behavioral factors (like alcohol consumption and drug abuse, unprotected sex and early sexual debut) or cleansing and sexual practices (like vaginal cleansing or douching, or use of sex enhancers inserted into the vagina) have also been shown to increase women's risk for HIV infection (Muula, 2008; Ramjee and Daniels, 2013, Idele et al., 2014). Due to the high prevalence of HIV in SSA, these young women are likely to be exposed frequently to HIV, making them a highly susceptible group.

1.3 Structure and immunity of the female reproductive tract

The female reproductive tract (FRT) has two main functions: to maintain a healthy, growing fetus; and to protect against pathogens (Wira et al., 2005a). The FRT can be divided into upper and lower sections. The upper FRT is comprised of the endocervix, uterus, fallopian tubes and ovaries (Figure 1.2). The lower FRT is made up of the vagina and the ectocervix. Both innate and adaptive immunity protect the FRT (Hickey et al., 2011). Innate immune mechanisms include the epithelium, innate immune cells (neutrophils and natural killer [NK] cells), mucus, complement, antimicrobial peptides (AMPs) and low pH, which are critical in preventing penetration of pathogens.

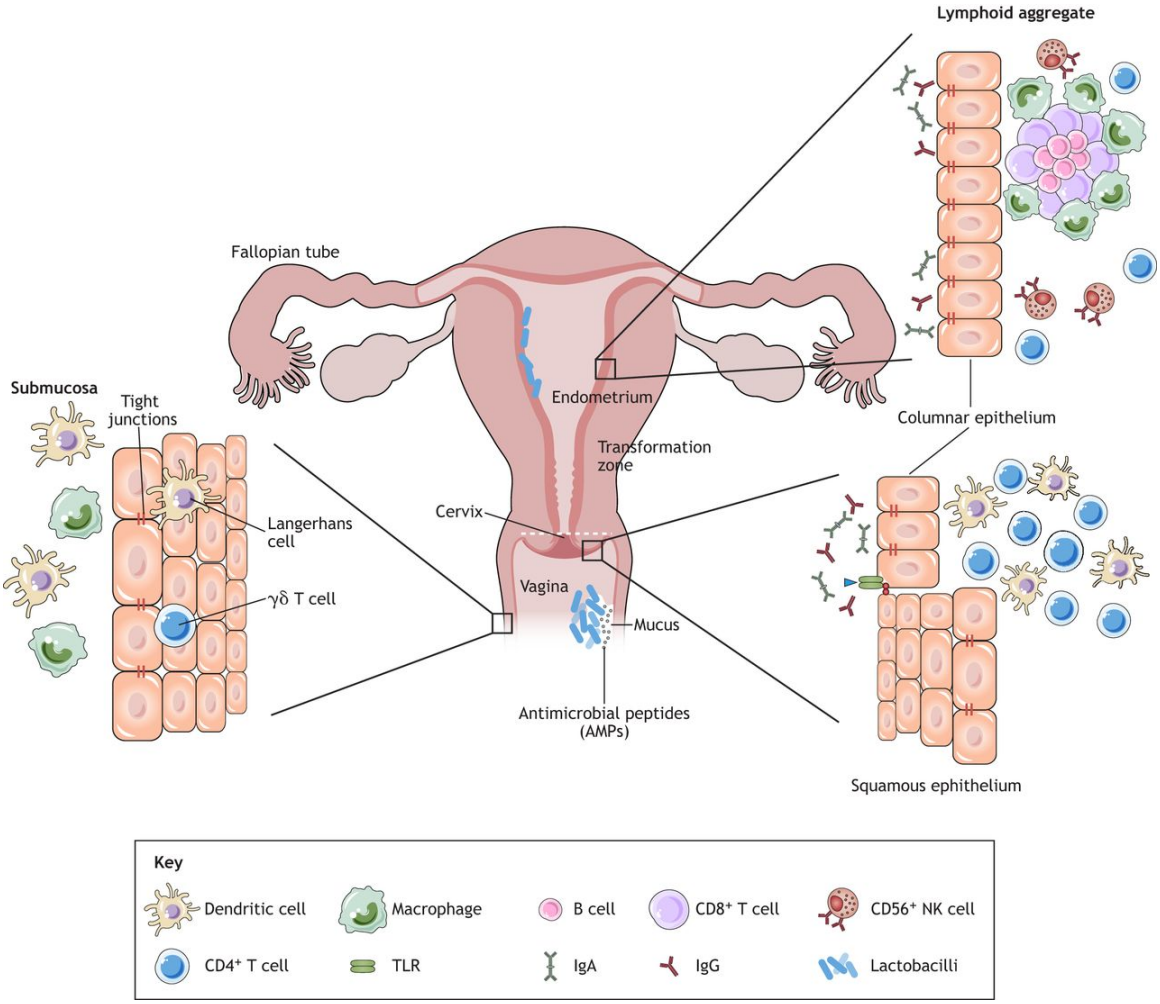

Figure 1.2. Anatomy and immune cells in the female reproductive tract. The upper FRT including the uterus and endocervix is lined by a simple columnar epithelium. The lower FRT including the vagina and ectocervix is lined by the stratified squamous epithelium. Target cells including macrophages and T cells are found in the lamina propria. Other immune protection mechanisms found in the vaginal epithelium include mucus, lactobacilli, AMPs, tight junctions, antibodies such as IgA and IgG. Lymphoid aggregates composed of B cells (inner) and CD8+ T cells (outer) are found in the endometrium, and they can be found surrounded by NK cells and CD4+ T cells. Figure taken from Wessels et al., (2018).

The epithelium provides a physical barrier to potential pathogens in the environment, without compromising critical reproductive functions like fertilization (Wira et al., 2005b). The upper FRT is lined with a monolayer of

columnar epithelial cells, which are tightly apposed to one another by tight junctions (Figure 1.2). This region of the FRT was originally thought to be sterile, and that infections are the result of ascending pathogens from the lower FRT. However, a more recent study in macaques showed that SIV could also infect CD4+ cells in the ovaries and other regions of the upper reproductive tract (Stieh et al., 2016). In addition, commensal bacteria have been detected in the uterus and fallopian tubes of healthy women (Chen et al., 2017). In contrast to the upper FRT, the lower FRT is lined by the multi-layer non-keratinized stratified squamous epithelium (Wira et al., 2005b). The region of the lower FRT where the multi-layer squamous epithelium of the ectocervix intersects with the simple columnar epithelium of the endocervix is known as the transformation zone (Pudney et al., 2005). The multi-layer squamous epithelium of the lower FRT and ectocervix also functions as a protective barrier against pathogens.

Epithelial cells making up the simple columnar and squamous epithelium express pattern recognition receptors (PRRs) which are important host receptors to initiate immune responses against pathogens, and include Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) receptors (Pioli et al., 2004; Ghosh et al., 2013b). In addition to providing a physical barrier to pathogens, *in vitro* studies have shown that epithelial cells can produce a variety of cytokines and chemokines (chemotactic cytokines) that recruit immune cells to mucosal surfaces. In addition, they produce anti-proteases and antimicrobial peptides (small peptides with the ability to directly neutralize and kill pathogens) which are important innate factors in immune defense and protecting epithelial cells from damage caused by inflammation (Fichorova et al., 2002; Wira et al., 2005a; Nguyen et al., 2014; Burgener et al., 2015; Nazli et al., 2018). Of the AMPs found in cervicovaginal secretions, the α- and β-defensins have been the best described (Dasari et al., 2007; Fan et al., 2008). Mucus, secreted by Bartholin glands in the vagina (located posterior and to the left and right of the opening of the vagina) and goblet cells in endocervical crypts, provides a natural physical barrier that can trap HIV and prevent the virus from penetrating through the epithelial cell layer (Fahey et al., 2005; Ferreira et al., 2014; Nguyen et al., 2014). The mucus also lubricates the vagina (primarily from Bartholin glands) to protect

against damage to the epithelial layer during sexual intercourse. Another important component of cervicovaginal mucus is its acidic pH (pH <4.5) that has been shown to slow down the rate of HIV diffusion (Lai et al., 2009). *Lactobacillus* species found in the healthy lower FRT produce lactic acid and hydrogen peroxide (H_2O_2), as bi-products of glycogen metabolism, are needed to maintain this acidic environment of the vagina (Antonio et al., 1999; Mirmonsef et al., 2011; Ravel et al., 2011).

Macrophages, NK cells, neutrophils and dendritic cells (DCs) are distributed in both the upper and lower FRT (Wira et al., 2005a). Macrophages tend to be detected at higher concentrations in the endometrium and myometrial tissues, while DCs tend to be found in the epithelial layer (Reis Machado et al., 2014). Neutrophils concentrations are reported to be higher in fallopian tubes (upper FRT) and decrease in concentration towards the vagina, where they respond to pathogens by phagocytosing them and/or by producing AMPs (Wira et al., 2005a).

Beneath the squamous epithelium is a structural layer made up of stromal fibroblasts that supports the tissues. These stromal cells contain a diverse population of immune cells, of which CD3+ T lymphocytes are the most abundant (Givan et al., 1997; Nguyen et al., 2014; Wessels et al., 2018). CD4+ T cells are distributed throughout the FRT (lower and upper; Stieh et al., 2014), although earlier studies suggested that these were present at higher concentrations in the cervical transformation zone than the vagina (Pudney et al., 2005). CD4+ T cells have been further sub-divided into several T helper (Th) subsets, including Th1 (which are defined by their ability to produce IFN-γ, IL-2 and TNF-α), Th2 (producing IL-4, IL-5 and IL-13), Th17 (producing cytokines like IL-17A, IL-17F, IL-21 and IL-22) and T regulatory (Treg) cells (producing IL-10, IL-9 and TGF-β; Hirahara and Nakayama, 2016). The differentiation of CD4+ T cells is directly or indirectly mediated by cytokines and is influenced by a number of factors, including antigen stimulation (Zhu and Paul, 2008; Golubovskaya and Wu, 2016).

1.3.1 HIV-1 infection within the FRT

Although it was generally thought that HIV infection of CD4+ cells within the FRT occurs through the vagina, ectocervix or endocervix (Shen et al., 2012), more recent evidence suggests that this can happen throughout the FRT, including CD4+ T cells resident in the ovaries and the fallopian tubes (Stieh et al., 2014, 2016). Experiments in macaques have suggested that vaginal SIV infection can be mediated by both cell-free and cell-associated viruses (Hladik and McElrath, 2008; Barreto-de-souza et al., 2014; Kolodkin-Gal et al., 2013), although little evidence exists for the latter.

HIV has been proposed to cross the FRT mucosal barrier through micro-abrasions in the FRT squamous epithelium, allowing direct infection of sub-mucosal CD4+ T cells, or via transcytosis of the virus across the columnar epithelium of the upper FRT (Nguyen et al., 2014). Other mechanisms that have been suggested include diffusive percolation in the squamous epithelium (Zhang et al., 1999; Carias et al., 2016). Following entry, HIV gains access to CD4+ immune cells like macrophages, DCs, Langerhans cells and T cells (Shattock and Moore, 2003). HIV primarily establishes infection, following sexual exposure, through CD4+ T cells expressing chemokine receptor CCR5 (Berges et al., 2008; Grivel et al., 2010). HIV has been shown to infect and replicate in sub-epithelial CD4+ mononuclear cells, after which these traffic to draining lymph nodes and subsequently are thought to spread systemically (Haase, 2011; Shen et al., 2012).

1.4 Th17 cells and Th17-related cytokines

Th17 cells were first described in mice, and later in humans (Park et al., 2005). They have been characterized by the expression of cell surface markers (like CCR6, CCR4, CD161, and IL-23R), transcription factors (like RORγt or RORC2), and production of cytokines (like IL-17; Annunziato et al., 2007; Singh et al., 2008). Th17 cells have been shown to play an important role in both anti-microbial and fungal immunity (Feinen et al., 2010; Conti and Gaffen, 2015; Li et al., 2018). Th17 cells recruit neutrophils and myeloid cells to effector sites by producing cytokines,

and they are also important in epithelial regeneration in mucosal tissues (Pelletier et al., 2014; Brockmann et al., 2017). Th17 cells differentiate from naïve CD4+ T cells following antigen priming, in the presence of cytokines like interleukin (IL)-6, IL-21, IL-23, IL-1β, IL-33, tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β (Khader et al., 2009; Korn et al., 2009). The functions of cytokines involved in Th17 differentiation (called Th17-related cytokines) have been best described in the mouse model (Harrison et al., 2008; Patel and Kuchroo, 2015; Wacleche et al., 2017). Both IL-6 and TGF-β are necessary to initiate Th17 differentiation (Sutton et al., 2006). IL-21 promotes Th17 differentiation, but is also produced by differentiated Th17 cells to regulate immune responses (Wei et al., 2007). IL-23 further commits the differentiated cells to the Th17 lineage (Revu et al., 2018). IL-1β, in combination with IL-6 and IL-23, is involved in differentiating CD4+ T cells into Th17 cells (Lasiglié et al., 2011). The function of IL-33, although not well defined, enhances Th17 cell differentiation by inducing DC maturation (Park et al., 2017). Overall, these cytokines also induce the expression of the transcription factors RORγt and RORα which are needed for Th17 differentiation (Awasthi and Kuchroo, 2009).

Studies of Th17 cells in the gut have suggested that differentiation of Th17 cells is influenced by the gut microbiota and the prevailing cytokine environment (Ivanov et al., 2008). Germ-free mice have reduced Th17 cells, although numbers of Th17 cells can be increased by colonizing their gut with conventional microbiota (Ivanov et al., 2008). Therefore, the composition of gut microbiota can directly or indirectly influence the development and phenotype of Th17 cells.

Th17 cells are characterized by their ability to produce IL-17A, IL-17F, IL-21 and IL-22 (Bettelli et al., 2008). Th17 cells producing IL-17 are the best described. Cytokines produced by Th17 cells have broad effects and can act in synergy with various other inflammatory cytokines (Korn et al., 2009). IL-17A and IL-17F are important in host defence and inflammation by inducing pro-inflammatory cytokine, chemokine and metalloproteinase production in various tissues (Hymowitz et al., 2001; Schofield et al., 2016). Moreover, IL-17A and IL-17F share a 50% amino acid sequence homology and bind to the same IL-17 receptor, and

have similar biological functions (Ishigame et al., 2009; Ouyang et al., 2012). Both of these IL-17 cytokines induce production of IL-6, several chemokines (like CXCL1 [GRO-1], CXCL2 [macrophage inflammatory protein {MIP}-2a], and CXCL5 [epithelial-derived neutrophil-activating {ENA} peptide 78]) and cytokines including granulocyte stimulating factors (granulocyte colony stimulating factor [G-CSF], granulocyte/macrophage colony stimulating factors [GM-CSF]). The released cytokines and chemokines promote different functions including the activation and migration of neutrophils to sites of inflammation (Bradley Forlow et al., 2001; Miyamoto et al., 2003). IL-17A and IL-17F also induce AMPs production including lipocalin and the β-defensins that prevent infection at mucosal surfaces (Liang et al., 2006; Aujla et al., 2008; Monteleone et al., 2011). Both are important for the clearance of extracellular bacteria such as *Staphylococcus aureus* and *Citrobacter rodentium* (Ishigame et al., 2009; Jin and Dong, 2013). Despite their similarity, IL-17F has a much lower cytokine-inducing potency than IL-17A (Sueki et al., 2008; Ouyang et al., 2012; Brembilla et al., 2018).

If not regulated, the inflammatory nature of Th17 cells cause pathology and Th17 cells have been associated with the development of autoimmune diseases and chronic inflammation (Kuwabara et al., 2017). Cytokines known to regulate Th17 cells include IL-25, IL-31, soluble CD40-ligand (sCD40L), IL-4, IL-10 and interferon (IFN)-γ (Martinez et al., 2008; Brockmann et al., 2017). Some of these cytokines regulate Th17 cells by increasing or decreasing their differentiation pathway signals. For example, sCD40L regulate Th17 cell differentiation by providing the optimal cytokine environment (Iezzi et al., 2009). IL-10, in contrast, directly suppresses cytokine secretion by T cells and macrophages, including IL-17 (Gu et al., 2008). IFN-γ and IL-4 negatively regulate Th17 responses by promoting Th1 and Th2 responses, respectively (Harrington et al., 2005). Furthermore, IL-25 has been shown to suppress Th17 immune responses by upregulating Th2 cytokines like IL-4 (Liu et al., 2016). IL-31, mainly produced by Th2 and mast cells (Zhang et al., 2008), has been found to affect the differentiation of Th17 cells. Still considered a novel cytokine (described for the first time in 2004; Dillon et al., 2004), IL-31 has been shown to limit Th2 inflammatory processes and secretion of IL-31 is triggered by IL-4 (Di Salvo et al., 2018). Furthermore, IL-31 is thought to regulate

cell proliferation, and to be involved also in tissue remodeling (Zhang et al., 2008). Th17-related cytokines are summarized in Figure 1.3 below.

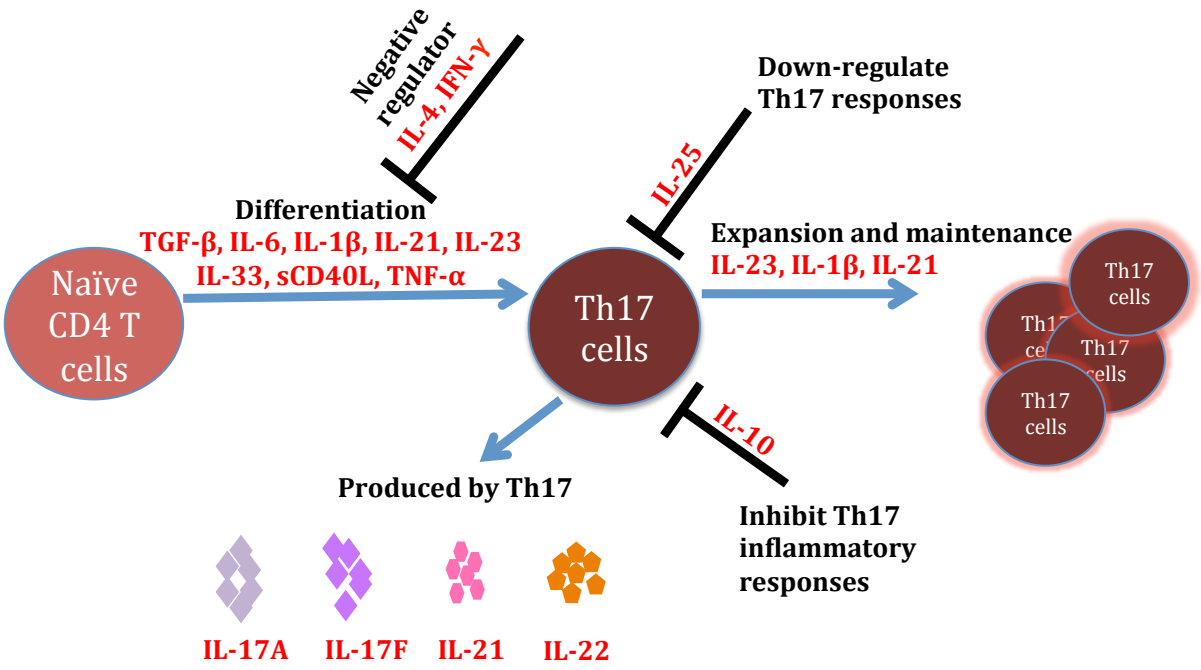

Figure 1.3. Cytokines considered to be related to Th17 cells and how they interact with Th17 cells. Naïve CD4+ T cells are primed by a combination of cytokines to differentiate into Th17 cells (including TGF-β, IL-6, IL-1β, IL-21, IL-23, IL-33, and sCD40L). IL-21, IL-1β and IL-23 further promote expansion, maintenance and survival of Th17 cells. Differentiation can be inhibited by IL-25, IL-4 and IFN-γ that favor Th2 and Th1 responses, respectively. Th17 cells directly produce IL-17A, IL-17F, IL-21 and IL-22, and the production of these cytokines can be directly inhibited by IL-10. Figure drawn by Iyaloo Konstantinus.

1.4.1 The role of Th17 cells at mucosal surfaces

Mucosal surfaces connect the human host with their environment, consisting of the respiratory, gastrointestinal, and genital tract mucosa. The role of Th17 cells in gastrointestinal and respiratory immunity has been well described in mice (Infante-Duarte et al., 2000; Khader et al., 2007; Atarashi et al., 2008; Ivanov et al., 2008). Th17 cells promote the formation of tight junctions between epithelial cells

needed to maintain the mucosal barrier integrity (Blaschitz and Raffatellu, 2010), and loss of Th17 cells have been linked with compromised mucosal barrier integrity (Kanwar et al., 2010; Hartigan-O'Connor et al., 2011; He et al., 2011). IL-17-producing cells are abundant in lamina propria of the small intestine (Ivanov et al., 2008). AMPs, like defensins, are induced through signaling pathways triggered by IL-17A and IL-17F at the intestinal mucosal surface, which have been shown to protect mice against enteric infections caused by bacteria (Rubino et al., 2012). In the lungs, IL-17 is important in protection against airborne diseases, with IL-17A playing a role in the defense against extracellular pathogens such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* in the airways (Ye et al., 2001; Kudva et al., 2011). In the oral mucosa, Th17 cells and IL-17 receptor signaling has been identified to be important in defense against oral candidiasis (Conti and Gaffen, 2010).

In the genital tract, Th17 immunity in mice has been shown to play an important role in protection against fungal and bacterial infections including chlamydia, gonorrhoea and candida (Feinen et al., 2010; Pietrella et al., 2011; Gladiator et al., 2013; Conti and Gaffen, 2015; Vicetti Miguel et al., 2016). *Ex vivo* studies using human T cells showed that *C. albicans* primed Th17 cells produce IL-17 and IFN-γ under the regulation of IL-1β (Zielinski et al., 2012). Th17 immunity might also mediate protection following intravaginal HSV-2 challenge (Anipindi et al., 2016). The role of Th17 immunity in the reproductive tract of women is not well defined, however, and more studies are needed to investigate the protective properties offered by these cells.

1.4.2 The role of Th17 cells in HIV infection

Th17 cells have been described to be the subset predominantly infected by HIV and SIV in rhesus macaques (Favre et al., 2009; Hed et al., 2010; Stieh et al., 2016). In macaques vaginally challenged with SIV, approximately 85% of all SIV-infected CD4+ T cells immediately after infection expressed the chemokine receptor CCR6, which binds to the cytokine MIP-3a (Stieh et al., 2016). Moreover, CCR6+ Th17 cells have been shown to harbour more HIV DNA compared to CCR6- CD4+ T cells

(Gosselin et al., 2010). Studies in HIV chronically infected adults have found that gastrointestinal tract Th17 cells were depleted compared to those found in HIV-uninfected individuals (Prendergast et al., 2010). Since CCR6 is a mucosal homing receptor, one of the explanations for the increased susceptibility of these cells to HIV infection might be due to their migration to areas where HIV readily infects, such as the genital tract (Rodriguez-Garcia et al., 2014; Stieh et al., 2016). Their role in mucosal epithelial repair also places them where barrier integrity is weakened, which are points at which HIV can easily enter.

Ex vivo experiments using mixed cell suspensions from tissues taken from the FRT of women found the highest number of HIV-infected Th17 cells in the ectocervix compared to endocervix and endometrium (Rodriguez-Garcia et al., 2014). Interestingly, Th17 cells at all the three sites expressed higher levels of CCR5 compared to CCR6- CD4+ T cells. Expression of CCR5 together with mucosal integrin α4β7, which binds to HIV-1 gp120 and enhances viral dissemination, may increase susceptibility to HIV (Sivro et al., 2018). Th17 cells were also dramatically depleted in the cervical mucosa after HIV infection, suggesting preferential infection (McKinnon et al., 2011; Masson et al., 2015b). Given the susceptibility of Th17 cells to HIV infection, there is a need to study the factors influencing the characteristics of this CD4+ T cell subset, especially in key risk groups like AGYW.

1.5 Biological factors associated with HIV infection in the FRT

Women have been suggested to be more biologically susceptible to HIV infection than men, because of their larger mucosal surface area of the lower FRT that can be exposed to HIV (Ramjee and Daniels, 2013; Carias et al. 2013). During sexual intercourse, the vagina is also exposed to infectious fluids and pathogens for longer periods of time compared to the penis (Hladik and Hope, 2009). The act of sex itself is also more likely to cause tissue injury in women than men, thereby facilitating greater exposure of target cells to pathogens including HIV (Ghosh et al., 2013a). Other biological factors that may influence HIV acquisition risk in women include endogenous and exogenous hormones, hormonal contraceptives

(HCs), genital inflammation, sexually transmitted infections (STIs), and bacterial vaginosis (BV). These will be discussed in the subsequent sections.

1.5.1 Inflammation and immune activation

Inflammation is a natural response to harmful pathogens or tissue damage (Helming, 2011; Leick et al., 2014). The inflammatory mechanism is needed for activating and recruiting immune cells to the affected area, to help fight the harmful organisms, or repair tissue damage. In addition to recruiting immune cells to an affected site, inflammation also promotes cellular activation and differentiation (Pape et al., 1997; Newton et al., 2012). Cytokines such as IL-17 contribute to various aspect of inflammation (Kolls and Lindén, 2004). IL-17 play a role in recruiting immune cells to the site of infection (including neutrophils and Th17 cells), and the recruited cells continue to produce cytokines and chemokines (Roussel et al., 2010; Ouyang et al., 2012). Hence, IL-17 and Th17 cells have been associated with inflammatory diseases (Miossec and Kolls, 2012; Schofield et al., 2016; Kuwabara et al., 2017).

Genital inflammation has been linked to increased HIV risk in women (Roberts et al., 2012; Masson et al., 2015). Women with elevated chemotactic cytokines including IP-10, MIP-1α and MIP-1β in the genital tract were found to be at a heightened risk of HIV acquisition (Mlisana et al., 2012; Morrison et al., 2014; Masson et al., 2014; McKinnon et al., 2018). Immune cells are elevated in women with high concentrations of inflammatory cytokines and chemokines in the FRT, including neutrophils, CD4+ and CD8+ T cells (Pudney et al., 2005; Nkwanyana et al., 2009). In macaques, it has been shown that chemokines recruit activated CD4+ T cells targeted by SIV to the vagina (Miller et al., 2005; Haase, 2011). Earlier studies suggested that “healthy” vaginal mucosal surfaces (in the absence of inflammation) are predominantly populated by resting CD4+ T cells, which do not support HIV replication (Tang et al., 1995). Activated CD4+ T cells express high levels of HLA-DR and glycoprotein CD38 on their surface. Expression of both HLA-DR and CD38 on CD4+ T cells have been associated with high levels of HIV co-receptor expression (CCR5) in lymph nodes (Meditz et al., 2011).

A number of conditions and practices contribute to genital inflammation, including STIs (section 1.5.2) and BV (section 1.5.3), douching, cleansing, using vaginal drying agents and lubricants (Myer et al., 2005; Alcaide et al., 2016), normal hormone cycling (section 1.6) and the use of HCs (section 1.7; Jabbour et al., 2009; Morrison et al., 2014; Guthrie et al., 2015; Tasker et al., 2017).

1.5.2 Sexually transmitted infections

STIs have been linked with a heightened risk of HIV acquisition (Steen et al., 2009; Ward and Rönn, 2011). Recently, SA adolescent girls were shown to bear a higher burden of STIs compared to their male counterparts (Francis et al., 2018). A meta-analysis done in SSA also reported a higher prevalence of STIs in AGYW aged 15-24 years than older women (Torrone et al., 2018). SA and most countries in SSA have adopted the WHO guidelines of syndromic management of STIs, in which only symptomatic STIs would be treated with antibiotics (WHO, 2004). However, the majority of STIs in women in particular are asymptomatic and therefore would not be treated (Barnabas et al., 2017). In most cases in resource-limited African countries, both symptomatic and asymptomatic STIs remain undiagnosed and therefore untreated (Barnabas et al., 2017; Torrone et al., 2018). In addition to increasing HIV acquisition risk, untreated STIs may result in several other adverse consequences including pelvic inflammatory disease (PID), adverse pregnancy outcomes and infertility.

Different pathogens cause STIs, including various bacterial (such as *Chlamydia trachomatis*, *Mycoplasma genitalium*, *Treponema pallidum* [causing syphilis] and *Neisseria gonorrhoeae*), protozoal (*Trichomonas vaginalis*), and viral pathogens (herpes simplex virus [HSV]-2, human papilloma virus [HPV], and HIV).

Of the bacterial STIs, *C. trachomatis* is probably the most common bacterial STI worldwide, especially in younger women <25 years of age (Newman et al., 2015). Despite being so prevalent and recurrent, it causes a “silent epidemic” because up to 70% of cases in women appear to be clinically asymptomatic despite *C.*

trachomatis causing severe damage to the upper FRT, including infertility. Infection with chlamydia initiates both innate and adaptive immune responses in the genital tract (Hafner et al., 2008; Vasilevsky et al., 2014), and CD4+ T cells have been shown to play a central role in controlling infection through IFN-γ secretion (Nagarajan et al., 2005; Nelson et al., 2005; Nogueira et al., 2017). Chlamydia has been suggested to increase the risk of acquiring HIV-1 by increasing pro-inflammatory cytokines and disrupting genital epithelial cells (Ficarra et al., 2008; Schust et al., 2012; Buckner et al., 2016). Moreover, chlamydia positive women were reported to have a dramatic increase in the number of CXCR4 and CCR5 chemokine receptors on their CD4+ T cells in the endocervix compared to chlamydia negative women, suggesting that they may be highly susceptible to HIV-1 infection (Schust et al., 2012). In mice, it has been shown that chlamydia also elicits Th1 and Th17 immune responses that may promote both pathogen clearance and genital tract damage (Vicetti Miguel et al., 2016).

M. genitalium is also often asymptomatic, and studies have found that these infections can persist for almost two years (Jensen, 2006; Cohen et al., 2007). *M. genitalium* has been associated with PID, cervicitis and other adverse reproductive health consequences. In a systematic review and meta-analysis, *M. genitalium* was associated with increased risk of HIV acquisition, and this association was stronger in SSA where the burden of HIV is greatest (Mavedzenge and Weiss, 2009). Several studies have reported that *M. genitalium* infection in women is associated with inflammatory responses, triggering the production of cytokines and the recruitment of activated HIV target cells (Zhang and Wear, 2000; Starnbach and Roan, 2008; Wu et al., 2008; Masson et al., 2014).

N. gonorrhoeae infection has also been associated with increased HIV susceptibility in women, that may be mediated by recruitment of activated immune cells or micro-ulceration associated with infection (Levine et al., 1998). *N. gonorrhoeae* is also a major cause of PID, and this can in turn facilitate with HIV transmission (Jarvis and Chang, 2012). In a murine model, Th17 cells were shown to be critical for the clearance of *N. gonorrhoeae* (Feinen et al., 2010).

Of the protozoal STIs, *T. vaginalis* is the most common non-viral and non-bacterial STI worldwide and recently there has been an alarming rise in the prevalence of this STI in adolescents (Fichorova et al., 2015). *T. vaginalis* is a flagellated unicellular organism, which adheres to vaginal epithelial cells and causes disruption of the epithelial layer, partially due to flagellation (Schwebke and Burgess, 2004; Midlej and Benchimol, 2010). Trichomonas has also been associated with increased susceptibility to HIV, as well as HSV-2 (Hirt and Sherrard, 2015).

HSV-2 is a prevalent viral STI that accounts for approximately 30% of all STIs in SSA (Looker et al., 2015). A recent meta-analysis reported HIV acquisition to be almost tripled in the presence of HSV-2 infection in both women and men (Looker et al., 2017). In adolescent girls between the ages of 16-18 years, a study in rural KwaZulu-Natal showed a prevalence of 10% (Karim et al., 2014). Following primary infection, HSV-2 remains latent in the sacral ganglia of infected persons and reactivates periodically, causing genital ulceration and viral shedding (Croen et al., 1991). Studies have also shown that women may shed HSV-2 in their genital secretions without any clinical symptoms (Cherpes et al., 2005). However, ulceration caused by HSV-2 has been associated with increased activated CD4+ T cells, providing more target cells for HIV (Rebbapragada et al., 2007), and these CD4+ T cells expressing CCR5 persist even in healed genital lesions (Zhu et al., 2009). The high burden of HSV-2 is thought to have contributed to the high prevalence of HIV in SSA (Wald and Link, 2002; Freeman et al., 2006).

1.5.3 The vaginal microbiome and bacteria vaginosis (BV)

The vaginal microbiota (VMB) is a collection of microorganisms lying superficial to the epithelial cells of the vagina (Ma et al., 2012), that consist of bacteria that colonize the vagina, and can play a protective role against pathogens (when healthy Lactobacilli spp. predominate) (Atashili et al., 2008; Linhares et al.,2011; Witkins et., 2017). These healthy commensal microbial communities are believed to constitute the first line of defense against invading pathogens (Dethlefsen et al.,

2007). Vaginal *Lactobacillus spp*, metabolize glycogen to produce lactic acid (both D- and L-isomers) that maintain the lower FRT pH <4.5 (Huang et al., 2014; Mirmonsef et al., 2014). While the lower FRT is typically colonized by Lactobacilli spp. during health, vaginal hygiene practices, sexual behaviours and even diet may cause disturbances in the VMB (Myer et al., 2005; Hutchinson et al., 2007; Thoma et al., 2011; Alcaide et al., 2015).

Disturbances in Lactobacilli colonization of the vagina is associated with a highly prevalent, recurrent or persistent vaginal dysbiosis – called BV – which typically results in the vaginal pH increasing to >4.5 (Ravel et al., 2011; Gajer et al., 2012). BV is further characterized by an overgrowth of endogenous bacteria communities (thought to originate from the GI tract or male partners) and a relatively low abundance of vaginal *Lactobacillus spp* (Ma et al., 2012). *Lactobacillus crispatus* has been described to be the most abundant bacteria in the healthy vagina specifically in women of European ancestry (Ravel et al., 2011). *L. crispatus* has been shown *in vitro* to inhibit *Candida albicans*, and protect HeLa cells from infection by chlamydia (Rizzo et al., 2013; Petrova et al., 2015; Nardini et al., 2016).

Although debate on the classification of VMB during health and BV states is ongoing, Ravel et al. (2011) initially suggested that these can be divided into five community types (CTs): characterized by the predominant bacteria spp of *L. crispatus* (CT I)*, L. gasseri* (CT II)*, L. iners* (CT III)*, L. jensenii* (CT IV)*,* and a diverse VMB dominated by anaerobes (CT V). More recently, Anahtar et al. (2015) suggested that this could be simplified into four CTs; with CT I dominated by *L. crispatus*, CT II dominated by *L. iners*, CT III dominated by *Gardnerella vaginalis*, and CT IV lacking consistent dominant bacterial species. Anahtar et al. (2015) further reported that only 37% of healthy (asymptomatic) South African young women had *a Lactobacillus*-dominated VMB. Researchers from our group subsequently suggested an alternate VMB classification in predominantly asymptomatic AGYW from two cohorts in South Africa, based on an unsupervised approach to the 16S rRNA gene sequence data using Fuzzy clustering with weighted UniFrac distances, in which CTs dominated by *Gardnerella vaginalis* and *Prevotella*-mixed groups would be grouped together (C1), while those dominated

by *L. crispatus* (considered C2), and *L. iners* (considered C3) would be grouped separately (Figure 1.4; Lennard et al., 2017).

Irrespective of geographical location, women with BV have bacterial communities dominated by a more diverse VMB (composed of *G. vaginalis*, *Atopobium vaginae*, *Prevotella bivia* and other facultative anaerobic gram-positive or negative bacteria; Ravel et al., 2011; Srinivasan et al., 2012; Anahtar et al., 2015; Petrova et al., 2015; Lennard et al., 2017). However, this classification of BV is not simple, as some healthy women do not have a lactobacilli-dominated vagina (Petrova et al., 2017; Bayigga et al., 2018).

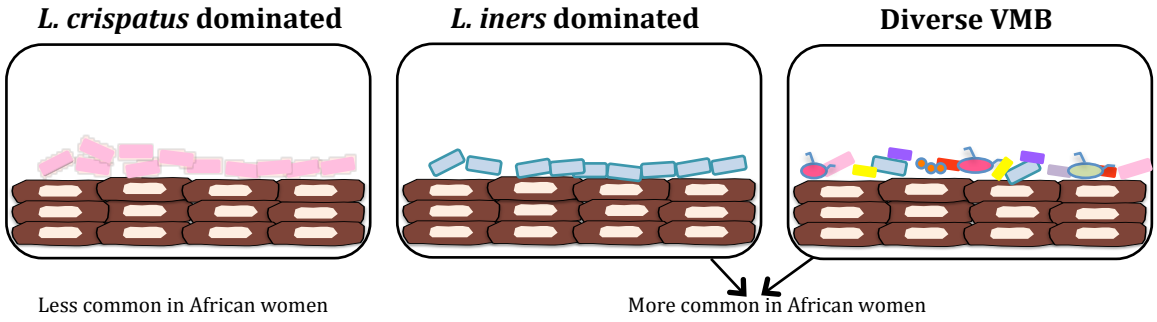

Figure 1.4. Composition of the vaginal microbiota according the community types describe in young SA women by Lennard et al (2017). A healthy vagina has been described as that dominated by *L. crispatus*. Healthy African women however mostly have *L. iners* and a diverse VMB with *Gardnerella* spp, *Prevotella* spp, and other facultative anaerobes. BV is characterized by overgrowth of anaerobes. Figure drawn by Iyaloo Konstantinus.

The current gold standard for laboratory diagnosis of BV is called Nugent scoring, and is based on evaluating the morphology and gram stain reactivity of bacteria (Nugent et al., 1991). This method assesses the relative abundance of gram-positive rods, gram-variable coccobacilli and gram-negative curved rods (Nugent, 1991). The scoring is based on a linear scale ranging from 0 - 10; a score between 0 - 3 is considered healthy, 4 - 6 is intermediate, and 7 - 10 is indicative of BV. Alternatively, Amsel criteria is more frequently used clinically, and assesses four

characteristics: (i) vaginal pH >4.5, (ii) the presence of clue cells on a saline wet mount, (iii) a homogenous and thin vaginal discharge, and (iv) a release of a fishy amine odor after adding potassium hydroxide. For a patient to be considered BV positive by Amsel criteria and receive antibiotic treatment, three of these four clinical characteristics have to be evident (Amsel et al., 1983).

BV increases HIV-1 risk significantly (Low et al., 2011; Cohen et al., 2012; Achilles and Hillier, 2013). Women with BV are at a heightened risk of HIV acquisition, and HIV positive women with BV are three times more likely to transmit the virus to their male partners (Coleman et al., 2007; Cohen et al., 2012). Moreover, BV is also associated with an up to 1.9-fold increased incidence of bacterial STIs (Wiesenfeld et al., 2003). The mechanism by which BV increases HIV risk in women has been attributed to several factors; including increased concentrations of pro-inflammatory mediators, such as IL-1α, IL-1β and TNF-α, in BV positive compared to BV negative women (Masson et al., 2014; Joag et al., 2018). The increase in these pro-inflammatory cytokines may facilitate HIV infection by disrupting the epithelial barrier (Bruewer et al., 2003), and by activating nuclear factor-κB, which binds to the HIV long terminal repeat and promotes HIV replication (Osborne et al., 1989; Stroud et al., 2009).

1.6 Endogenous female sex hormones

The hormones involved in the regulation of the female reproductive system include follicle-stimulating hormone (FSH), Gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), estrogen (E2) and progesterone (P4; Wira et al., 2015). The menstrual cycle occurs as a result of changes in these hormones that are in turn regulated by the hypothalamic-pituitary axis. During the follicular phase of the menstrual cycle, FSH and LH released from the hypothalamus travel in the blood to the ovaries to stimulate growth of about 15-20 eggs and the production of estrogen. An increase in E2 inhibits the production of FSH to allow the body to limit the number of follicles that mature (Pessina et al., 2006). This is followed by the production of LH needed for the eggs to be released from the ovaries, also known as ovulation. Ovulation requires the follicle to rupture so that

the ovum can be released into the fallopian tube. The ruptured follicle becomes the corpus luteum, which produces both E2 and P4 needed to maintain optimum conditions for fertilization and implantation during this luteal phase. In the absence of pregnancy, the corpus luteum shrinks and levels of E2 and P4 start decreasing. P4 plays a role in maintaining pregnancy, whereby increased levels lead to inhibition of ovulation during pregnancy, while decreased levels lead to menstruation and endometrial repair (Challis et al., 2009).

1.6.1 Effect of endogenous hormones on FRT microenvironment

Endogenous hormones play an important role in regulating immunity and the microenvironment of the FRT (Tan et al., 2015; Wira et al., 2015). Susceptibility to pathogens can vary depending on the menstrual cycle due to hormonal fluctuations. E2 and P4 regulate various aspects of the immune system in the FRT, both directly or indirectly, including mucosal cellular composition, chemokines and cytokines, homing of immune cells, epithelial cell receptor expression and epithelial barrier integrity (Wira et al., 2005b; Heldring et al., 2007; Fahey et al., 2008). Although not yet fully characterized, P4 receptors are found on plasmacytoid DCs, macrophages, CD4+ and CD8+ T cells (Wira et al., 2015; Hapgood et al., 2018). E2 receptors are also expressed by many immune cells, including neutrophils, T cells, B cells and NK cells (Fish, 2008). E2 modulates signaling pathways of pro-inflammatory receptors and PRRs (Jorgenson et al., 2005; Aflatoonian et al., 2007; Lin et al., 2009). P4 also regulates some immunological pathways, including modulating cytokine production, up-regulating HIV-1 receptor CCR5 expression, and increasing expression of PRRs by fibroblasts (Hirata et al., 2007).

Antibodies in cervical mucus have been shown to decrease during ovulation, or are diluted out by increased mucus secretion, but increase in concentration during the secretory and proliferative phases of the menstrual cycle (Wira et al., 2011). Cytotoxic T lymphocytes (CTL), sampled by endometrial biopsy, were found to be less cytotoxic during the luteal phase, and more cytotoxic during the follicular phase of the menstrual cycle (White et al., 1997a). In addition, expression of CXCR4

(also an HIV co-receptor, that is the receptor for the chemokine stromal cell-derived factor 1 [SDF-1]) on human epithelial cells lining the endometrium in the FRT increases during the proliferative phase (Yeaman et al., 2003).

The concentration of AMPs and cytokines also fluctuates during the menstrual cycle. For example, levels of human beta-defensins (HBD)-1, -2 and -3 peak during the secretory phase of the menstrual cycle (King et al., 2003). *In vitro* studies found that endogenous P4 was associated with lower genital pro-inflammatory cytokines production (including TNF-α, IFN-γ and IL-12) and increased production of the anti-inflammatory cytokine IL-10 (Butts et al., 2007; Kyurkchiev et al., 2007; Jones et al., 2010; Grandi et al., 2016). These genital tract pro-inflammatory cytokines attract immune cells, including HIV targets cells CD4+ T cells, to the FRT (Arnold et al., 2016).

In addition, hormonal changes also affect the integrity of the FRT epithelial barrier. Proteases involved in degradation of the epithelial barrier have been found to be elevated during the luteal phase (Bradley et al., 2018). Conversely, concentrations of anti-proteases are elevated during ovulation and the follicular phase of the menstrual cycle (Birse et al., 2015). Anti-proteases are important for reducing inflammation and have been shown to inhibit binding and replication of HIV (Aboud et al., 2014). Therefore, epithelial barrier integrity likely depends on the phase of menstrual cycle as endogenous E2 promotes proliferation of epithelial cells, while P4 has been associated with thinning of the vaginal epithelium in animal models (Marx et al., 1996; Patton et al., 2000). These alterations in mucosal barrier permeability during the menstrual cycle might lead to differences in susceptibility to pathogens, including HIV. Figure 1.5 summarizes some of the known effects of the menstrual cycle on the microenvironment of the FRT.

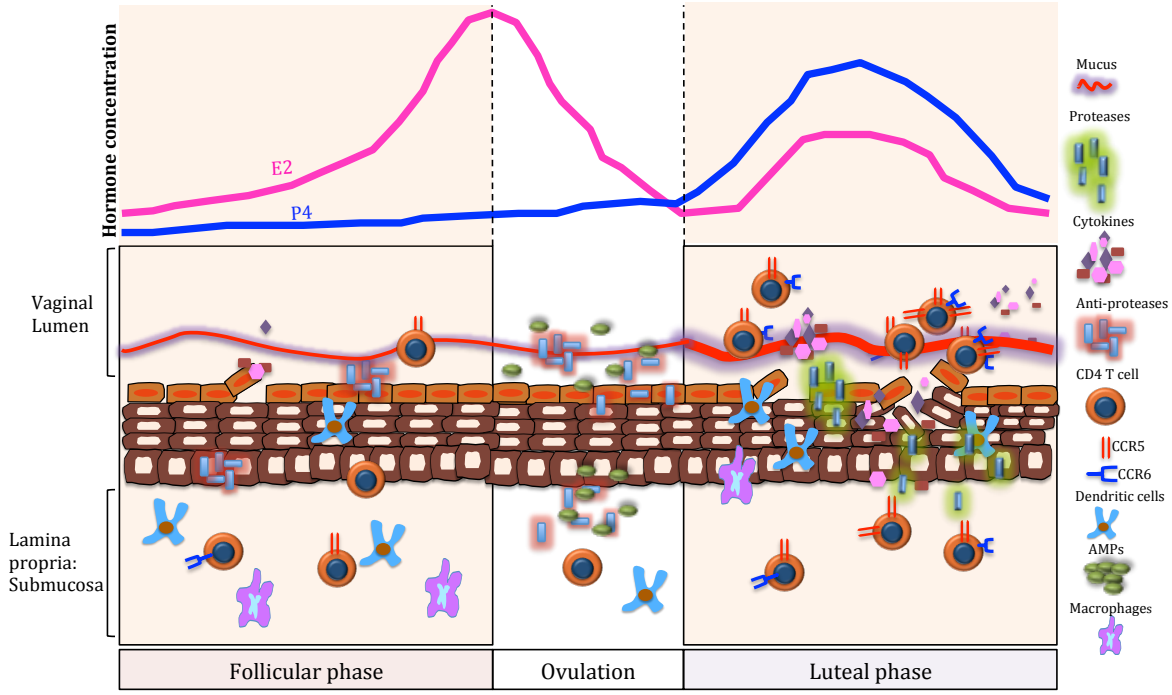

Figure 1.5. The influence of endogenous hormone on immunity and the integrity of the epithelial barrier in the FRT. The follicular phase is characterized by increases in anti-proteases, anti-inflammatory cytokines and an intact epithelial barrier. Similarly, during ovulation there is a high level of anti-proteases in addition to AMPs. The luteal phase has high levels of pro-inflammatory cytokines causing an influx of highly activated CD4+ T cells expressing CCR6 and CCR5. Although the mucus layer is thick during this time, the epithelial barrier integrity is weakened. Figure drawn by Iyaloo Konstantinus.

1.7 Hormonal contraceptives

It is estimated that hormonal contraceptives are used by more than 140 million women worldwide (UNDESA, 2015). About a quarter of women in SSA use modern contraceptive methods (Chersich et al., 2017). A wide range of HCs are available for women to choose from, including: combined oral contraceptive pills (COCPs), contraceptive patches, levonorgestrel intrauterine devices (IUDs), levonorgestrel (LNG) implants, combined contraceptive vaginal rings and long-acting injectable contraceptives. In SSA, the long-acting injectable progestins [depot-medroxyprogesterone acetate (DMPA) and norethisterone enanthate (NET-EN)] are the most commonly used methods, making up about 47% of all contraceptive choices in SA (Figure 1.6; UNDESA, 2015). In addition, non-hormonal methods are

also available, including: male and female condoms, male and female sterilization, copper IUDs (Cu-IUDs), the withdrawal and rhythm methods. A study conducted in SA young women between 20 and 29 years found that their contraceptive choices were largely determined by partner's opinion, although availability and side effects of contraceptives were also important (Oluwole and Skaal, 2016).

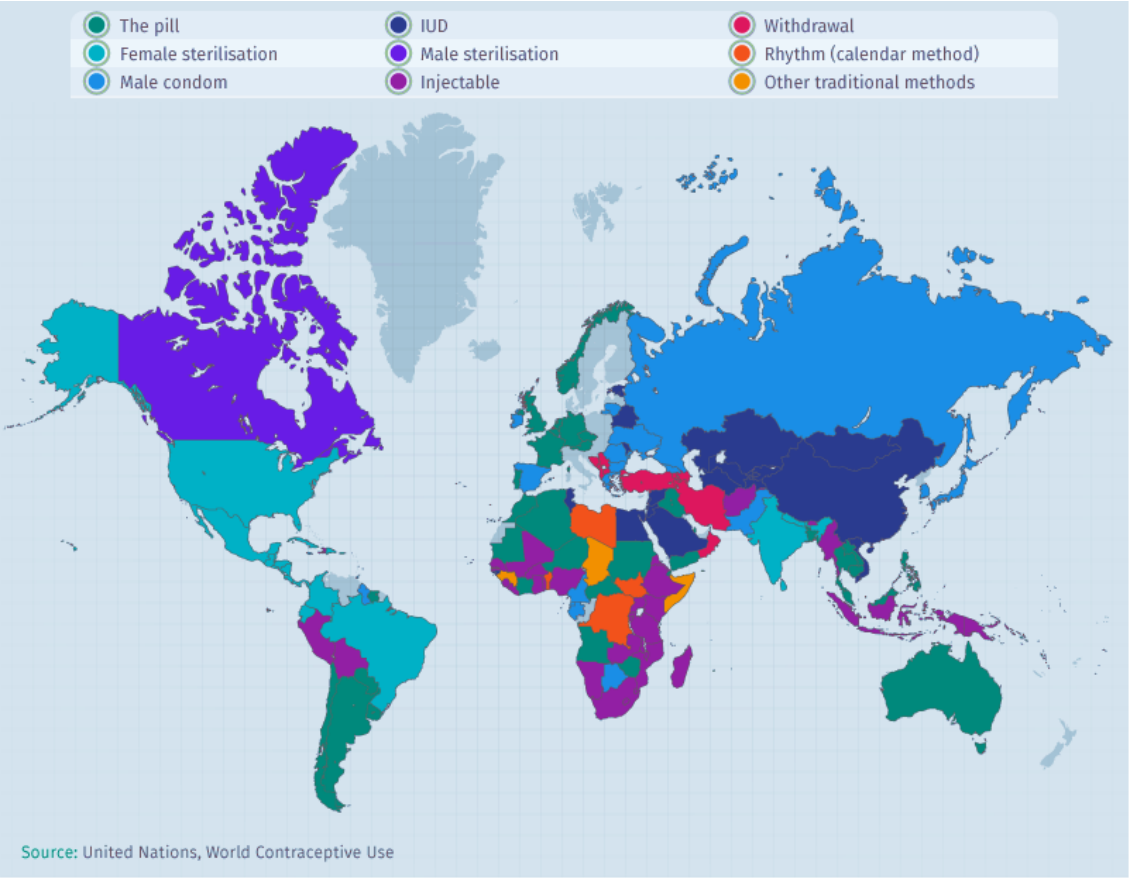

Figure 1.6. World map of the most popular contraceptive method by country. Permanent methods such as male (deep purple) and female sterilization (turquoise) are common in North America and Asia. In Europe, women mostly use the pill (green), while condoms are widely used in Russia (medium blue). The southern African region popular method is the injectable contraceptives (royal purple). Image taken from United Nations Department of Economic and Social Affairs Population Division, 2015 (Available at: https://onlinedoctor.superdrug.com/birth-control-around-the-world/).

1.7.1 DMPA and NET-EN

Injectable contraceptives are popular in developing countries because they are affordable, long-acting and relatively private. DMPA and NET-EN are both long-acting reversible methods of contraception (Jacobstein and Polis, 2014). DMPA, first discovered in 1951 by the Upjohn Company, is administered as a 150mg intramuscular injection of the synthetic 17-hydroxyprogesterone derivative of progesterone as an aqueous microcrystalline suspension, that is slowly released into the blood stream; and administered every three months. Following administration, DMPA plasma concentrations increase steadily to plasma levels 0.2ng/ml within 24 hours (Mishell, 1996). In contrast, NET-EN, first used in 1957 by the Schering Company, is delivered as a 200mg intramuscular injection of an oily solution of 17C-alpha-ethinyl-17-beta-heptanoyloxy-4-esterene-3-one progesterone derivative, administered every two months. Following administration, NET-EN plasma concentrations reach a peak of 5.5 to 11ng/ml in about 10 days. Therefore, with both, there is a sharp increase in progestogen blood concentration in the first two days, followed by a decline over the following weeks. Both injectable contraceptives bind to the progesterone receptor (PR) on fibroblasts, smooth muscles and epithelial cells in the upper FRT (Kurita et al., 2000; Rękawiecki et al., 2011; Kowalik et al., 2013). However, the pharmacokinetics profiles of DMPA and NET-EN are different from those of the endogenous P4. Both have higher binding affinities to the PR than endogenous progesterone, but bind more weakly to the androgen receptor than P4 (Schindler et al., 2003). MPA also binds with high affinity to the glucocorticoid receptor (GR) while the endogenous progesterone and NET-EN only bind weakly to this receptor (Sedgh et al., 2016; Hapgood et al., 2018). The GR receptor has immunomodulatory effects, whereby binding of an agonist leads to transactivation, whereas binding of an antagonist results in transrepression (Africander et al., 2011).

1.7.2 NuvaRing

The NuvaRing® combined contraceptive vaginal ring (CCVR) is a self-administered HC product that can be used monthly, which was first marketed in 2001 but only

became available in the private health sector in South Africa in 2013 (Figure 1.7; Roumen, 2008). It is made of the inert copolymer evatane, and it measures 54mm in diameter and 4mm in thickness, that contains a combination of ethinylestradiol (EE) and etonogestrel (ENG) which are equally dispersed inside the ring (Mulders and Dieben, 2001). NuvaRing® slowly releases a low dose of progestin ENG and estrogen EE over three weeks, which are absorbed through the vaginal epithelium. On average, it is estimated that 120µg of ENG and 15µg of EE are released daily from the ring (Timmer and Mulders, 2000; Oddsson et al., 2005). The maximum concentration of EE (approximately 35pg/ml) is attained within 2-3 days, while the maximum concentration of ENG (approximately 1700pg/ml) is attained after 1 week of ring insertion. The manufacturers recommend that the three continuous weeks of use be followed by a ring free week, during which withdrawal bleeding normally occurs. The main advantages of NuvaRing are the lower doses of contraceptive hormones released and convenience of using it for a whole month. The daily controlled-release of hormones also avoids fluctuation in hormone levels. NuvaRing has also be shown to produce superior cycle control compared to COCPs with the same hormone formulation (Bjarnadóttir et al., 2002).

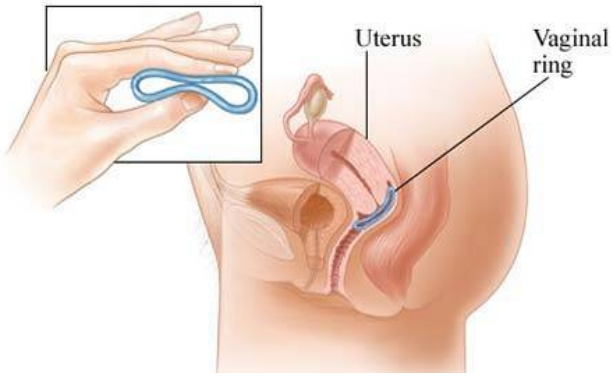

Figure 1.7. Illustration of how NuvaRing is placed inside the vagina. The clear plastic ring is folded and pushed into the vagina. This ring stays for three weeks and it is replaced afterwards. Image taken from https://www.obgynecologistnyc.com/procedures/nuvaring-birth-control-vaginal-ring/.

1.7.3 COCPs

COCPs are taken orally each day to prevent pregnancy, being the third most popular HC method globally (UNDESA, 2015). Early COCPs regimens consisted of a 28-day pill pack, which included 7 placebo pills taken at the end of the 21 days to initiate withdrawal bleeding (Nappi et al., 2016). Other regimens with shorter hormone free days (2-4 days) are now available on the market, as they have lighter and shorter withdrawal bleeds (Ahrendt et al., 2009; Mansour et al., 2011). Of the many COCPs available on the market, Nordette® and Trisaphil® birth control pills are commonly available in South Africa, and contain LNG and EE. Nordette® COCPs contains 0.15mg of LNG [d(-)-13 betaethyl-17-alpha-ethinyl-17-beta-hydroxygon-4-en-3-one], and 0.03mg of EE, [19-nor-17α-pregna-1,3,5 (10)-trien-20-yne-3,17-diol]. In contrast, Trisaphil® has a triphasic hormonal regimen of three different drugs: phase 1 is comprised of 6 tablets each containing 0.05mg of LNG, and 0.03mg of EE; phase 2 is comprised of 5 tablets, each containing 0.075mg LNG and 0.04mg EE; and phase 3 is comprised of 10 tablets, each containing 0.125mg LNG and 0.03mg EE. A major drawback of COCPs is that daily intake makes compliance more difficult, and also the occurrence of daily fluctuations of hormonal plasma levels.

1.7.4 Public health perspective on HCs and HIV acquisition

HCs have had profound health benefits for women and their infants worldwide, with significantly reduced mortality and morbidity (WHO, 2017). Despite the enormous gains of HCs, current data suggests that injectable HCs may increase HIV risk in women. SSA has the highest injectable contraceptives use, which coincide with the highest prevalence of HIV in the world (Butler et al., 2013). CCVRs are currently not available in public clinics in SSA (Kestelyn et al., 2018), and there is therefore not enough data on the CCVRs and risk of HIV acquisition. The impact of NuvaRing on the vaginal microenvironment is of particular interest given the interest in combining hormonal contraceptives with microbicides or ART in these rings.

Several observational and recent meta-analyses have shown that DMPA significantly increases a woman's risk of HIV-1 infection (Hild-Petito et al., 1998; Trunova et al., 2006; Morrison et al., 2015; Wall et al., 2015; Polis et al., 2016; Butler et al., 2016). In contrast, there is still no convincing evidence on HIV risk from observational studies on NET-EN and COCPs, and insufficient studies on NuvaRing, HC patches, or HC implants (Polis et al., 2016). Because the available epidemiological data vary in quality, the WHO have resisted changing guidelines on use of the injectable DMPA formulation. There is a risk that taking DMPA off the market may be more harmful to infant and maternal health, as women may experience unintended pregnancies as a consequence. To improve confidence in the observational risk data, a randomized controlled trial has just been completed – called the "Evidence for Contraceptive Options and HIV Outcomes (ECHO)" study - a multi-centre, open-label, randomized clinical trial comparing HIV incidence in women using DMPA, LNG, and Cu-IUD, and the results will be communicated during 2019 (http://echo-consortium.com).

1.8 Biological mechanisms by which HCs increase risk for HIV infection

Several mechanisms to explain the relationship between certain HCs and increased HIV risk have been proposed (Blish and Baeten, 2011; Hickey et al., 2016). HCs have been suggested to thin the vaginal epithelium in animal models (Marx et al., 1996; Butler et al., 2016). In addition, HCs cause extension of the endocervical columnar epithelium into the ectocervix, also known as cervical ectopy (Hild-Petito et al., 1998). HCs have also been shown to alter both the composition and metabolism of the VMB (Achilles and Hillier, 2013). HCs might initiate changes in systemic or local immunity by modulating the levels of defense proteins and soluble mediators, including chemotactic cytokines (Morrison et al., 2014, Ngcapu et al., 2015; Deese et al., 2015), which are likely to influence recruitment of HIV target cell and increased expression of chemokine receptors needed for HIV to infect cells. Some of the proposed mechanisms are summarized in Figure 1.8.

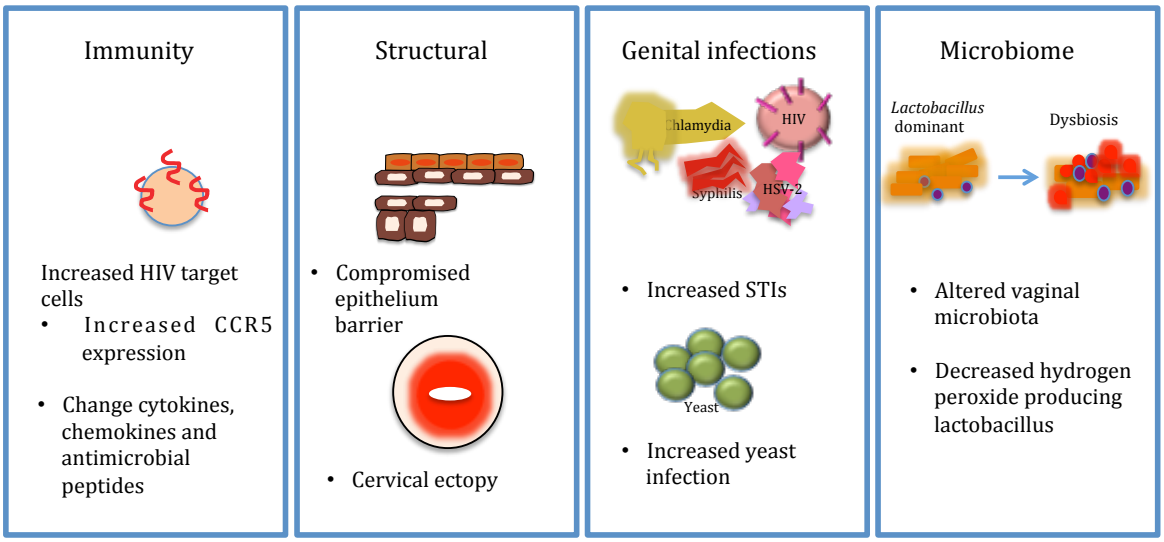

Figure 1.8. Summary of proposed mechanisms by which HCs contribute to HIV-1 transmission. HCs alter immunity, disrupt vaginal structures, increase genital infections, and alter the VMB. Figure drawn by Iyaloo Konstantinus.

1.8.1 Impact of HCs on structural features of the FRT

HCs have been suggested to affect the epithelial barrier of the FRT, although published reports have been conflicting. Some studies have found a decrease in thickness, while others found no changes (Bahamondes et al., 2000; Eschenbach et al., 2000; Mitchell et al., 2014). Thinning of the vaginal epithelium has been observed in rhesus macaques treated with high doses of DMPA (Hild-Petito et al., 1998). A study comparing a progesterone-based IUD and COCPs found that women on both HC methods had comparable epithelial thickness, but thickness was lower compared to women not using any HCs (Tjernlund et al., 2015). Interestingly, one study found that HCs (DMPA, COCPs and LNG-implant) generally induced hyperplasia of the vaginal epithelium in women (Ildgruben et al., 2003). Using proteomics, Chandra et al. (2013) found changes in adherens (protein complexes that occur at cell–cell junctions in epithelial tissues) and epithelial tight junctions proteins in vaginal biopsies of women following 12 weeks of DMPA use. Furthermore, DMPA was associated with a downregulation of many genes involved in the maintenance of the mucosal barrier while COCPs were not (Zalenskaya et al., 2018). Therefore, HCs might affect intraepithelial tight junctions

and compromise the integrity of the epithelial barrier. In a South African study, women with clinically signs of a disrupted genital epithelium and abnormal vaginal discharge were at the highest risk for HIV acquisition (Hazard ratio: 4.30, 95% CI: 2.55, 8.22; Abbai et al., 2016).

HCs have been reported to cause cervical ectopy (Venkatesh and Cu-uvin, 2013), with COCPs being associated with the largest areas of cervical ectopy, but not DMPA (Bright et al., 2014). Ectopy in turn is associated with a heightened HIV risk (Moss et al., 1991). Adolescents are more likely to have larger areas of ectopy due to metaplasia, which can occur during puberty (Moscicki et al., 1999). It is also during this time that adolescents seek HCs.

1.8.2 Impact of HCs on immunity

Several studies have reported that DMPA impacts production of cytokines and chemokines in the FRT, although some suggested that cytokines were suppressed (Ngcapu et al., 2015), others showed differential effects (Fichorova et al., 2015), while others suggested upregulation in these (Deese et al., 2015).

Cervical swabs from women on DMPA had higher levels of RANTES but pro-inflammatory cytokines including IL-1β and IL-6 did not differ from women not on contraceptives (Fichorova et al., 2015). DMPA users were further reported to have elevated MIP-1α, MIP-1β, IL-6, IL-8, IP-10 and RANTES; while NET-EN users had elevated IL-6, IL-8 and RANTES concentrations (Deese et al., 2015). In a recent study, DMPA users were found to have elevated RANTES and lower concentrations of biodefensin-2 (BD-2; Morrison et al., 2018). Thus, cytokines associated with DMPA use in observational studies include those that have been found to be associated with increased HIV acquisition risk, MIP-1α, MIP-1β, IL-6, IL-8, IP-10 and RANTES (Mlisana et al., 2012; Morrison et al., 2014; Masson et al., 2015a). Inflammatory cytokines may cause activation of HIV target cells and their recruitment to the genital mucosa. In agreement with this, increased frequencies of activated cervical CD4+ T cells were observed in women using DMPA (Byrne et al.,

2016; Chandra et al., 2013). Use of COCPs was found to increase expression of IFN-γ, IL-12 and IL-10 in cervical secretions (Barousse et al., 2007).

In contrast to these studies showing increased cytokine production associated with HC use, an observational study conducted in South African women found lower cervicovaginal concentrations of cytokines IL-12 and IL-15, and chemokines MCP-1, MDC, eotaxin and fractalkine in those using DMPA (Ngcapu et al., 2015). The current understanding of the impact of HCs on cytokine production in the genital mucosa thus remains limited, as most studies are observational and few included data collected during randomized trials.

Use of DMPA may facilitate HIV-1 infectivity by causing an increase in HIV target cells and through the regulation of their co-receptors (Baeten et al., 2001; Ghanem et al., 2005; Chandra et al., 2013). Epithelial tissue biopsies from women using DMPA showed an increase in macrophages and T cells expressing CCR5 (Chandra et al., 2013). Another study in South Africa found that women using DMPA had more cervical CCR5+ CD4+ T cells compared to women not using long-term contraceptives (Byrne et al., 2016). Interestingly, Byrne et al. (2016) found that women in the same study who were in the luteal phase of menstruation also had higher CCR5 expression compared to women in the follicular phase. Women on COCPs were also found to have elevated CCR5 expression on their T cells in the cervical epithelium (Prakash et al., 2002). In contrast, women using the progestin based IUD had reduced expression of CCR5 on their cervical T cells (Achilles et al., 2014).

In contrast to the other HC products, there have been comparatively few studies assessing the effect of NuvaRing use on immunity in the FRT. Michel et al. (2015) reported that NuvaRing use was associated with decreased CD207+ Langerhans cells in the vaginal epithelium compared to DMPA users.

1.8.3 Impact of HCs on STIs

A number of studies have identified associations between the use of HCs and acquisition of STIs (other than HIV). COCP use has been reported to increase risk for chlamydia, and HPV infection (Cottingham and Hunter, 1992; Borgdorff et al., 2015). DMPA, similarly, was shown to increase susceptibility to HSV-2 and decrease immune responses to HSV-2 in mice (Gillgrass et al., 2003). In women, DMPA use was found to be at double the risk of acquiring HSV-2 compared to those not using HCs (Grabowski et al., 2015). Baeten et al. (2001) reported that both injectable HCs and COCPs were associated with increased risk of chlamydia infection. However, no association was observed between DMPA use and risk for syphilis and gonorrhoea infection (Baeten et al., 2001; Harris et al., 2009; Marks et al., 2011; Noguchi et al., 2014).

1.8.4 Influence of HCs on the VMB

Studies investigating the impact of HCs on the composition and stability of the VMB have yielded conflicting results. A recent meta-analysis that included more than 50 studies found that women using different HCs were at up to 25% decreased risk for both prevalence and incidence of BV compared to women not using HCs (Vodstrcil et al., 2017). The use of HCs has been found to alter the normal microbiota in women according to age, favoring *Lactobacillus fermentum* and *E. coli* in women between the age of 20 – 30 years, and *L. fermentum*, *Candida* and *E. coli* in women between the age of 31 – 40 years (Kazi et al., 2012).

A study on CCVRs (NuvaRing) in Rwandan women reported an association between vaginal dysbiosis and increase in the biomass associated with the used ring, which was predominantly colonized by *Lactobacillus* and several BV-associated bacteria (Crucitti et al., 2018). Another study comparing the levonorgestrel-releasing IUD (Mirena®) with COCPs observed a trend towards an increased abundance of BV-associated bacteria in women using the former, and an increase in *Lactobacillus* in women using the latter (Brooks et al., 2017).

DMPA use has been associated with decreased concentrations of H_2O_2-producing lactobacilli, but also decreased concentrations of *G. vaginalis* and total bacterial load (Mitchell et al., 2014; Roxby et al., 2016). The hypo-estrogenic effect of DMPA (and the effects this lack of estrogen has on important nutrients needed to sustain protective lactobacilli in the vagina) has been proposed to be the mechanism by which this HC method alters the VMB (Wessels et al., 2018).

1.9 Study aims and objectives

The overall aim of this dissertation is to investigate the impact of several HC methods on the immune microenvironment of the FRT of South African adolescent girls (15-19 years) at risk for HIV acquisition, in a randomized crossover study.

Rationale for this study

Young women in South Africa are extremely vulnerable to both HIV infection and unintended pregnancies (Cowan and Pettifor, 2009; UNAIDS, 2017). Epidemiological data suggests that some forms of HCs used to prevent pregnancy may increase HIV risk, although reports have been conflicting. Given that adolescence is the time when most young women seek access to HCs, during early time points in their sexual maturity, it is important to evaluate how HCs modulate the immune microenvironment of the vagina. Despite the possible association between long-acting injectable HCs and increased risk of HIV acquisition from observational studies, the WHO concluded in 2016 that they still do not have sufficient evidence to change the current guidelines that indicate "no restriction" for use of progestin-only injectable contraceptives, until more randomized trials provide quality data for all available forms of contraception. To address this gap, this randomized crossover trial evaluating the use of NuvaRing, NET-EN and COCPs on the immune microenvironment of the vagina in adolescent girls at risk for HIV acquisition was conducted in Cape Town, South Africa – called the uCHOOSE study.

Objective 1: To characterize cervical CD4+ T cells, Th17 cells and Th7-related cytokines in the female reproductive tract of young adolescents and to investigate the impact of genital infections and STIs on these cervical HIV-susceptible T cell subsets.

Objective 2: To investigate whether BV and the vaginal microbiota alter Th17 cell abundance and activation and related cytokines in the genital tracts of AGYW.

Objective 3: To investigate the impact of NuvaRing, NET-EN and COCPs on immune cells including CD4+ T cells, Th17 cells, Th17-related cytokines, and CD8+ T cells.

Chapter 2

Characterizing the immune microenvironment in the lower reproductive tract of adolescent girls

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2.1 Abstract

Since the FRT is the first site for HIV transmission during heterosexual intercourse, studies characterizing immune cells and HIV target cells at this mucosal site are critical. CD4+CCR5+ T cells are the primary HIV target cells that become infected following sexual exposure. Of this subset, Th17 cells have recently been suggested to be the most susceptible to infection. Although described to make up a large proportion of the CD4+ T cells in the FRT, little is known about their presence and their characteristics in adolescent girls. The aim of this part of the study was to characterize the immune microenvironment in the FRT, focusing on CD4+ T cells, with particular focus on Th17 cells, and Th17-related cytokines in South African adolescents. Cervical Th17 cells, defined by expression of CCR6 and CCR10 by FACS analysis, were identified to be a major CD4+ T cell subset (making up 54% of total CD4+ T cells) in cervical cytobrushes collected from a cohort of 151 AGYW (15-19 years), assessed cross-sectionally into the uCHOOSE study. In addition, higher frequencies of Th17 cells than CCR6-CCR10- CD4+ T cells expressed CCR5 similarly to what has been shown by previous studies (68% versus 56%, respectively). The concentrations of Th17-related cytokines were measured by Luminex. Of these, the Th17 regulatory cytokine IL-31 had the highest median concentration (32 pg/ml) while the inflammatory cytokine IL-1β (associated with the differentiation of Th17 cells) was detected at the highest maximum concentration (8040 pg/ml). Adolescents with yeast infection had lower cervical Th17 cell frequencies compared to those without yeast, although genital cytokine concentrations were similar. On the contrary, asymptomatic bacterial STIs did not appear to influence the frequency and activation status of cervical Th17 cells, although concentrations of IL-1β and IL-17A were elevated. Only 4.8% of adolescent girls were contraceptive naïve at baseline in this cohort. However, previous contraceptive use did not seem to affect cervical Th17 cell frequencies or Th17-related cytokines compared to the HC naïve group.

2.2 Introduction

The FRT has evolved to meet the unique requirements of balancing immune protection against harmful pathogens, while tolerating allogeneic spermatozoa, the developing semi-allogenic fetus and commensal VMB (Wira et al., 2005a). Only recently have we started to understand the complexity of the immune system in the FRT and how it interacts with pathogens. The mucosal microenvironment, including immune cells in the FRT is regulated by sex hormones including endogenous estradiol and progesterone (Wira et al., 2010).

Mucosal CD3 T lymphocyte cells are distributed in the upper and lower reproductive tract of the FRT (White et al., 1997b; Lee et al., 2015a). CD4+ T cells in the reproductive tract are the primary targets for sexual transmission of HIV to women (Saba et al., 2010; Shen et al., 2012; Stieh et al., 2014). These cells may express CCR5, a chemokine receptor that is needed for HIV infection. Although HIV can infect resting memory CD4+ T cells, the efficiency of infection is much higher in activated CD4+ T cells, which have been defined by expression of CD38 and HLA-DR (Zhang et al., 1999; Meditz et al., 2011; Joag et al., 2016). CD4+ T cells are a heterogeneous T cell subset and are defined by their chemokine receptor expression patterns and the cytokines they produce. Different T cells subsets differ in their susceptibility to HIV infection (Cicala et al., 2009; Gosselin et al., 2010; Buzon et al., 2014), and Th17 cells have recently been suggested to be more susceptible to HIV infection than other subsets (including Th1, Th2 and Treg cells; Monteiro et al., 2011; McKinnon et al., 2015; Stieh et al., 2016). Moreover, Th17 cells were found to be selectively depleted in the gut mucosa of HIV-infected individuals (Prendergast et al., 2010).

There is now accumulating evidence that the inflammatory cytokines IL-1β, IL-33 (related to IL-1), IL-23 (related to IL-12), IL-6, and TNF-α are involved in differentiation of naïve CD4+ T cells to Th17 cells (Stockinger and Veldhoen, 2007; Korn et al., 2009). In turn, Th17 cells produce IL-17A, IL-17F, IL-21 and IL-22, and are critical for immune defense against both fungal and bacterial infections, particularly at mucosal surfaces (Ghilardi and Ouyang, 2007; Aujla et al., 2007;

Awasthi and Kuchroo, 2009; Sugimoto et., 2008; Guglani and Khader, 2010). A key role for Th17 cells is to recruit neutrophils to the site of infection through the co-production of IFN-γ and TNF-α (Pelletier et al., 2014). Since Th17 cells are inflammatory, they are closely regulated by the anti-inflammatory and regulatory cytokines IL-4, IL-10, IL-25, IL-31, IFN-γ and sCD40L (Li et al., 2018; Sandquist and Kolls, 2018).

Th17 cells selectively express the transcription factor RORγt and receptors including CCR6, IL-23R, CD161, and CCR4 (Annunziato et al., 2007; Prendergast et al., 2010). CCR6 is a homing receptor to mucosal surfaces and has been established as a robust marker for human Th17 cells (Singh et al., 2008; Wang et al., 2009; Stieh et al., 2016; Lee and Körner, 2017). CCR6, together with its ligand MIP-3a (CCL20), are both important in the pathogenesis of and immunity to HIV infection (Lee and Körner, 2017). Furthermore, MIP-3a has also been shown *in vitro* to facilitate efficient HIV-1 integration in resting CD4+ T cells, by binding to CCR6 together with other cytokines (Cameron et al., 2010).

The presence of Th17 cells at the genital mucosa has previously been described in adult women (Rodriguez-Garcia et al., 2014), although little is known about the frequency of these highly HIV susceptible CD4+ T cells at the genital mucosa of adolescents at high risk for HIV infection. Development of a better understanding of the factors that influence recruitment and activation of these highly susceptible HIV target cells at the genital mucosa is important for the development of HIV prevention strategies. The aim of this Chapter was therefore to evaluate characteristics of CD4+ T cells and Th17 cells and profile Th17-related cytokines in AGYW, in relation to reproductive health and in response to infection with STIs.

2.3 Materials and Methods

2.3.1 Study participants

The uCHOOSE study was a randomized crossover HC trial to compare acceptability of the CCVR NuvaRing compared to NET-EN and COCPs (Triphasil or Nordette) that screened a total of 156 healthy, HIV-negative sexually active adolescents between the ages of 15-19 years, who were seeking family planning services at the Desmond Tutu HIV Foundation Youth Centre Sexual Reproductive Health clinic in Masiphumelele, Cape Town (Table 2.2). Eligibility criteria included being contraceptive naïve or wanting a method change, residing in Masiphumelele, having had no symptomatic STI within the prior 40 days, negative urine pregnancy test and no intent to become pregnant in the next 8 months, normal pap smear within the last 12 months, no contra-indications to any of the study products, willing to refrain from inserting any non-study vaginal products or objects into the vagina throughout the duration of study participation, and willing to use condoms for anal and vaginal sex. From these 156 AGYW that were screened, 151 met the eligibility criteria, and from these, a total of 130 adolescents were enrolled. Assent was obtained from the adolescents <18 years of age, while consent was obtained from their legal guardians. AGYW ≥18 years provided written informed consent. Demographic, sexual risk behaviours and clinical data were collected at enrolment and follow up visits using structured questionnaires administered by a trained counselor at the Youth Centre. This study was approved by the Faculty of Health Sciences Research Ethics Committee of the University of Cape Town (HREC REF 801/2014). In addition, UCT HREC approved this study as a part of this PhD (REC 818/2016; Konstantinus). Results in this chapter will focus on adolescents from the screening visit only who met the eligibility criteria (n=151), and will be cross-sectional. More details on the randomized trial and follow-up visits are provided in Chapter 4, section 4.4.1.

2.3.2 Sample collection

The following genital tract samples were collected in the following order:

1. Menstrual cup (MC): Cervicovaginal secretions were collected by inserting a menstrual cup (Softcup®) for 30 minutes. These were used to analyse Th17-related cytokines by Luminex (section 2.3.8 and 2.3.9).
2. STI testing: A vulvovaginal swab was collected for STI testing using an in house validated multiplex PCR assay, which was performed at the STI Reference Laboratory at the NICD, Sandringham as previously described (Lewis et al., 2012; section 2.3.4).
3. BV Nugent scoring: Posterior fornix and lateral wall swabs were collected to prepare a wet mount slide for BV Nugent scoring by Gram staining, and for detection of yeast hyphae and spores (section 2.3.4). Nugent scoring was performed at the STI Reference Laboratory at the NICD, Sandringham, Johannesburg.
4. Vaginal microbiome: A lateral vaginal wall swab was collected for 16S rRNA and shotgun metagenomic sequencing, performed by Christina Balle (Dept Immunology, UCT). The microbiome data will not be included in this dissertation as it formed part of Christina Balle's PhD dissertation, which is currently being examined.
5. Flow cytometry: An endocervical cytobrush (Digene® Corporation, MD, USA) was collected for cervical mononuclear cells (CMCs), which were analysed using flow cytometry (section 2.3.6).

2.3.3 Blood specimens

Blood specimens were collected using a Serum Separator Tube (SST) for the measurement of endogenous hormones: estradiol (E2), luteinizing hormone (LH) and follicle stimulating hormone (FSH). These measurements were performed by the Bioanalytical Research Corporation laboratories (BARC; Cape Town, South Africa). HSV-2 serology was performed on serum by the BARC laboratory, directed against antibodies to HSV-2 glycoproteins G-1 and G-2.

2.3.4 Reproductive tract infections and vaginal pH

Cervical swabs were sent to the National Institute of Communicable Diseases for screening for STIs, yeast (hyphae on Gram stain slide) and BV (Nugent scoring; NICD, Johannesburg, South Africa). For BV diagnosis, FLOQSwabs™ (Copan Diagnostics, CA, USA) were collected from the lateral vaginal wall and posterior fornix, smeared onto a glass slide and Gram stained for Nugent scoring. According to Nugent scores, women were categorized as being BV-negative (Nugent scores 0–3), having intermediate microbiota (Nugent scores 4–6), or being BV-positive (Nugent score 7–10). For STI testing, a vulvovaginal swabs (Dryswabs™, Medical Wire and Equipment, England) were collected and screened for *Neisseria gonorrhoeae, Mycoplasma genitalium, Chlamydia trachomatis* and *Trichomonas vaginalis* by multiplex PCR at the National Health Laboratory Services STI Surveillance laboratory in Sandringham, Johannesburg, as previously described (Lewis et al., 2012). For measurement of vaginal pH, swabs were collected from the posterior fornix and lateral vaginal wall and then rolled onto pH indicator strips and compared with colour zones representing different pH values (pH range: 3.6-8.2; Düren, Germany).

2.3.5 Processing of cervical cytobrushes

Cervical mononuclear cells were collected using a Digene® cervical sampler [Digene Corporation, MD, USA; (Gumbi et al., 2008)]. Briefly, the cervical cytobrush was inserted into the endocervical os under speculum examination, rotated through 360° and immediately placed into a 15ml tube containing 3ml of cold transport medium [RPMI 1640 (Life Technologies Corporation, CA, USA) medium supplemented with 5mM glutamine, fungazone, penicillin, streptomycin and 10% fetal calf serum (FCS)]. The cervical cytobrushes were transferred to 4°C in a Nalgene bench-top cooler (Rochester, NY, USA) and transported to the laboratory within 4 hours of collection.

Cervical cytobrushes were processed by gently rotating them against the sides of the 15ml tubes to dislodge cells. The transport medium was then flushed through

the cytobrush 30 times using a sterile Pasteur pipette. The cytobrush was removed and discarded. The cell suspension was centrifuged at 320g for 10 minutes. The supernatant was collected and then stored at -80°C for other assays. The pelleted cells were dislodged, resuspended in 100µl phosphate buffered saline (PBS) and immediately prepared for flow cytometry.

2.3.6 Flow cytometry

As part of this study, a 9-colour flow cytometry panel was designed and optimized to measure CCR6+ CCR10- Th17 cell subsets in cervical cytobrush preparations from the uCHOOSE AGYW (Table 2.1). Cervical cells were transferred to a 96 v-bottom well plate (Corning® Incorporated, Kennebunk, USA). The cells were phenotyped using monoclonal antibodies to detect the following markers: CD3, CCR6, CCR5 (BD Biosciences, Plymouth, UK); CD4, CD19, CD14 (Invitrogen, Carlsbad, USA); CD38 (eBioscience, CA, USA); and CD8, CCR10, HLA-DR (Biolegend, CA, USA). All antibodies were titrated to optimize the noise/signal ratio. Viable cells were identified using a LIVE/DEAD® ViVid stain (Life Technologies). Fluorescence minus one (FMO) controls were used to set the gates. Data were acquired on a BD FORTESSA™ cytometer and analyzed with FlowJo Software (version 9.9.5; Tree Star, Ashland, OR, USA). Rainbow beads were used daily to calibrate the FORTESSA to ensure that the fluorescence measurements were comparable in all the experiments.

For staining, the CMCs resuspended in 100µl of PBS were transferred from the 15ml tubes to a 96-well plate (Corning, MA, USA). The cells were centrifuged at 1215g at 4°C for 5 minutes, the supernatant discarded and the cell pellet was resuspended in 50µl of ViVid and incubated in the dark at room temperature (RT) for 20 minutes. The cells were washed twice with PBS (100µl and 150µl, respectively) for 5 minutes at 1215g at 4°C, and stained with chemokine antibodies (CCR6, CCR10 and CCR5) at 37°C for 30 minutes. The cells were then washed twice in PBS (100µl and 150µl, respectively) for 5 minutes at 1215g at 4°C, and thereafter stained with a cocktail of antibodies (CD14, CD19, CD3, CD4, CD8,

CD38, HLA-DR) by incubating them in the dark at RT for 30 minutes. Cells were washed as previous, resuspended in 150µl Cellfix and transferred to a 5ml FACS tube and acquired within 24 hours using the BD FORTESSA™ cytometer. Compensation tubes were prepared with every acquisition.

Table 2.1 Summary of antibodies included in the optimized 9-colour panel

Marker	**Fluorochrome**	**Laser**	**Function**
CD14 CD19 ViVid	Pacific blue	Violet	Dump channel
CD3	APC-H7	Red	T lymphocytes cell marker
CD4	PE-Cy5.5	Green	CD4 T cell marker
CD8	BV711	Violet	CD8 T cell marker
CD38	PE-Cy7	Green	Activation marker
HLA-DR	Alexa fluor 700	Red	Activation marker
CCR5	APC	Red	Co-receptor
CCR6	BV605	Violet	Homing marker
CCR10	PE	Green	Memory like skin-resident T cells

2.3.7 Gating strategy

A summary of the gating strategy used to identify T cell populations and activation of these cells is shown in Figure 2.1. A time gate was set to establish a stable flow stream and exclude any inconsistency, followed by a singlet gate to exclude cells clumped together. A dump channel was set to exclude CD14 (monocytes), CD19 (B cells) and dead cells. Live CD3 were gated on to exclude other cells, and CD4+ and CD8+ cells were gated on from the CD3 population.

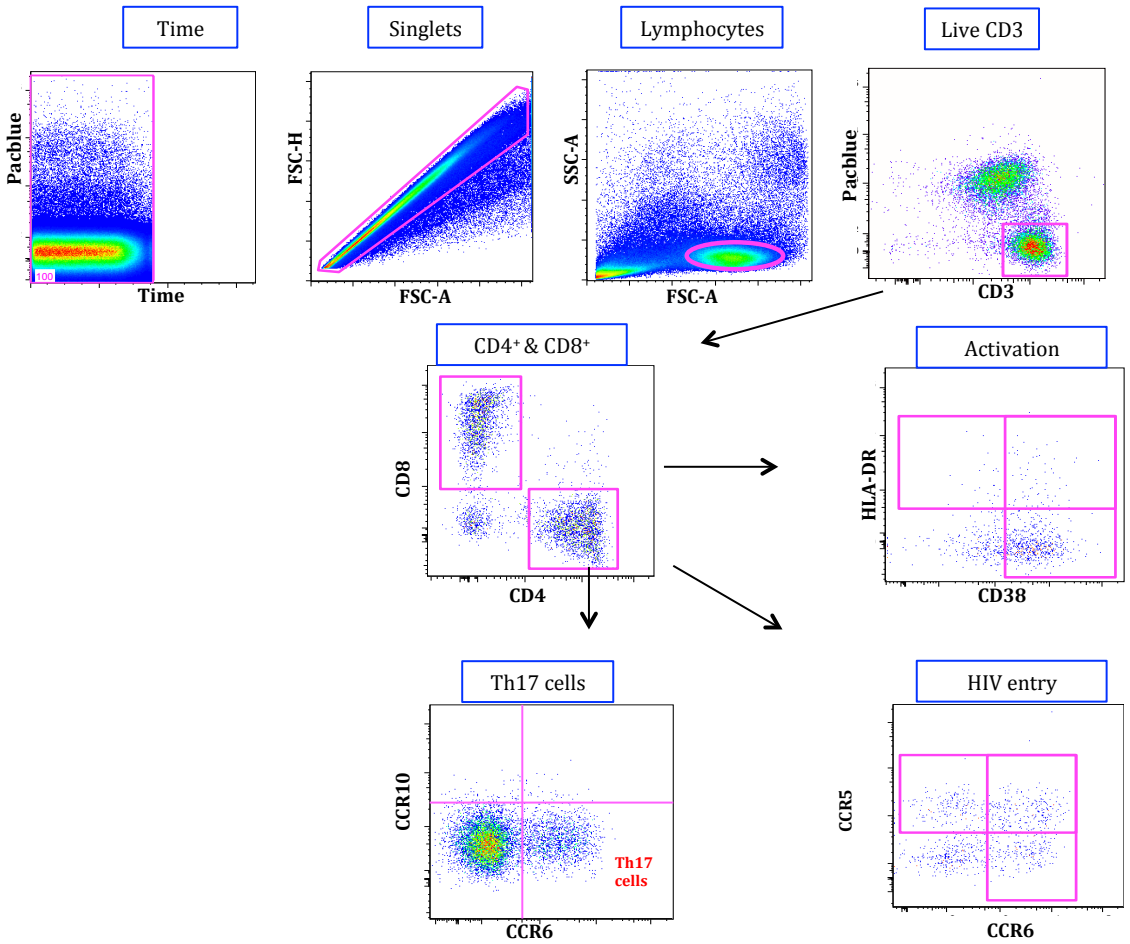

Figure 2.1. Gating strategy for T cell phenotyping from cervical cytobrush-derived T cells. A time gate was set followed by a singlet gate to exclude doublets. Next, live CD3 cells were gated by excluding dead lymphocytes (Pacblue positive), on followed by CD4+ and CD8+ T cell gates. Chemokine receptors CCR6 and CCR10 were stained to identify different CD4+ T cell subsets including Th17 cells. Activated cells were defined as those expressing CD38 or/and HLA-DR. The CCR5 chemokine receptor needed for HIV-1 entry was also stained.

The median numbers of CD3+ cells that were recovered per cytobrush from 151 samples was 769 (range 10 – 19695). Furthermore, 21/151 cytobrushes had <100 CD3 counts per cytobrush and were therefore not included in final FACS analysis as they were considered not to have adequate CD3+ cell numbers for Boolean gating (Liebenberg et al., 2011a).

2.3.8 Menstrual cup processing

Cervicovaginal secretions were collected by inserting a Softcup® menstrual cup [(MC), Evofem Inc, CA, USA] for 30 minutes. The MC containing genital fluid was placed into a labelled sterile 50ml polypropylene tube (Greiner Bio One, Frickenhausen, Germany), transferred to 4°C in a Nalgene (Rochester, NY, USA) bench-top cooler and transported to the laboratory for further processing within 4 hours of collection. In the laboratory, the MC was weighed and the volume of the secreted fluid was calculated by subtracting the average weight of the 50ml tube containing an unused MC (Jaumdally et al., 2017). The MC plastic membranes were pushed inside out using a sterile pipette tip to allow the collected secretions to flow out of the MCs into the tubes. The tubes were centrifuged at 453g for 10 minutes at 4°C with brakes off. The MCs were removed after centrifugation and discarded. The MC secretions were diluted five-fold with PBS according to the calculated weight of the collected secretions, and stored at -80°C to measure cytokines using Luminex assays.

2.3.9 Measurement of Th17-related cytokines by Luminex

The concentrations of IL-17A, IL-22, IL-21, IL-1β, IL-4, IL-6, IL-10, IL-17F, IL-23, IL-25, IL-31, IL-33, IFN-γ, sCD40L and TNF-α were measured using Bio-Plex Pro™ Human Th17 cytokine Luminex kits (Bio-Rad Laboratories Inc, CA, USA). Briefly, diluted MC secretions were thawed overnight at 4°C. To separate the mucous pellets from the fluid supernatants, the secretions were transferred to a Spin-X 0.2μm filter tubes (Corning® Incorporated), centrifuged at 1950g for 10 minutes and the supernatants removed. After filtering the supernatants, samples were transferred to the Luminex plates. Luminex assays were performed according to the manufacturer Bio-Plex Pro™ Human Th17 cytokine assays instruction manual. Data was collected using a Bio-Plex™ Suspension Array Reader (Bio-Rad Laboratories Inc), and a 5-parameter logistic regression formula was used to calculate sample concentrations from the standard curves. To assess inter-plate variability, specimens from six participants were run across all plates and a total of six plates were run. Cytokine concentrations that were below the detection limit of

the assay were reported as the mid-point between the lowest concentrations measured for each cytokine and zero. Overall, detection of IL-4 and IL-10 was low, with only 37% and 42% of samples having detectable levels, respectively; see Appendix Table A1.

2.3.10 Statistical analyses

Statistical analysis was performed using Prism version 6.0 (GraphPad Software, CA, USA), STATA™ version 12 (StataCorp, TX, USA) and R Studio. Baseline demographic, clinical and behavioral characteristics were summarized with descriptive statistics including frequencies, medians with interquartile ranges (IQR). For categorical variables, the Fisher's exact test was used to compare groups. Non-parametric Mann-Whitney U test and Wilcoxon Signed Rank test were used for unmatched and paired samples, respectively. For comparison between more than two groups, the Kruskal-Wallis one-way analysis of variance was used. Correlations were performed using the Spearman Rank test. A p-value of ≤0.05 was considered to be statistically significant.

2.4 Results

A total of 156 adolescent girls were screened, and 151 HIV negative adolescents who met the eligibility criteria (see section 2.3.1) were included in this study. Five girls were not included because of medical conditions [anemic (n=1), being treated with antibiotics for tuberculosis (n=1), being hypertensive (n=1), having raised liver function (n=1)] or because they were below the target age for this study (n=1). The median age of adolescents at enrolment was 17 years (range 15-19) (Table 2.2), with a median BMI of 25kg/m^2, which falls just above a weight that is considered normal (18.5-24.9; https://www.nhlbi.nih.gov/health/educational). The prevalence of STIs at the screening visit was 56.2% (85/151), with infections with *C. trachomatis* being the most prevalent (33.1%; 50/151). Despite expecting high rates of asymptomatic infections, it was unexpected that all of the STIs in this adolescent cohort, diagnosed using laboratory testing, were asymptomatic. The prevalence of BV (Nugent score 7-10) was 43.0% (65/151), while 14.6% (22/151) AGYW had evidence of yeast infections. The age of sexual debut in this group of young women was 15 (14-16), and 9.3% of the AGYW reported to have had multiple sexual partners. The majority of the adolescents had been on some form of HC prior to enrolment.

Table 2.2 Characteristics of South African AGYW included in the study

Characteristic	**Median (IQR) or %(n/N)**
N	151
Age [median years (range)]	17 (15–19)
Sexual debut [median years (range)]	15 (14-16)
Multiple partners [% (n/N)]	9.3% (12/128*)
Vaginal cleansing [% (n/N)]	12.8% (19/148*)
BMI (kg/m^2) [median (range)]	25.0 (21.8 - 28.9)
Serum hormone conc. [median (range)]	
E2 (pmol/l)	100.0 (74.7 - 141.8)
S-FSH (U/L)	4.9 (3.6 - 6.0)
LH (IU/L)	4.2 (2.0 - 6.0)
Prior contraceptive method [% (n/N)]	
None	4.8% (7/144*)
DMPA	19.4% (28/144*)
NET-EN	65.3% (94/144*)
COCPs	8.3% (12/144*)
Implanon	2.1% (3/144*)
Genital infections [% (n/N)]	
No STI or BV	19.2% (29/151)
N. gonorrhoeae	12.0% (18/151)
T. vaginalis	8.6% (13/151)
C. trachomatis	33.1% (50/151)
M. genitalium	2.7% (4/151)
HSV-2 seropositive	27.1% (41/151)
Presence of yeast hyphae	14.6% (22/151)
BV Nugent scoring [% (n/N)]	
BV negative (Nugent 0-3)	45.7% (69/151)
Intermediate (Nugent 4-6)	11.3% (17/151)
BV positive (Nugent 7-10)	43.0% (65/151)

*Missing values for those n ≠ 151 due to data not provided by some participants
BV, bacterial vaginosis; BMI, body mass index; E2, estradiol; S-FSH, follicle stimulating hormone; LH, luitenizing hormone

2.4.1 Characteristics of cervical T cells in adolescents

Of the CD3+ T cells found in cervical cytobrushes of adolescents, 44.9% were CD4+ while 22.8% were CD8+ (Figure 2.2A). A greater proportion of CD8+ than CD4+ T cells expressed the activation markers CD38 ($p=0.0005$) and CD38/HLA-DR ($p=0.03$; Figure 2.2B). Higher frequencies of CD8+ than CD4+ T cells expressed CCR5 ($p=0.01$, Figure 2.2C), although there was a strong positive correlation between frequencies of CD4+ and CD8+ T cells expressing CCR5 ($r=0.76$, $p<0.0001$; Figure 2.2D). In contrast, significantly higher frequencies of CD4+ T cells than CD8+ T cells expressed CCR6 ($p<0.0001$). There was also a positive correlation between CD4+ and CD8+ T cells expressing CCR6 ($r=0.31$, $p=0.0006$; Figure 2.2D).

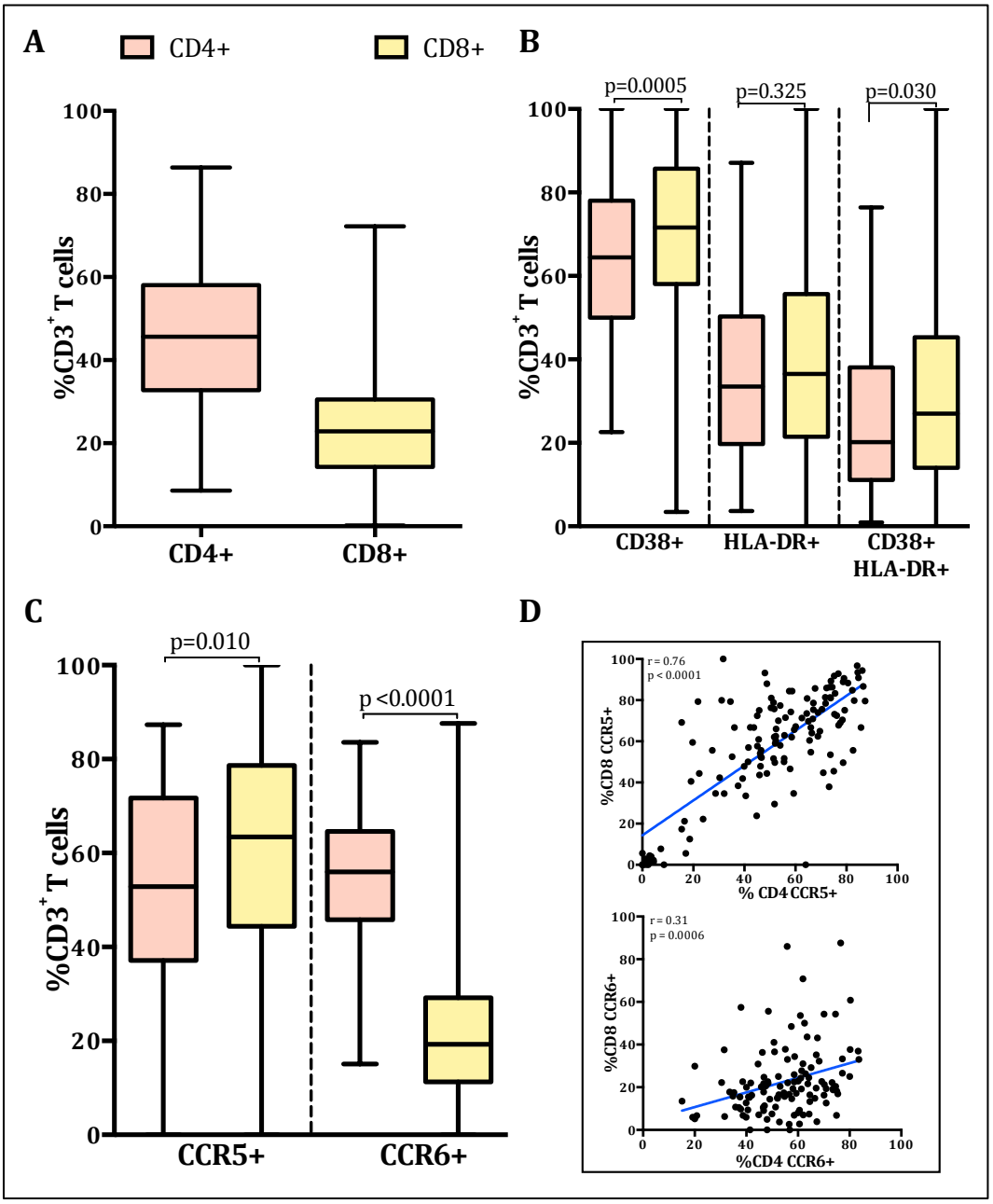

Figure 2.2. Characterization of phenotype and activation status of cervical T cell in adolescents. (A) Proportion of CD4+ (peach) and CD8+ (yellow) T cells. (B) Proportion of CD4+ and CD8+ cells expressing activation markers CD38 and HLA-DR. (C) Frequency of CD4+ and CD8+ T cells expressing chemokine receptors (CCR5 [left panel], CCR6 [right panel]). (D) Correlation plots between CD4+ and CD8+ expressing CCR5 (top panel) and CCR6 (bottom panel). Box and whisker plots represent the median, IQR and range. Mann-Whitney U test was used to compare CD4+ and CD8+ T cell frequencies. Spearman rank test was used to test correlations. P≤0.05 were considered significant.

2.4.2 Phenotype and activation of cervical Th17 cells

Th17 cells have previously been reported to be a major T cell subset in the FRT, using CCR6 expression by CD4+ T cells to define this helper cell subset (Rodriguez-Garcia et al., 2014). Although several studies have shown that Th17 cells can express a number of different chemokine receptors (including CCR4), the majority have been shown to express CCR6, which mediates mucosal homing in response to MIP-3a (Acosta-Rodriguez et al., 2007; Singh et al., 2008). For this study, Th17-like cells were considered to be CD4+ T cells that express CCR6. Furthermore, the chemokine receptor CCR10 was used to differentiate Th17 from Th22 cells known to express higher levels of this chemokine (Ye et al., 2012). Although IL-17 production directly by these cells was not measured, these cells have been referred to as Th17 cells throughout this study.

More than half of cervical CD4+ T cells were considered Th17-like (54.4%; CCR6+CCR10-), and CCR6-CCR10- CD4+ T cells (representing Th1 and Th2 cells [Zhong et al., 2017]) were the next most abundant subset; 42.2%) (Figure 2.3A). Cervical Th17 cells were similar across all age groups, and frequencies in younger adolescents (15-17 years; median 50.0%) were the same as in older adolescents (18-19 years; median 56.0%).

As HIV preferentially infects activated CD4+ T cells and CCR5 is the preferred co-receptor for viral entry (Joag et al., 2016; Stieh et al., 2016), expression of activation markers HLA-DR/CD38 and co-receptor CCR5 were evaluated on adolescent cervical Th17 cells (Figure 2.3). More Th17 cells expressed CD38 than HLA-DR (Figure 2.3B). The frequency of CCR5 expressing Th17 cells was significantly higher than CCR6-CCR10- CD4+ T cells ($p<0.0001$; Figure 2.3C). Co-expressions of CCR5/CD38 were also more frequent for Th17 cells compared to CCR6-CCR10- CD4+ T cells ($p=0.0008$). These data suggest that cervical Th17 cells in adolescents were highly activated, and expressed higher levels of CCR5 than Th1- and Th2-like cells in the FRT.

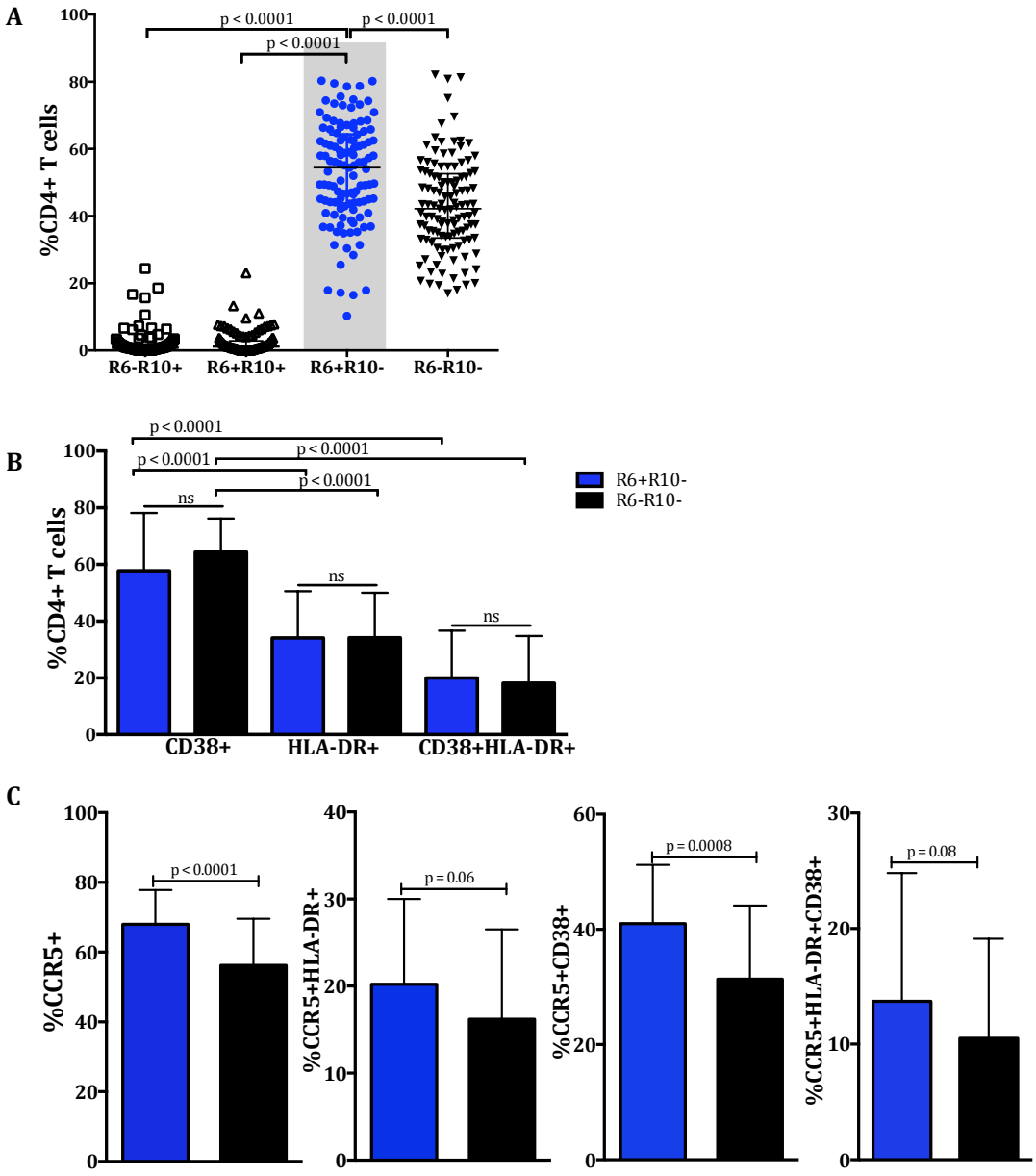

Figure 2.3. Cervical CD4+ T cells in young adolescents. (A) Proportions of different subsets of CD4+ T cells defined by CCR6 and CCR10 chemokine receptors. Th17 cells (CCR6+CCR10-) are represented by the blue dots, CCR6-CCR10+ CD4+ T cells are shown in clear squares, CCR6+CCR10+ CD4+ T cells are shown in clear triangles, and CCR6-CCR10-CD4+ T cells are shown in black inverted triangles. (B) Frequencies of CD38+ and HLA-DR+ Th17 cells (CCR6+CCR10-; blue bars) and Th1/2-like T cells (CCR6-CCR10-; black). (C) Expression of CCR5 alone and in combination with CD38 and/or HLA-DR by Th17 cells (CCR6+CCR10-; blue bars) and Th1/2-like T cells (CCR6-CCR10-; black). Mann-Whitney U tests were applied to compare the markers between the groups, and $p \leq 0.05$ were considered significant.

2.4.3 Evaluating genital Th17-related cytokines

Fifteen Th17-related cytokines were measured in softcup secretions, including two family members of IL-17 produced by Th17 cells: IL-17A and IL-17F (Figure 2.4). For analysis, the cytokines were divided into those produced by Th17 cells (IL-17A, IL-17F, IL-21, IL-22), those involved in the differentiation of Th17 cells (IL-6, IL-1β, IL-23, IL-33, TNF-α), and those involved in the regulation of Th17 cells (IL-4, IL-10, IL-25, IL-31, IFN-γ, sCD40L) (Martinez et al., 2008; Maeda, 2013; Patel and Kuchroo, 2015). Although they are considered the defining cytokines produced by Th17 cells, both IL-17A and IL-17F were detected at low concentrations in cervical secretions from these adolescents (median concentration of 1.3 pg/ml and 3.5 pg/ml, respectively), which was close to the detection limit of the assay (1.2 pg/ml and 3.0 pg/ml, respectively). Concentrations of IL-17A and IL-17F positively correlated ($r=0.77$, $p<0.0001$; Figure 2.5A). Of the cytokines produced by Th17 cells, IL-22 was present at the highest median concentration of 6.4 pg/ml, and correlated positively with IL-17A ($r=0.67$, $p<0.0001$), IL-17F ($r=0.75$, $p<0.0001$; Figure 2.5A), and IL-21 concentrations ($r=0.58$, $p<0.0001$).

IL-1β, IL-6, TNF-α, IL-23, and IL-33 all tended to be co-regulated and positively correlated with one another (Figure 2.5B). Furthermore, these inflammatory cytokines also correlated positively with production of IL-17A and IL-17F (data not shown). Similarly, IL-25, IL-4, IL-10, IFN-γ and sCD40L also correlated with one another (Figure 2.5C) as well as with IL-17A and IL-17F concentrations (data not shown). Cervical concentrations of Th17-related cytokines did not differ by age group (data not shown).

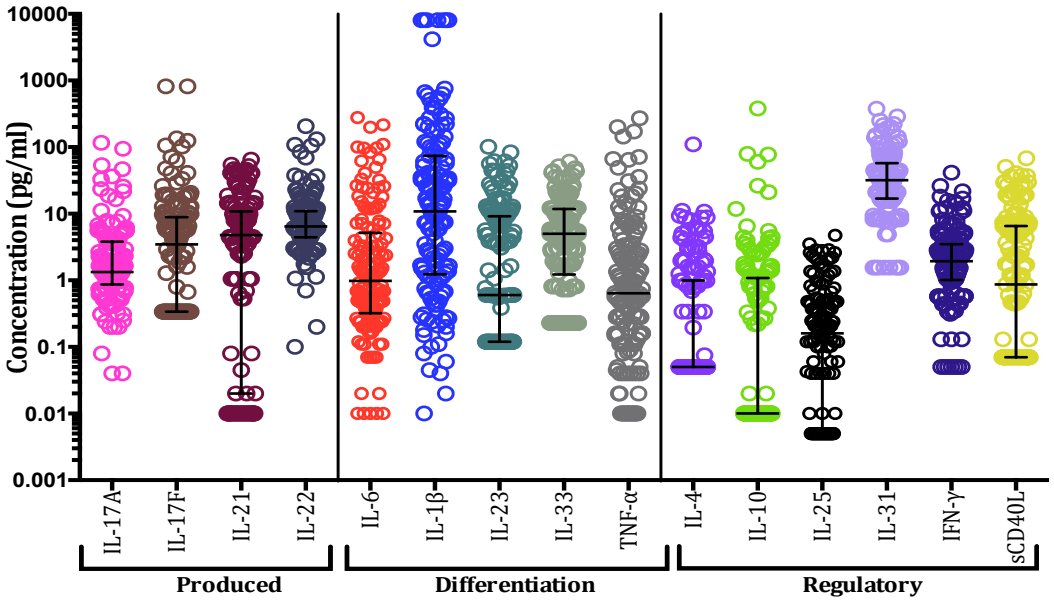

Figure 2.4. Abundance of Th17-related cytokines in the lower reproductive tract (LRT) of adolescent girls. Cytokines were grouped into three groups according to their primary Th17-related functions; including those produced by Th17 cells (IL-17A, IL-17F, IL-21, IL-22), those involved in Th17 differentiation (IL-6, IL-1β, IL-23, IL-33, TNF-α), and those involved in regulating Th17 cells (IL-4, IL-10, IL-25, IL-31, IFN-γ, sCD40L). The median (middle lines) and IQR (error bars) are plotted on the graph.

A. Cytokines produced by Th17 cells

	IL-17A	IL-17F	IL-21	IL-22
IL-17A	1	0.77	0.53	0.67
IL-17F	0.77	1	0.53	0.75
IL-21	0.53	0.53	1	0.58
IL-22	0.67	0.75	0.58	1

B. Cytokines involved in the differentiation of Th17 cells

	IL-6	IL-1β	IL-23	IL-33	TNF-α
IL-6	1	0.83	0.57	0.69	0.79
IL-1β	0.83	1	0.61	0.70	0.86
IL-23	0.57	0.61	1	0.73	0.59
IL-33	0.69	0.70	0.73	1	0.76
TNF-α	0.79	0.86	0.59	0.76	1

C. Cytokines involved in the regulation of Th17 cells

	IL-4	IL-10	IL-25	IL-31	IFN-γ	sCD40L
IL-4	1	0.46	0.75	0.68	0.69	0.63
IL-10	0.46	1	0.58		0.61	
IL-25	0.75	0.58	1	0.70	0.82	0.68
IL-31	0.68		0.70	1	0.72	0.86
IFN-γ	0.69	0.61	0.82	0.72	1	0.63
sCD40L	0.63		0.68	0.86	0.63	1

Figure 2.5. Correlation between Th17-related cytokines in genital secretions of adolescent girls. Cytokines were grouped into three groups according to their primary Th17-related functions; including those produced by Th17 cells (IL-17A, IL-17F, IL-21, IL-22), those involved in Th17 differentiation (IL-6, IL-1β, IL-23, IL-33, TNF-α), and those involved in regulating Th17 cells (IL-4, IL-10, IL-25, IL-31, IFN-γ, sCD40L). Only significant correlation coefficients (Rho scores) are displayed in blue (representing positive correlations).

2.4.4 Relationship between Th17 cell frequencies and Th17-related cytokines

The relationship between Th17 cells and Th17-related cytokines was investigated. Frequencies of cervical Th17 cells in cytobrushes did not appear to correlate with concentrations of the Th17-related cytokines (data not shown). However, the extent of Th17 activation (CD38+) tended to correlate positively with genital concentrations of IL-17A (r=0.22, p=0.01), IL-17F (r=0.17, p=0.05), IL-6 (r=0.21, p=0.02), IL-10 (r=0.25, p=0.004), and sCD40L (r=0.19, p=0.04; Figure 2.6), although none of these associations remained significant after adjusting for multiple comparisons. Interestingly, there was a weak negative correlation between the frequency of Th17 cells expressing CCR5 and IL-17A (p=0.02, r=-0.20) (data not shown).

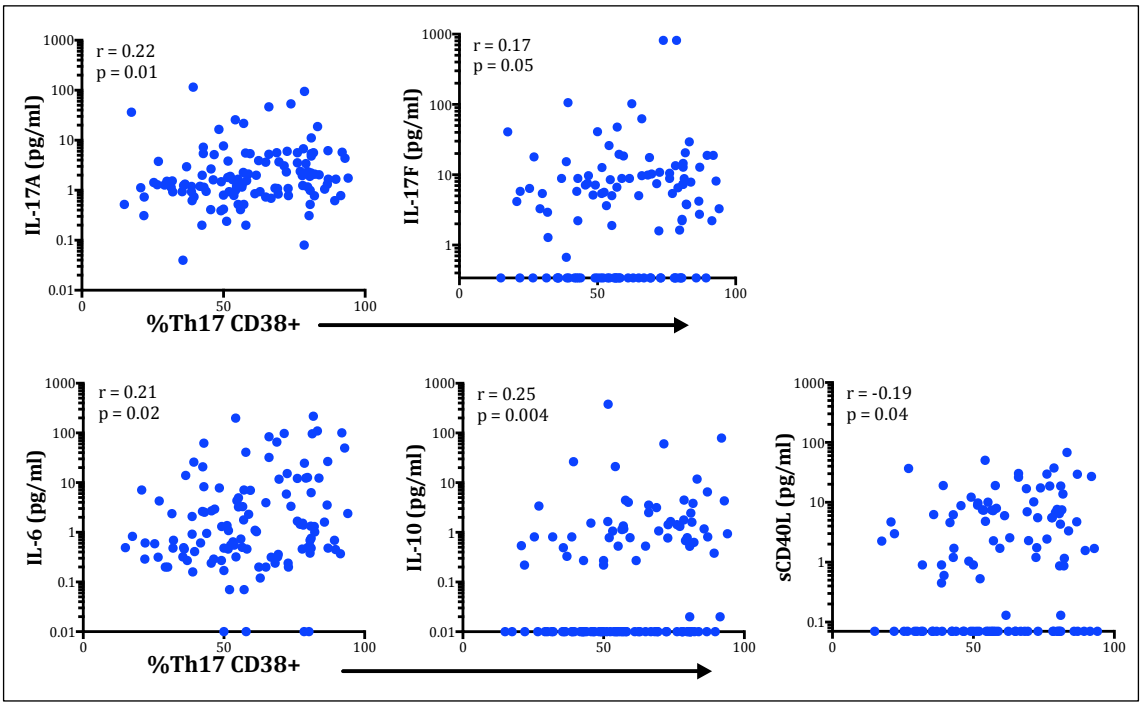

Figure 2.6. Correlation between the frequencies of activated (CD38+) Th17 cells and Th17-related cytokines (IL-17A, IL-17F, IL-6, IL-10 and sCD40L). Spearman Rank test was applied to test the relationships between cervical Th17 cells (R6+R10- CD4+) T cells and Th17-related cytokines in the lower reproductive tract. P≤0.05 were considered significant, and only significant relationships are shown.

2.4.5 Endogenous hormones and Th17 cells

The relationship between endogenous hormone levels and cervical Th17 cell frequencies and state of activation was next investigated. While FSH did not appear to change activation or frequency of cervical Th17 cells, E2 correlated negatively with frequencies of activated Th17 cells expressing HLA-DR (r=-0.23, p=0.01; Table 2.3) and those co-expressing CD38/HLA-DR (r=-0.22, p=0.01). There were also weak negative correlations between LH and Th17 cells expressing CD38 (r=-0.22, p=0.01), CD38/HLA-DR (r=-0.20, p=0.02), CCR5/CD38 (r=-0.19, p=0.03), CCR5/HLA-DR (r=-0.19, p=0.04) and CCR5/CD38/HLA-DR (r=-0.19, p=0.03). The relationship between Th17-related cytokines and endogenous serum hormones was also investigated, however there were no statistically significant correlations (data not shown).

Table 2.3 Relationship between endogenous hormone concentrations and cervical Th17 cell activation

	E2	***FSH***	***LH***
	Spearman correlation p-value (rho)		
%Th17	0.842 (-0.018)	0.030 (0.748)	0.333 (0.090)
CCR5+	0.069 (0.169)	0.279 (-0.101)	0.545 (-0.056)
CD38+	0.057 (-0.177)	0.197 (-0.121)	0.015 (-0.225)
HLA-DR+	0.010 (-0.237)	0.102 (-0.153)	0.089 (-0.159)
CD38+HLA-DR+	0.013 (-0.229)	0.160 (-0.132)	0.025 (-0.208)
CCR5+CD38+	0.394 (-0.080)	0.317 (-0.094)	0.035 (-0.196)
CCR5+HLA-DR+	0.105 (-0.152)	0.055 (-0.178)	0.041 (-0.190)
CCR5+CD38+HLA-DR+	0.088 (-0.159)	0.241 (-0.110)	0.038 (-0.193)

2.4.6 Impact of yeast infection on cervical Th17 cells and cytokines

Th17 cells have been reported to play a pivotal role in defense against infection with *Candida albicans* (Hernandez-Santos et al., 2013; Levitz, 2009). Here, the effect of yeast infection, indicated by the present of fungal hyphae or spores on Gram stained slides, on the frequency and activation status of Th17 cells was investigated. For this analysis, only 23/151 adolescents with no STIs (chlamydia, gonorrhoeae, mycoplasma, or trichomonas) also excluding co-infections were included. Adolescents who had evident yeast infections (n=16) had a lower frequency of Th17 cells compared to those without yeast (n=7; p=0.02; Figure 2.7A). On the contrary, the proportion of CCR6-CCR10- CD4+ T cells (Th1 and Th2 cells) was higher in yeast+ adolescents (p<0.001, Figure 2.7C). Although both yeast+ and yeast- adolescents had similar frequencies of Th17 cells that were activated or expressed CCR5, there was a trend towards a decrease in these markers in yeast+ adolescents (Figure 2.7B). Similarly, CCR5, HLA-DR and CD38 (p<0.0001) on CCR6-CCR10- CD4+ T cells tended to be lower in yeast+ girls (Figure 2.7D). In contrast, Th17-related cytokines did not appear to differ significantly in adolescents with or without genital yeast being evident (Figure 2.8).

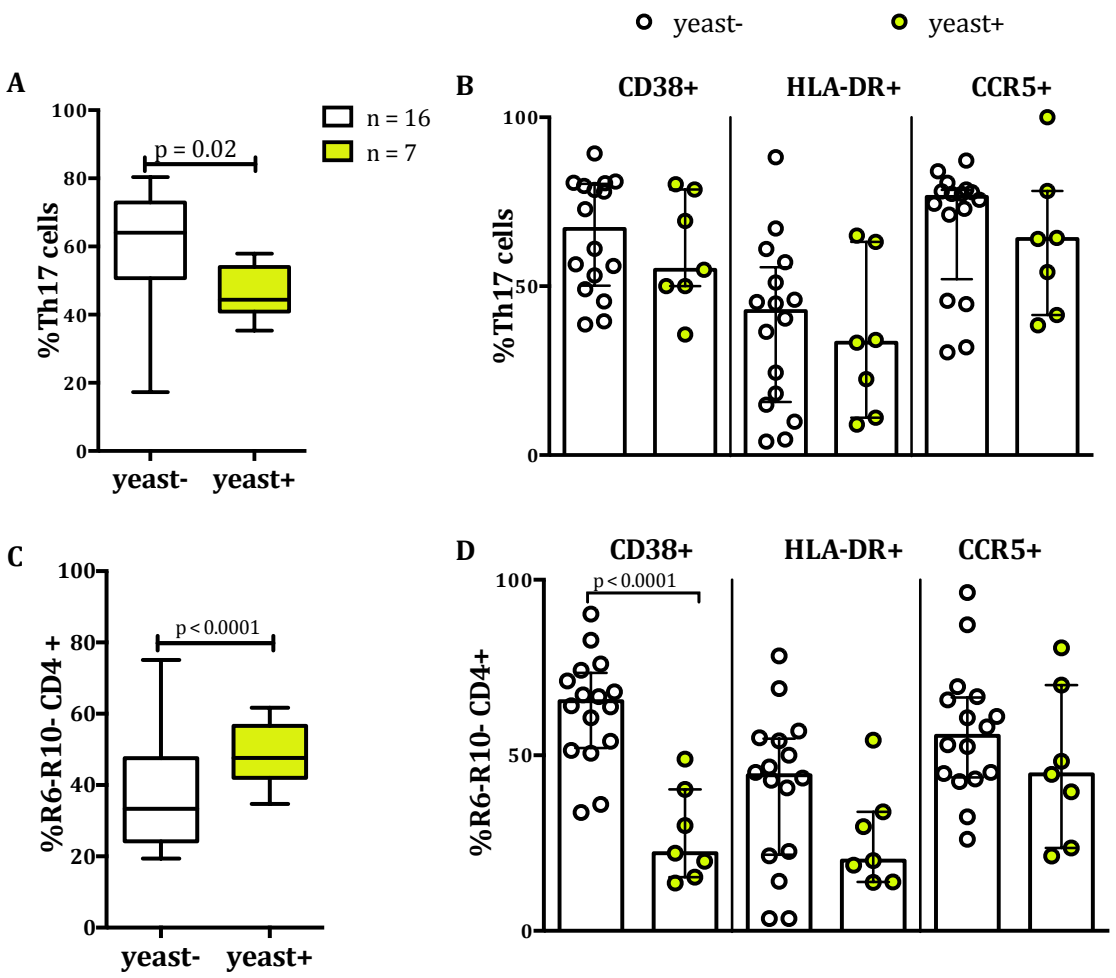

Figure 2.7. Impact of yeast infection on frequency and activation status of cervical Th17 cells in adolescents. (A) Frequency of Th17 cells in yeast- (clear box) and yeast+ (green box) adolescents. (B) Expression of CD38, HLA-DR and CCR5 on Th17 cells, stratified by yeast infection status. (C) Frequency of CCR6-CCR10- CD4+ T cells between yeast- and yeast+ adolescents. (D) Expression of CD38, HLA-DR and CCR5 on CCR6-CCR10- CD4+ T cells stratified by yeast infection status. Box and whisker plots show the median (middle line), IQR (box) and range (whiskers). Mann-Whitney U tests were used to compare the groups and P≤0.05 were considered significant.

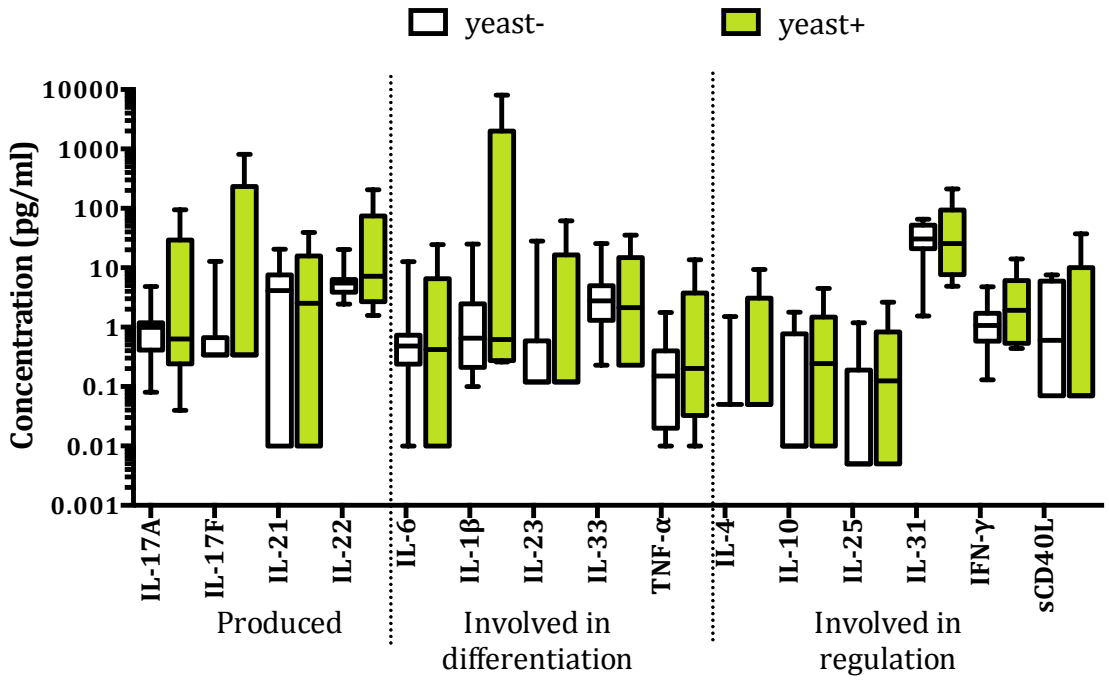

Figure 2.8. Impact of yeast infection on genital tract Th17-related cytokine concentrations. Adolescents without yeast infections are shown as clear bars, while those with a yeast infection are shown as green bars. Mann-Whitney U tests were used to compare the groups, and $p \leq 0.05$ were considered significant.

2.4.7 Impact of STIs on Th17 cells and cytokines

Th17 cells are thought to play an important role in regulating immunity against pathogens in the FRT (Feinen et al., 2010; Wazen et al., 2013). To ascertain whether the frequency and/or activation status of Th17 cells was influenced by STIs, like *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium* and *T. vaginalis,* these cells were compared between STI- (n=22) and STI+ (n=31) adolescents who did not have a yeast infection or BV (Nugent 7-10). Median Th17 event counts (per cytobrush) in STI- and STI+ adolescents did not differ significantly (246 vs 283 respectively, Figure 2.9A left panel), and the frequency of cervical Th17 cells and their activation status (CD38 and HLA-DR) did not differ by STI status (Figure 2.9A right panel and 2.9B, respectively). Although not significant, higher frequencies of activated (CD38+) cervical Th17 cells expressed CCR5 in STI+ compared to STI- adolescents (p=0.09; Figure 2.9C).

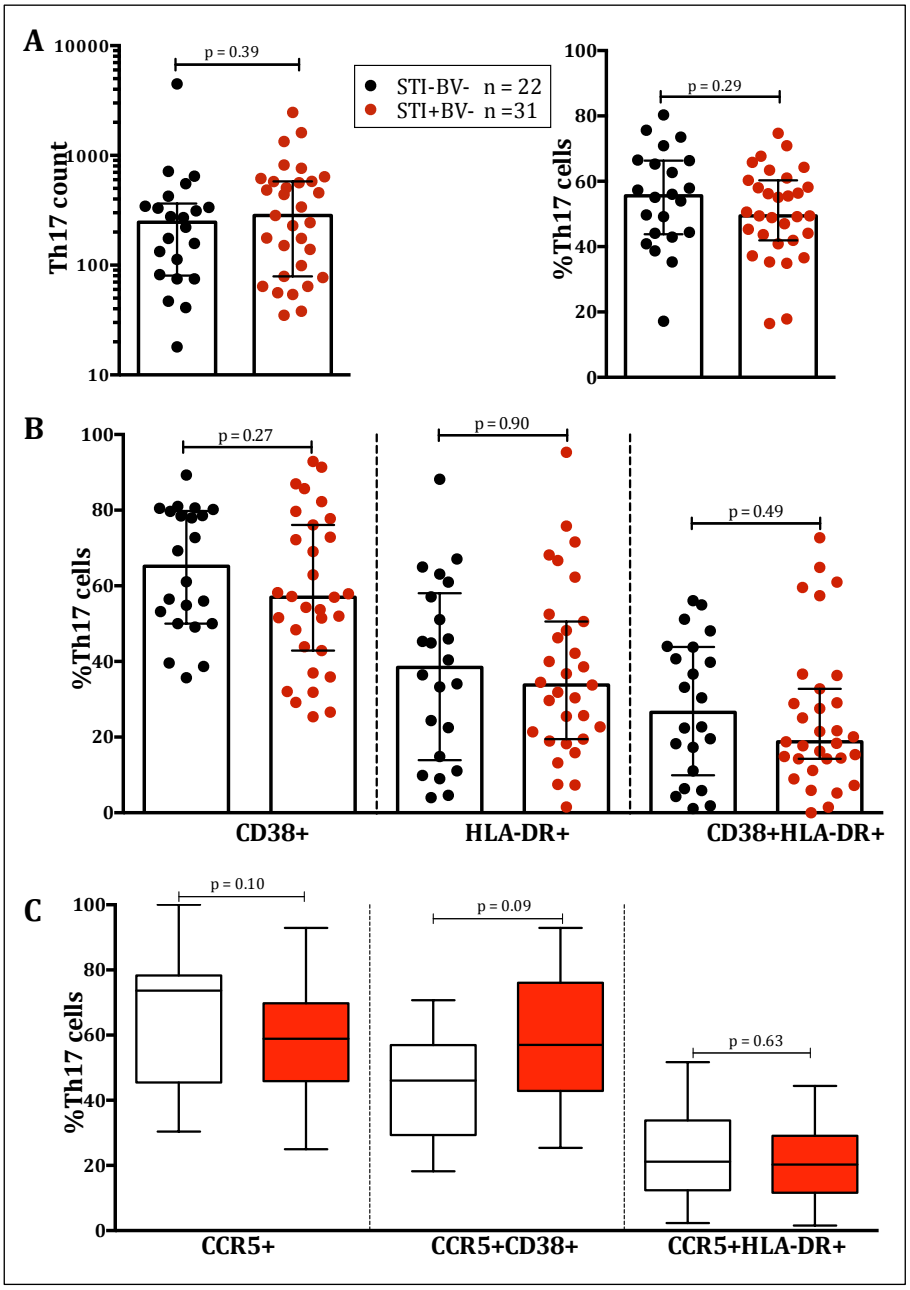

Figure 2.9. Impact of STIs (including chlamydia, gonorrhoeae, mycoplasma and trichomonas) on Th17 cell frequencies and activation status. (A) Th17 counts (left panel) and frequencies (right panel) in STI-BV- (black dots) and STI+BV- (red dots) adolescents. Bars indicated median values, and error bars indicate IQR. (B) Activation of Th17 cells as measured by markers CD38 and HLA-DR (STI-BV- [black dots] and STI+BV- [red dots]; bars indicated median values, and error bars indicate IQR). (C) Frequencies of CCR5 expression, alone (left panel) or in combination with activation markers (CD38, middle panel; and HLA-DR, right panel). Box and whisker plots show the median (middle

line), IQR (box) and range (whiskers). Mann-Whitney U-tests were used to compare the groups, and P≤0.05 were considered significant.

Research from our group and others have shown that certain STIs are associated with the induction of several cytokines, including IL-1β and IL-17 which were measured in this study (Crowley-Nowick et al., 2000; Mlisana et al., 2010; Masson et al., 2014; Deesa et al., 2015; Masson et al., 2015b). In this adolescent cohort, IL-17A (p=0.001) and IL-1β (p=0.02) were significantly elevated in adolescents who had an STI compared to those with no STI (Figure 2.10). IL-1β remained significant, after adjusting for multiple comparisons.

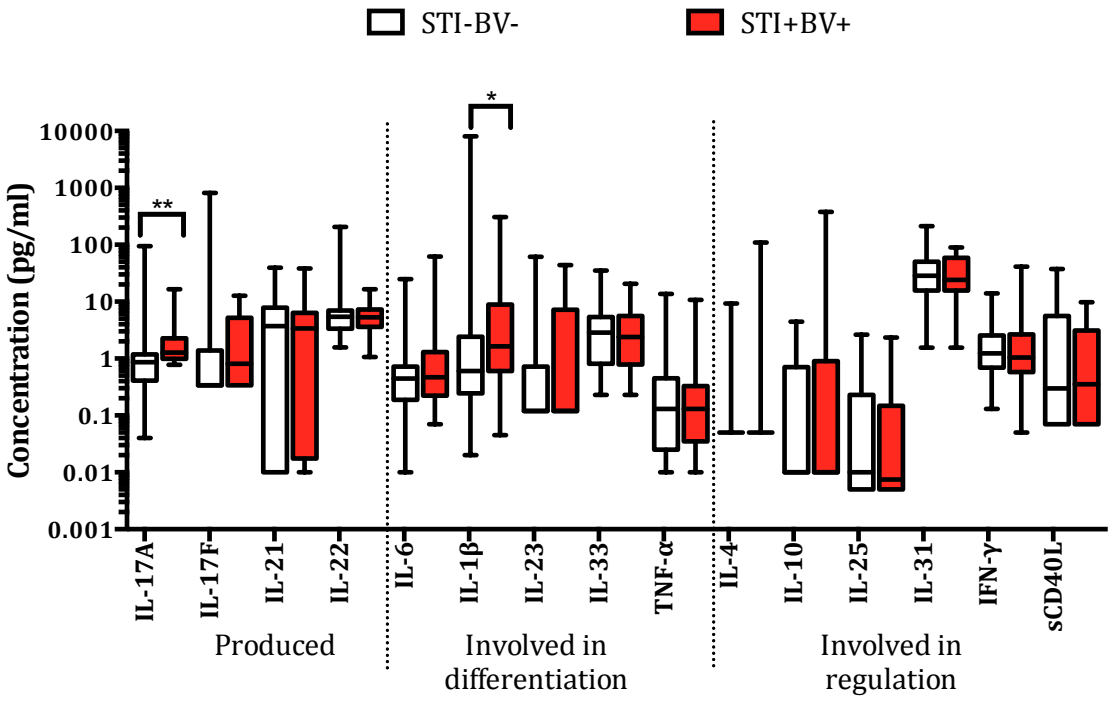

Figure 2.10. Impact of STIs on genital concentrations of Th17-related cytokines, in adolescents with (red box) and without STIs (clear boxes). Box and whisker plots show the median (middle line), IQR (box) and range (whiskers). Mann Whitney U-tests were used to compare the groups. *p<0.05; **p<0.01.

Since chlamydia infection was highly prevalent in this cohort (33%, Table 2.2), its effect alone on cervical Th17 cells and Th17-related cytokines was also investigated. For this analysis, 13 adolescents with asymptomatic chlamydia only (BV+C-) were compared to 22 adolescents without an STI or BV (BV-C-). Chlamydia did not alter the frequencies and activation status of Th17 cells (Figure 2.11A). On the contrary, IL-17A was significantly elevated in adolescents with chlamydia compared to those without (p=0.004, Figure 2.11B).

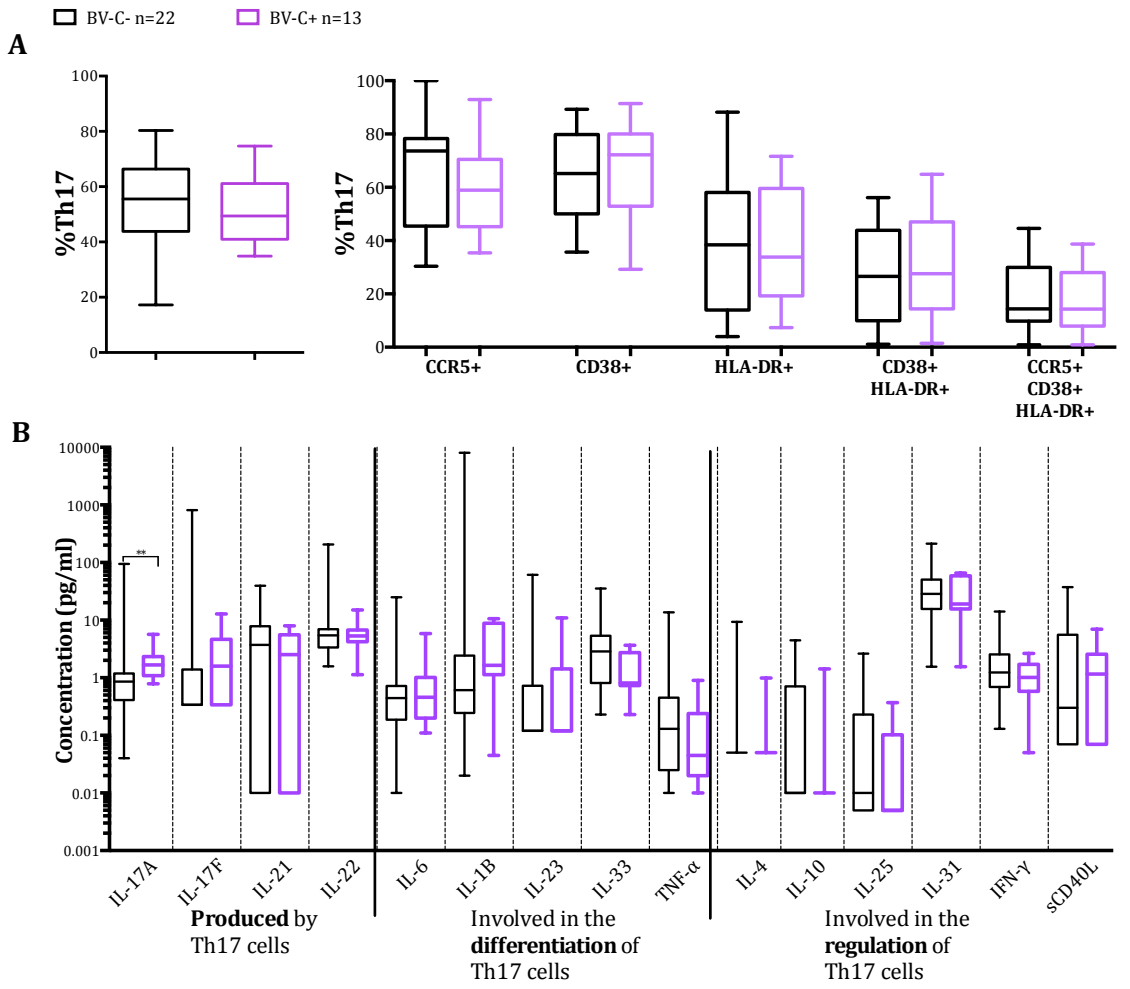

Figure 2.11. Impact of asymptomatic chlamydia on Th17 cell frequencies, activation status and concentrations of Th17-related cytokines, in adolescents with (black box) and without chlamydia (purple boxes). (A) Th17 frequencies (left panel) and expression of CCR5, CD38 and HLA-DR on Th17 cells (right panel). Concentrations of Th17-related cytokines in adolescents with and without chlamydia. Box and whisker plots show the median (middle line), IQR (box) and range (whiskers). Mann-Whitney U-tests were used to compare the groups, and P≤0.05 were considered significant. **p<0.01.

2.4.8 Impact of previous HC use on Th17 cells and cytokines

Since only 4.8% of adolescents were contraceptive naïve at baseline, Th17 cells between contraceptive naïve versus contraceptive users were compared. For this analysis, samples were available from 112 adolescents (naïve=6, NET-EN=75, COCPs=10 and DMPA=21). Frequencies of Th17 cells were not significantly different between contraceptive naïve adolescents and those who had previously used NET-EN, COCPs, or DMPA (Figure 2.12). The proportion of Th17 cells expressing CCR5 did not differ significantly between contraceptive naïve and adolescents using any contraceptives. However, adolescents who had used NET-EN had significantly lower proportions of Th17 cells expressing CCR5 compared to those using DMPA or COCPs ($p=0.04$ and $p=0.001$, respectively). In addition, adolescents who previously used COCPs had significantly higher frequencies of CCR5+ Th17 cells compared to those using DMPA ($p=0.01$). Expression of activation markers by Th17 cells did not differ significantly between contraceptive naïve and contraceptive users. However, a greater proportion of cervical Th17 cells from AGYW using NET-EN expressed HLA-DR and HLA-DR/CD38 compared to those using COCPs or DMPA.

In contrast to Th17 cell frequencies or activation status, Th17-related cytokines did not differ significantly between adolescents who had previously used different forms of hormonal contraception compared to those who were naïve to HCs at enrolment (Figure 2.13).

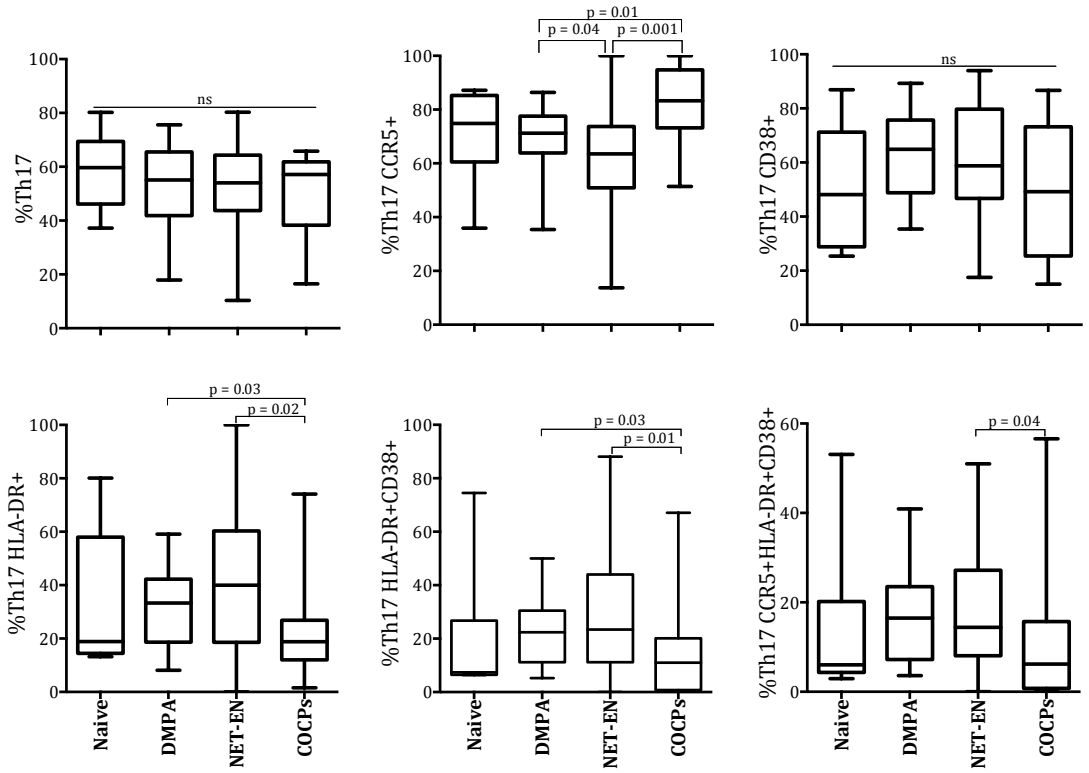

Figure 2.12. Effect of previous contraceptive use on cervical Th17 cell activation status and CCR5 expression. Frequencies of activated Th17 cells were compared between groups of naïve (n=6), DMPA (n=21), NET-EN (n=75) and COCPs users (n=10). Box and whisker plots show the median (middle line), IQR (box) and range (whiskers). P≤0.05 was considered significant.

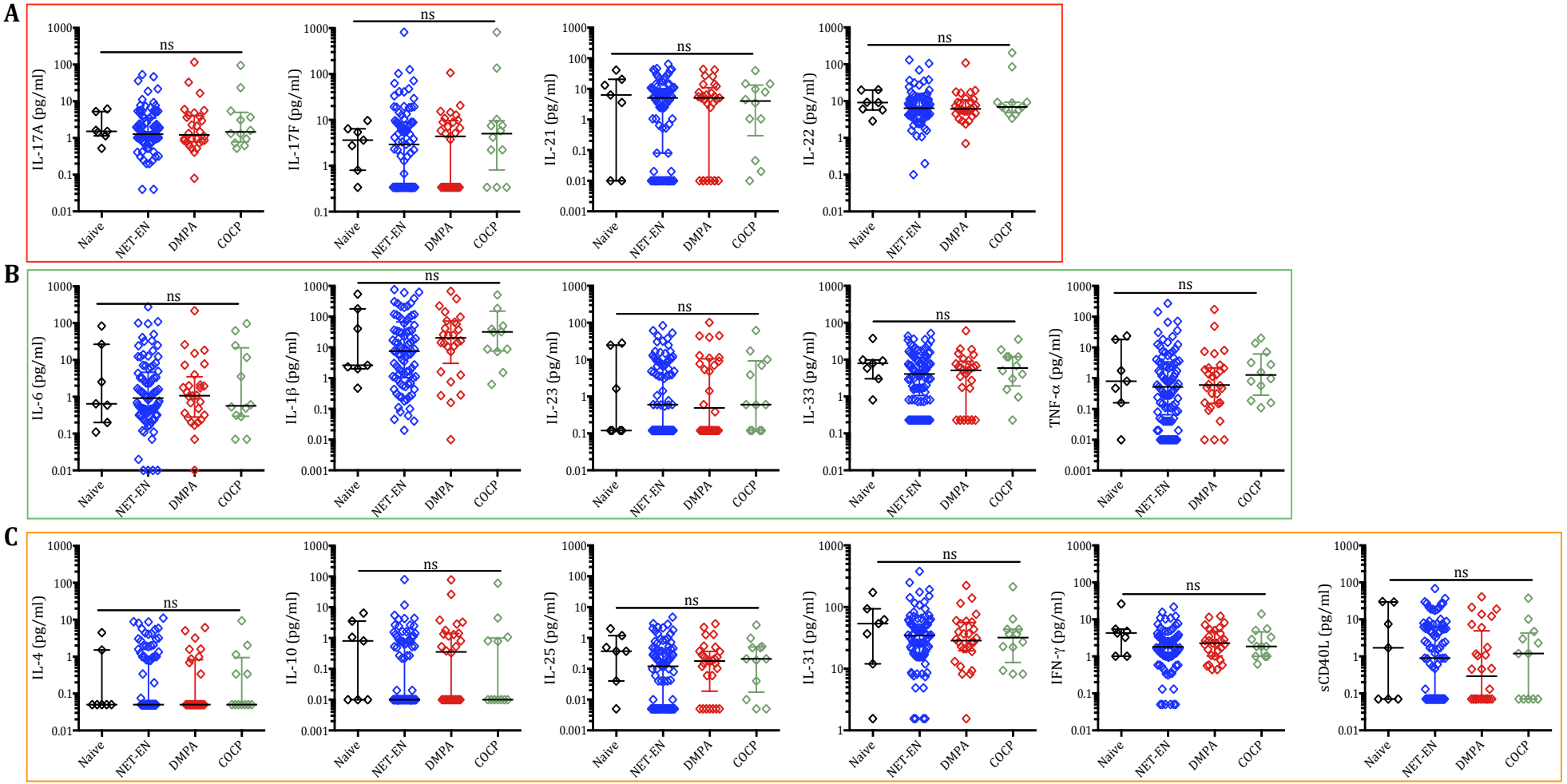

Figure 2.13. Th17-related genital tract cytokines stratified by previous use of hormonal contraceptives. (A) Cytokines produced by Th17 cells. (B) Cytokines involved in the differentiation of Th17 cells. (C) Cytokines involved in the regulation of Th17 cells. Kruskal-Wallis and Mann-Whitney U tests were used to compare cytokine concentrations according to HC group, and $P \leq 0.05$ were considered significant.

2.5 Discussion

Characterization of HIV target cells in the FRT of AGYW remains an important gap in our understanding of mucosal predictors of HIV risk. The purpose of this chapter was to characterize the immune microenvironment in adolescent girls, with a focus on Th17 cells and Th17-related cytokines. As previously described in adult women (Nkwanyana et al., 2009), cervical CD4+ T cells were more abundant in cervical cytobrush samples than CD8+ T cells. While more CD8+ T cells expressed CCR5 (the major HIV co-receptor for heterosexual transmission as well as the cognate receptor for MIP-1a, MIP-1b and RANTES; Zang et al., 2001), more CD4+ T cells expressed CCR6 (mucosal homing receptor, the cognate receptor for MIP-3a, and marker for Th17 cells; Lee et al., 2017).

In this study, cervical Th17 cells were characterized by expression of CCR6 of CD4+ T cells, but lack of CCR10 expression (Acosta-Rodriguez et al., 2007; McKinnon et al., 2015; Stieh et al., 2016). Using this chemokine receptor definition of Th17 cells (CCR6+CCR10-), Th17 cells were found to be the major CD4+ T cell subset in the FRT of adolescent girls (median frequency of 54.4%), followed by CCR6-CCR10- CD4+ T cells (Th1/Th2; Zhong et al., 2017). Rodriguez-Garcia et al. (2014) previously reported that genital tract Th17 frequencies ranged between 11.3% - 23.7% depending on anatomical site, including the cervix. This current study included adolescents for the first time, although age did not appear to influence the frequency of Th17 cells (comparing cervical Th17 frequencies in younger [15-17 years] versus older [18-19 years]). A previous study from our group characterized IL-17 producing cervical cytobrush-derived CD4+ T cells (by intracellular staining for IL-17 following stimulation with PMA/ionomycin) in adult South African women (+18 years), which were detected at more than half the frequencies of those reported in this study (Masson et al., 2015b). In women from Kenya, another study reported that higher frequencies of IL-17-producing Th17 cells were detected in cervical cytobrushes compared to blood, where Th17 cells were also defined by intracellular staining of IL-17 following PMA/ionomycin stimulation (McKinnon et al., 2011). While PMA/ionomycin stimulation and intracellular IL-17 staining are likely to underestimate Th17 cell numbers, it is also possible that defining this functional subset using chemokine receptor expression

(CCR6+CCR10-) may overestimate this subset. Furthermore, it is possible that expression of CCR6 on immune cells is not static, as it is stored intracellularly, or is continually recycled between the cytoplasmic and cell surface compartments (Ebert et al., 2002). Ebert and colleagues (2002) found that ~50% of the CD4+ T cells stained for CCR6 once permeabilized, suggesting that a subpopulation of cells constitutively express CCR6 intracellularly in the absence of surface expression. Following activation, however, some (but not all) of these cells begin expressing CCR6 on their surfaces (Ebert et al., 2002).

Susceptibility of Th17 cells to HIV infection has been documented by a number of recent studies, with some attributing this to higher expression of the HIV CCR5 co-receptor by this subset compared to other CD4+ T cells (El Hed et al., 2010; McKinnon et al., 2011; Rodriguez-Garcia et al., 2014; McKinnon et al., 2015; Stieh et al., 2016). In adolescents, this study did find that higher frequencies of CCR6+CCR10- Th17 cells expressed CCR5 compared to CCR6-CCR10- CD4+ T cells. While frequencies of highly activated Th17 and CCR6-CCR10- CD4+ T cells were similar, significantly higher frequencies of highly activated Th17 cells expressed CCR5 than CCR6-CCR10- CD4+ subset. Since it is well established that HIV preferably infects highly activated CD4+ T cells (Bégaud et al., 2006; Koning et al., 2005; Meditz et al., 2011; Joag et al., 2016), it is likely that this finding would contribute to HIV risk in these adolescents. Moreover, expressing the chemokine receptor CCR6 by Th17 cells draws them to mucosal surfaces like the genital tract as this chemokine receptor mediates mucosal homeostatic and inflammatory responses (Schutyser et al., 2003).

For naïve CD4+ T cells to differentiate into Th17 cells, different combinations of cytokines and chemokines are involved (Martinez et al., 2008; Spolski and Leonard, 2009; Monteleone et al., 2011; Jin and Dong, 2013; Brockmann et al., 2017). The 15-plex Th17 panel that was used to measure cytokines in this study included IL-17A and IL-17F, which are both produced by Th17 cells, in addition to IL-21 and IL-22. Of these, IL-22 was detected at the highest concentrations in genital secretions, and is known to induce expression of AMPs (Liang et al., 2006). Of the two IL-17 isomers, IL-17F was detected at higher concentrations than IL-17A. Most studies to date measured IL-17A (also known as IL-17) in the FRT, but not IL-17F (McKinnon et al., 2011; Masson et al.,

2015b; Boily-Larouche et al., 2017). Since these two subtypes differ in some aspects including less potency of IL-17F to induce cytokines, understanding their inflammatory roles and that of other IL-17 subtypes not measured in this study in the FRT is important. IL-6, IL-1β, IL-21, IL-23, IL-33 and TNF-α play a role in the differentiation of Th17 cells (Hebel et al., 2011; Lasigliè et al., 2011; Patel and Kuchroo, 2015). Of these, IL-1β thought to amplify the differentiation of Th17 cells was measured at the highest concentrations. Of the cytokines that are involved in regulating Th17 cells (including IL-4, IL-10, IL-25, IL-31, IFN-γ and sCD40L), IL-31 was detected at the highest concentrations. IL-31 belongs to the IL-6 cytokine family, and it can positively and negatively affect Th17 differentiation (Zhang et al., 2008). Furthermore, genital IL-31 in these adolescents strongly correlated positively with sCD40L, a cytokine that provides an optimal cytokine environment for the differentiation of Th17 cells (Iezzi et al., 2009). A similar positive correlation between IL-31 and sCD40L was found in healthy serum (de J. Guerrero-García et al., 2018). IL-10, a major regulatory cytokine produced by Treg cells, was found to correlate, albeit relatively weakly, with about half of the Th17-related cytokines (including IL-21, IL-23, IL-33, TNF-α, IL-4, IL-25 and IFN-γ). In mice, IL-10 has been shown to negatively regulate RORγt (expressed by Th17 cells) and Th17-related cytokine production by macrophages and T cells (Gu et al., 2008). While genital tract concentrations of IL-17A and IL-17F correlated positively with higher frequencies of activated (CD38+) cervical Th17 cells, IL-17A appeared to correlate negatively with frequencies of CCR5+ Th17 cells. While none of the chemokines that bind to CCR5 were measured as part of this study (such as MIP-1a, MIP-1b and RANTES), these CCR5-binding chemokines do correlate positively with inflammatory cytokines like IL-1β and IL-6 that were measured (Masson et al., 2014). Since others from our group have found that CCR5 expression by cervical CD4+ T cells in adult women are negatively associated with genital concentrations of CCR5-binding chemokines (such as RANTES), and that higher concentrations of RANTES in the FRT is associated with CCR5 receptor internalization (Liebenberg et al., 2011), it is also possible therefore that some of the CCR5+ frequency loss that was noted in this study of adolescent reproductive health was related to high levels of inflammation and CCR5-binding chemokines.

Endogenous hormones such as E2 and P4 play an important role in host immunity such as regulating induction of cytokines and chemokines (Polese et al., 2014; Khan and

Ansar Ahmed, 2016). Adolescence is a time of dramatic hormonal changes, and therefore the impact of E2, FSH and LH on Th17 cells and Th17-related cytokines was evaluated. Endogenous E2 and LH levels were negatively associated with the frequency of highly activated (CD38+HLA-DR+) Th17 cells, suggesting that these endogenous female hormones may modulate cellular activation. However, no association was found between genital concentrations of any of the Th17-related cytokines and serum hormone levels. Although the direct effects of E2 and LH on Th17 cells or IL-17 secretion were not addressed in this study and are not known, studies in postmenopausal women showed an inverse correlation between E2 deficiency and IL-17A serum levels (Molnar et al., 2014).

The involvement of genital tract Th17 cells to controlling vaginal yeast infections has not been elucidated, although both Th1 and Th17 responses are thought to be important in immune defense against *C. albicans* infections (Kagami et al., 2014; Pietrella et al., 2011). *Candida albicans* is the most commonly fungal pathogen of humans and is frequently found on the mucosal surfaces of the body, including the lower FRT (Richardson and Moyes, 2015). In this study, adolescents who had vaginal yeast hyphae evident on their wet mounts had lower frequencies of cervical Th17 cells than yeast negative adolescents. Furthermore, IL-17A, IL-17F and IL-22 are also thought to be critically important for immune protection against *C. albicans* infection (Conti et al., 2015). However, Th17-related genital tract cytokines appeared to be similar in yeast positive and yeast negative AGYW. Masson et al. (2015b) previously reported that adult women who had evidence of vaginal candidal pseudohyphea had lower concentrations of genital IL-17 compared to women who did not have detectable yeast infections. In addition, *in vitro* stimulation of human PBMCs with *C. albicans* down-regulated IL-17 production (Cheng et al., 2010).

While vaginal yeast appeared to be associated with lower frequencies of Th17 cells, the presence of STIs did not appear to similarly influence Th17 frequencies or activation status. However, concentrations of both IL-17A and IL-1β were elevated in adolescents with an STI, and this is similar to what has been described in adult women (Masson et al., 2015b). It is possible that cells other than CD4+ T cells are producing IL-17A, such as γδ T cells (Price et al., 2012), which are also abundant at mucosal surfaces such as the

FRT (Alcaide et al., 2016; Strbo et al., 2016). Alternatively, it is also possible that elevated inflammatory cytokines like IL-1β may influence CCR6 internalization and cycling, by predicting increased production of MIP-3a (the cognate chemokine for CCR6). MIP-3a, which was not measured in this study, can be induced following treatment of immune cells with IL-1β (Li et al., 2013). However, the small sample size of the adolescents in this study who had single infections are likely to have influenced this outcome as previous studies have suggested that genital tract Th17 cells are increased in women with laboratory-diagnosed STIs. For example, gonorrhoea had been reported to initiate Th17 responses associated with recruitment of neutrophils and a pro-inflammatory milieu (Feinen et al., 2010; Feinen and Russell, 2012).

High BMI has previously been shown to increase frequencies of blood Th17 cells in children (Schindler et al., 2017). Similarly, high BMI in adults has been linked to higher concentrations of IL-17 in genital secretions, as well as IL-6, IL-12, and IL-1RA (Ventolini et al., 2017). However, BMI did not appear to influence cervical Th17 frequencies or IL-17 concentrations in adolescents in this study, although the median BMI for the cohort was >25, which would be considered high by international standards.

Most of the adolescents recruited into the study were not contraceptive naïve at enrolment, although information collected on how long they had been using HCs prior to enrolment was limited. Therefore, the relationship between previous contraceptive use and Th17 cells was important to evaluate. Despite this limitation, contraceptive naïve adolescents had similar cervical Th17 frequencies and level of activation as the adolescents who were using HCs. However, differences were observed when the different HC products were compared at enrolment. In adolescents that had been using in NET-EN prior to enrolment, expression of CCR5 on Th17 cells was significantly lower compared to those reporting DMPA and COCPs use. In contrast, expression of HLA-DR and HLA-DR with CD38 was lower in adolescents reporting to previously have used COCPs compared to NET-EN and DMPA users. Th17-related cytokines in genital secretions did not appear to differ significantly between adolescents who had never used HCs compared to those who had.

Less than 20% of the adolescents in this study had no BV or STI (chlamydia, gonorrhoeae, mycoplasma and trichomonas). STIs were associated with significantly increased concentrations of IL-17A and IL-1β in genital secretions, as has previously been described (Masson et al., 2014). In contrast, cytokines like IL-10 and IL-4 that negatively regulate Th17 cells were only detected at low levels in the genital tract. This finding fits with previous observations that inflammatory responses suppress anti-inflammatory responses and vice versa (Zhang et al., 2007).

The analyses in this Chapter have some limitations that should be acknowledged. Th17 cells were not measured in blood so it was not possible to determine the relationship between blood and genital tract Th17-related cytokines. Furthermore, there were no older women in this cohort to compare Th17 cells and Th17-related cytokines in younger versus older women. However, the distribution of these cells remained comparable between the participants when stratified by age.

In conclusion, highly activated Th17 cells were found to be abundant in the FRT of adolescents, which expressed high levels of CCR5. Th17-related cytokines, known to be involved in different Th17 pathways, were detected in the FRT, and appeared to be induced by several factors like STIs. The presence of Th17 cells and Th17-related cytokines in adolescents could indicate protective immune responses in the lower FRT that are important for maintaining immunological homeostasis. However, these increased Th17 frequencies could also render adolescents more susceptible to HIV.

Chapter 3

Relationship between Th17 cells, BV, and vaginal microbiota

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3.1 Abstract

BV is a common vaginal dysbiosis among reproductive-aged women, caused by changes in vaginal microbial communities, which results in inflammatory changes in the lower FRT. Th17 cells are known to be important for defense against bacterial infections, although their interaction with the vaginal microbiota and BV is not yet understood. Adolescence is a time of increased risk for BV and Th17 cells are abundant in the genital tracts of these young women. The aim of this study was to evaluate whether BV and the vaginal microbiota were associated with changes in cervicovaginal Th17 cell populations and activation status in AGYW, as well as with the genital tract cytokines related to these cells. For this study, BV was characterized by Nugent score (Nugent 7-10 were considered BV+), vaginal pH was measured using Macherey-Nagel pH strips (pH>4.5 considered high), and 16S rRNA gene sequencing of cervical swabs was used to define community types using unsupervised clustering: C1 being more diverse; C2 predominantly made of *Lactobacillus crispatus*; and C3 predominantly made up of *Lactobacillus iners*. While frequencies and the activation status of CCR6+CCR10- Th17 cells were similar in BV+ and BV- AGYW (by Nugent scoring) and by vaginal community types (C1, C2 and C3), Th17-related cytokines were significantly elevated in BV+ compared to BV- adolescents, and in C1 versus C2 and C3 adolescents. These findings suggest that microbial changes in the lower FRT of adolescents and BV-associated changes do influence the profile of Th17-related cytokines although not Th17 cells.

3.2 Introduction

BV is a highly prevalent, recurrent and complex vaginal dysbiosis characterized by a shift in the vaginal microbiota from one dominated by *Lactobacillus* spp. (including *L. crispatus, L. jensenii, L. gasseri, L. johnsonii*, and *L. mucosae*) to more diverse non-*Lactobacillus* anaerobic bacterial communities dominated by various species, including *Prevotella, Gardnerella, Atopobium*, and *Sneathia* (Vitali et al., 2007; Brotman et al., 2010; Xia et al., 2016). A recent review has highlighted some of the challenges that the international scientific community has faced in developing a universally relevant definition for BV (McKinnon et al., 2019). McKinnon et al. (2019) summarize some of the major reasons for these challenges being that "normal" bacterial profiles may differ markedly between women, particularly women of different ethnicities (Ravel et al., 2011); that the methodologies for diagnosing BV have differed quite widely between studies; and there is still no consensus on what constitutes a "pathogenic" versus "pathobiont" bacterial community with regards to BV. Currently, BV may be defined clinically as symptomatic BV (Klebanoff et al., 2004), by high vaginal pH levels (pH>4.5), as "Amsel-BV" (Amsel et al., 1983), "Nugent-BV" (Nugent et al., 1991) or "molecular-BV" (McKinnon et al., 2019), each of which depends on the method used for diagnosis.

Symptomatic BV is non-specifically diagnosed if women present with vaginal discharge, a fishy odor and vaginal itching or burning (Amsel et al., 1983). However, most women with BV are asymptomatic (Mlisana et al., 2011). BV is often diagnosed in the clinical setting using Amsel's criteria according to the release of a fishy amine odor (on addition of potassium hydroxide), presence of squamous cells coated with bacteria (known as clue cells), and high vaginal pH levels (Amsel et al., 1983; White et al., 2011; Nelson et al., 2015). The sensitivity and specificity of Amsel's criteria have ranged from 37% to 70% and 94% to 99%, respectively, compared to Nugent scoring (Coleman and Gaydos, 2018). The current international gold standard for diagnosing BV is the Nugent scoring system (Nugent, 1991). However, this method is not used frequently in the clinical setting as experienced laboratory staff and relatively specialized

equipment (microscopes and gram stains) are needed. “Molecular-BV” describes dysbiosis identified using any molecular technique. Due to the complexity of BV, bacterial community types (CT) have become useful for the characterization of vaginal dysbiosis (Ravel et al., 2011; Ma et al., 2012). Vaginal CTs characterizes the microbiota based on the dominant microbial species, although several groups have suggested different classifications within each CT: Ravel et al. (2011) first recommended five CTs in a large study of more than 300 asymptomatic North American women (including African, Caucasian, Asian and Hispanic American women); Anahtar et al. (2015) subsequently suggested that a four CT system was best to describe vaginal bacterial communities in 146 asymptomatic South African women aged 18-23 years; and authors from our group later argued for a three CT system with Cluster 1 (C1) being most diverse, Cluster 2 (C2) being dominated by *L. crispatus*, and Cluster 3 (C3) being dominated by *L. iners* (Lennard et al., 2017).

Vaginal microbiome studies have found a strong correlation between high-diversity microbial communities and BV diagnosed by Nugent scoring (Van de Wijgert et al., 2014). Despite differences in how the vaginal bacterial communities in health and disease should be clustered, all studies have agreed that *L. crispatus* and *L. iners* tend to be the most prevalent vaginal lactobacilli in healthy women (Ravel et al., 2011; Anahtar et al., 2015; Gosmann et al., 2017; Lennard et al., 2017; McClelland et al, 2018), although molecular studies have shown *L. crispatus* to be associated with better health outcomes than *L. iners* (Ravel et al., 2011; Gosmann et al., 2017).

Nugent-BV has been associated with a 1.5–3.0-fold increased risk of acquiring STIs, including HIV (McClelland et al., 2018; Low et al., 2011), HPV (Gillet et al., 2011), *C. trachomatis* and *N. gonorrhoeae* (Brotman et al., 2010; Bautista et al., 2017), as well as a 3-fold increased risk of HIV-1 transmission to a male partner (Cohen et al., 2012; Klatt et al., 2017). This is particularly concerning in young SA women, as HIV is exceptionally prevalent in this group and recent data estimates the prevalence of BV in these women to be around 42% (Kharsany and Karim, 2016; Torrone et al., 2018). The association between BV and HIV acquisition is

possibly attributable to mucosal inflammation, which increases the abundance of HIV target cells, and reduces epithelial barrier integrity (Spear et al., 2007; Dandekar et al., 2010; Arnold et al., 2016; Klatt et al., 2017). Previous studies have shown that endogenous microbiota play a major role in the differentiation of Th17 cells in the small intestine, and that antibiotic treatment reduces the number of Th17 cells in the gut (Ivanov et al., 2008). Different microbes may induce the production of cytokines involved in the differentiation of Th17 cells, including IL-6, IL-1β, IL-23, IL-33 and TNF-α (Guglani and Khader, 2010). Th17 cells, in turn, produce inflammatory cytokines, such as IL-17A, IL-17F and IL-22, which may contribute to the inflammatory cascade and promote HIV infection (Elhed and Unutmaz, 2010).

The role of Th17 cells and related cytokines in promoting immunity against or in response to vaginal dysbiosis is unknown. Although Th17 cells are critical for the clearance of bacterial pathogens, their susceptibility to HIV is of major concern. Therefore, the aim of this chapter was to evaluate the relationship between BV (and related vaginal dysbiosis) with the presence and activation status of Th17 cells and Th17-related cytokines in the lower FRT of adolescents.

3.3 Materials and Methods

3.3.1 Study participants

As described in Chapter 2, a total of 151 HIV-negative adolescent girls screened at baseline were included in this cross-sectional analysis of the relationship between BV and Th17 cell frequencies (and/or their activation status; see Chapter 2, section 2.3.1). Demographic, behavioral and clinical data was collected as described in Chapter 2 and the same AGYW were included in this analysis.

3.3.2 BV diagnosis

A lateral vaginal wall/posterior fornix swab was collected from each adolescent for Gram staining and Nugent scoring (for Nugent scoring), which was scored at the National Institute of Communicable Diseases (NICD, Sandringham, Johannesburg). According to Nugent score criteria, AGYW were categorized as being BV negative (Nugent scores 0–3), having intermediate microbiota (Nugent scores 4–6), or being BV positive (Nugent scores 7–10). Vaginal pH was measured using colour-fixed indicator strips (Macherey-Nagel, Duren, Germany), and normal vaginal pH was considered pH≤4.5 (Money et al., 2005).

3.3.3 Processing of cervical cytobrushes and flow cytometry

Cervical cytobrushes were collected as described in Chapter 2 (section 2.3.5), and processed within 4 hours. The freshly collected cervical cells were stained using a flow cytometry panel as described in Chapter 2 (section 2.3.6), which was optimized for this study.

3.3.4 Measurement of Th17-related cytokines

The concentrations of Th17-related cytokines (IL-17A, IL-17F, IL-21, IL-22, IL-6, IL-1β, IL-23, IL-33, TNF-α, IL-4, IL-10, IL-25, IL-31, IFN-γ and sCD40L) were measured by Luminex, as described in Chapter 2 (section 2.3.9).

3.3.5 Vaginal 16S rRNA gene amplification and Illumina miSeq sequencing

Vaginal lateral wall swabs were collected for microbiome analysis by 16S rRNA amplicon sequencing, by Christina Balle as part of the larger uChOOSE study (Christina Balle PhD thesis), and the method had been described elsewhere (Lennard et al., 2017). Briefly, available swabs from 150 participants were thawed and treated with an enzyme cocktail consisting of mutanolysin (25kU/ml, Sigma Aldrich, Modderfontein, SA), lysozyme (450kU/ml, Sigma Aldrich), and lysostaphin (4kU/ml, Sigma Aldrich) for 1 hour at 37°C. The microbial DNA was extracted using *Quick*-DNA™ Fungal/Bacterial Miniprep kits (Zymo Research) and prepared for sequencing. The V4 hypervariable region of the bacterial 16S rRNA genes was amplified by PCR using modified universal primers (Pearce et al., 2014; Lennard et al., 2017). Samples with ≥5000 reads were selected for downstream analyses. The operational taxonomic unit (OTU) table was normalized and filtered such that each OTU had at least 10 counts in at least 20% of samples or a relative abundance of at least 0.001% (as previously described; Lennard et al., 2017).

3.3.6 Statistical analyses

Baseline characteristics were summarized using descriptive measures, such as median, frequencies and IQR. For categorical variables, the Fisher's Exact test was used to compare groups. Non-parametric Mann-Whitney U test and Wilcoxon Signed Rank test were used for unmatched and paired samples respectively. For comparison between more than two groups, the Kruskal-Wallis one-way analysis of variance was used. Correlations were performed using the

Spearman Rank test. Multivariate logistic regression was used to assess associations between BV and log_{10}-transformed cytokine concentrations after adjusting for possible confounders. Microbiota community clusters were established by Fuzzy clustering using the R package 'cluster' (Maechler et al., 2017) with optimal k, a membership exponent of 1.25 and weighted UniFrac as the dissimilarity measure. Statistical analysis was performed using Prism version 6.0 (GraphPad Software, CA, USA), R Studio and STATA™ version 12 (StataCorp, TX, USA). A p-value of ≤0.05 was considered to be statistically significant. The Benjamini-Hochberg method was used to adjust for multiple comparisons (Benjamini and Hochberg, 1995).

3.4 Results

Of the 151 adolescents screened for the uCHOOSE study (Table 2.2, Chapter 2), 46% (69/151) were BV negative (Nugent 0-3), 11% (17/151) had intermediate vaginal microbiota (Nugent 4-7), and 43% (65/151) were BV positive (Nugent 7-10; Table 3.1). Adolescents with BV (Nugent scores 7-10) did not differ from those without BV (Nugent scores 0-3) in serum hormone levels of FSH and LH, although E2 serum levels tended to be higher in those with BV (p=0.03) or intermediate Nugent scores (p=0.07). Behavioural characteristics and previous contraception use were similar between the groups (Table 3.1). Prevalence of STIs was high overall (Table 2.2, Chapter 2), but did not differ significantly according to BV status (Table 3.1). However, the prevalence of evident vaginal yeast infections was significantly higher in BV- adolescents than BV+ or intermediate AGYW (p=0.02), while reported condom use was higher in BV- versus BV+ or intermediate groups (p=0.03). As has previously been described (Money et al., 2005; Hemalatha et al., 2013), vaginal pH was significantly higher in BV+ compared to BV- adolescents (pH5.0 [4.7 - 5.3] versus pH4.7 [4.4 - 5.0], respectively, p<0.0001), and those with intermediate microbiota had pHs similar to BV+ adolescents (pH=5.3 [4.8 - 5.6]).

Table 3.1 Participant characteristics according to Nugent-BV status

	BV negative n=69	**Intermediate n=17**	**BV positive n=65**	
	n/N (%), Median (IQR)			
				p-value
Median age	17 (16 - 18)	17 (16 - 19)	17 (16 - 18)	0.355
BMI (kg/m²)	25.1 (22.2 - 28.6)	23.9 (21.4 - 25.3)	25.8 (21.7 - 30.4)	0.164
Serum hormone levels				
E2 (pmol/l)	93.0 (74.0 - 137.5)	87.0 (57.5 - 129.5)	107.5 (82.3 - 157.8)	0.053
S-FSH (U/L)	5.0 (3.3 - 6.0)	5.3 (2.9 - 5.9)	4.8 (3.6 - 6.1)	0.973
LH (IU/L)	4.0 (2.0 - 5.1)	4.8 (2.3 - 6.5)	4.9 (2.0 - 6.7)	0.111
Vaginal pH	4.7 (4.4 - 5.0)	5.3 (4.8 - 5.6)	5.0 (4.7 - 5.3)	**<0.0001**
Genital infections				
No STI	29/69 (42.0%)	10/17 (58.8%)	26/65 (40.0%)	0.367
N. gonorrhoeae	8/69 (11.6%)	1/17 (5.8%)	9/65 (13.8%)	0.990

T. vaginalis	5/69 (7.2%)	4/17 (23.5%)	5/65 (7.7%)	0.165
C. trachomatis	22/69 (31.9%)	5/17 (29.4%)	23/65 (35.3%)	0.999
M. genitalium	2/69 (2.9%)	0/17 (0%)	2/65 (3.1%)	0.601
HSV-2 serology	16/69 (23.2%)	2/17 (11.8%)	19/65 (29.2%)	0.440
Presence of yeast hyphae	16/69 (23.2%)	2/17 (11.8%)	5/65 (7.7%)	**0.020**
Behavioural characteristics				
Age of sexual debut	15 (14 - 16)	15 (14 - 16)	15 (14 - 16)	0.938
Condom use always	43/61 (70.5%)	9/12 (76.9%)	26/54 (47.2%)	**0.030**
Ever washed vagina	3/69 (4.3%)	2/18 (11.1%)	8/65 (12.3%)	0.217
Previous contraceptive use				
None	3/69 (4.3%)	1/17 (5.8%)	3/61 (4.9%)	0.981
DMPA	13/69 (18.8%)	2/17 (11.7%)	13/61 (21.3%)	0.608
NET-EN	45/69 (65.2%)	12/17 (70.6%)	37/61 (60.6%)	0.531
COCP	5/69 (7.2%)	1/17 (5.8%)	6/61 (9.8%)	0.799
Implanon	0/69 (0%)	1/17 (5.8%)	2/61 (3.2%)	0.234

BV, bacterial vaginosis; BMI, body mass index; E2, estradiol; S-FSH, follicle stimulating hormone; LH, luitenizing hormone

3.4.1 Vaginal microbiota according to Nugent-BV status

To evaluate the vaginal microbial composition in AGYW at baseline, the variable region 4 (V4) of the bacterial 16S rRNA gene was sequenced to assess bacterial relative abundance (Anahtar et al., 2015). Others from our group have previously reported that observed bacterial communities in vaginal samples from adolescents in the same community clustered into three distinct CTs (C1, C2, C3) by Fuzzy clustering, with weighted UniFrac distances (Lennard et al., 2017). This characterization of vaginal communities was adopted in this chapter, whereby C1 represents a more diverse microbiota with low *Lactobacillus* abundance, while C2 and C3 were made up of predominantly *L. crispatus* and *L. iners,* respectively (Figure 3.1B). This analysis was conducted by Christina Balle as part of her PhD (submitted). Of the 151 baseline samples collected, 141/151 (BV negative n=64, intermediate n=14, BV positive n=63) had sequence of high enough quality to analyze (Balle, Jaspan et al., personal communication).

At baseline, 52% (73/141) of the participants had a non-*Lactobacillus* dominated microbiota, while 48% (68/141) had a *Lactobacillus*-dominated microbiota. Of

those with a *Lactobacillus*-dominant microbiota, 21% (30/141) had primarily *L. crispatus* and 27% had *L. iners* (38/141). When these clusters were stratified according to Nugent criteria (Figure 3.1A), 97% of BV+ adolescents grouped into the C1 CT (61/63), with the most abundant bacterial species being *G. vaginalis*, *BVAB1*, *Megasphaera* spp., *L. iners* and *Prevotella* spp. Of those with intermediate Nugent scores, 71% were classified into C1 (10/14). Of those who were considered BV negative, 44% had *L. crispatus*-dominant CTs (28/64) and 53% had *L. iners*-dominant CTs (34/64).

A

	BV negative	Intermediate	BV positive	
	n/N (%)			
Community types				
C1	2/64 (3.1%)	10/14 (71.4%)	61/63(96.8%)	<0.0001
C2	28/64(43.8%)	2/14 (14.3%)	0/63 (0%)	<0.0001
C3	34/64 (53.1%)	2/14 (14.3%)	2/63 (3.2%)	<0.0001

B

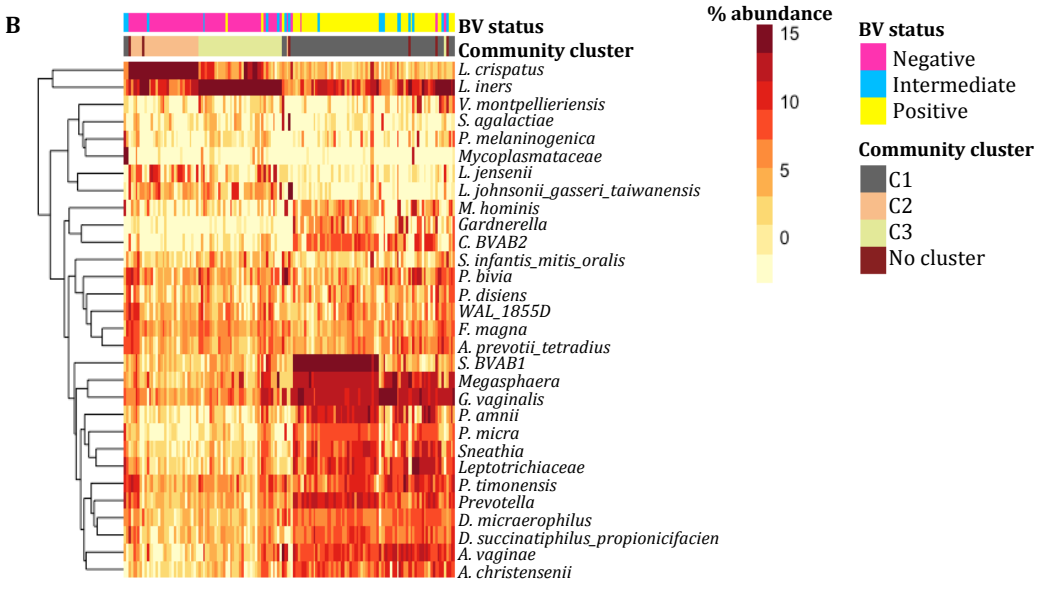

Figure 3.1. Vaginal microbial composition in adolescents at baseline. (A) Table showing community types stratified according to BV status. (B) Unsupervised hierarchical clustering of bacterial taxa in vaginal lateral wall swabs identified by 16S rRNA sequencing. Adolescents are labeled according to BV status and community cluster. Community types were identified using fuzzy clustering based on the dominant bacterial species: C1 (more diverse anaerobic bacteria), C2 (*L. crispatus*) and C3 (*L. iners*) (according to the method described by Lennard et al., 2017). The dendrogram on

the left of the figure shows the relatedness of different bacterial taxa. Figure 3.1B provided by Christina Balle.

3.4.2 Relationship between BV status and Th17 cell frequencies

Since several studies have shown that Th17 cells are important in protection against bacterial and fungal pathogens (Pietrella et al., 2011; Cooper, 2014), the relationship between BV status and cervical Th17 cell frequencies and/or activation status was assessed. Both the median frequencies of Th17 cells, their relative counts, and the proportion expressing CCR5 or activation markers did not differ by BV status (Figure 3.2), irrespective of whether STIs were excluded from this analysis (data not shown). In addition, no significant differences according to BV status in overall CD4+ T cell frequencies or activation status was observed (data not shown).

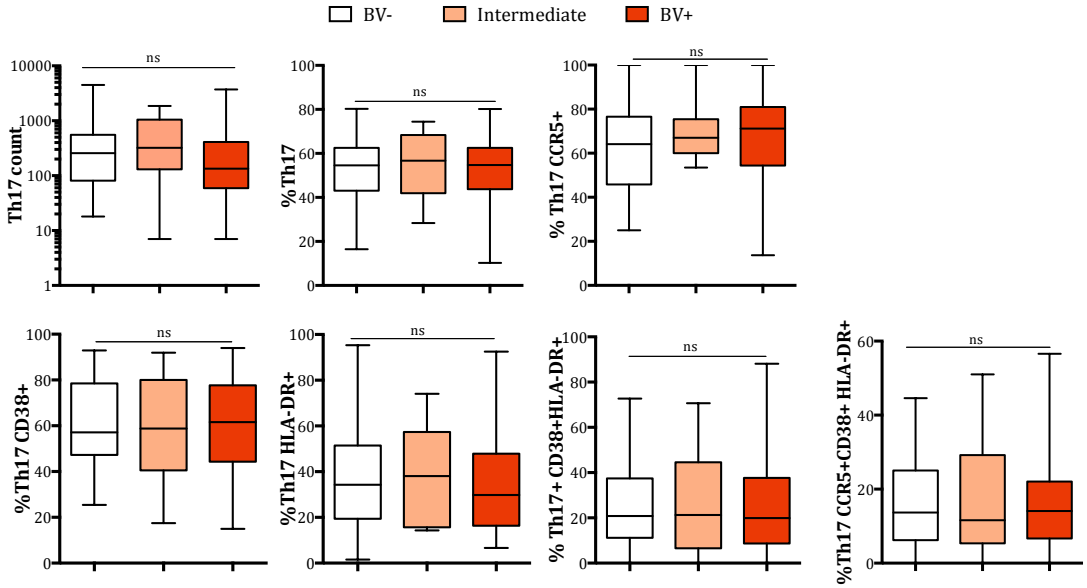

Figure 3.2. Proportion and phenotype of Th17 cells stratified according to BV status (by Nugent scoring). (Top panel) Box and whisker plots (median, IQR and range) showing Th17 counts (first graph), Th17 frequencies (second graph) and CCR5 expression on Th17 cells (third graph) according to BV status. (Bottom panel) Expression of activation markers CD38 and HLA-DR, and co-expression of CCR5 with

activation markers on Th17 cells. Mann-Whitney and Kruskal-Wallis tests were used to compare groups. P<0.05 were considered significant. ns=not significant.

3.4.3 Relationship between Th17 cell frequencies, activation status and vaginal community types

BV-associated microbial diversity in the vagina has been reported to be associated with increased activated CD4+ T cell frequencies in some cohorts (Gosmann et al., 2017), but not others (Lennard et al., 2017). Th17 cell frequencies and activation were thus compared between AGYW according to CTs to determine whether vaginal CTs in adolescents influence mucosal T cell frequencies or function. The frequencies and counts of Th17 cells were found to be evenly distributed across the three CTs (Figure 3.3). Similarly, expression of CCR5, CD38 and HLA-DR on Th17 cells was not significantly different between the groups. In addition, overall changes in CD4+ T cell frequencies and activation did not significantly differ by vaginal CT category (data not shown).

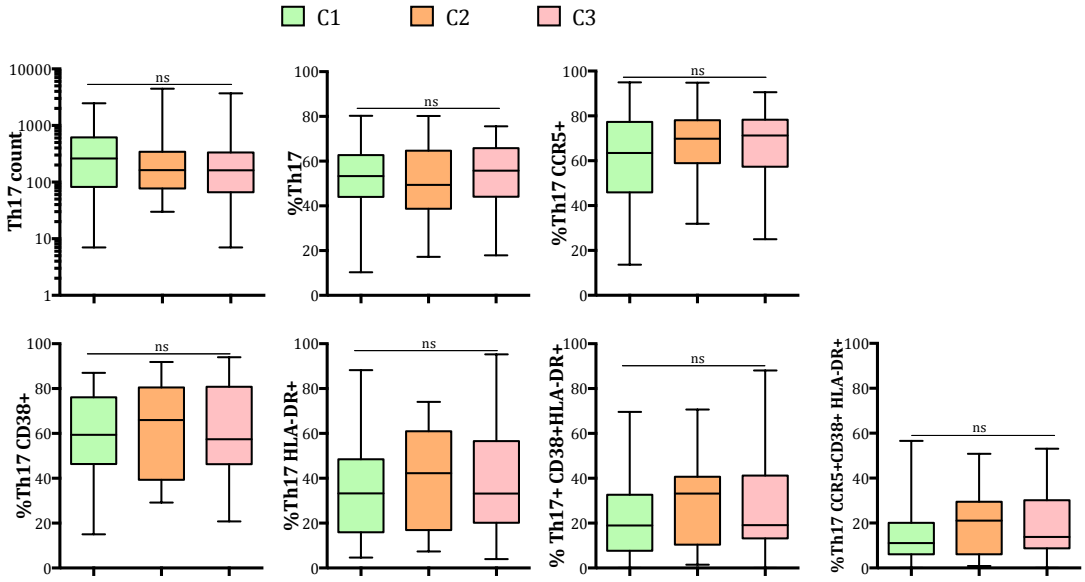

Figure 3.3. Proportion and phenotype of Th17 cells stratified according to community types (C1: diverse microbiome, C2: *L. crispatus*, C3: *L. iners*) determined using 16S rRNA sequencing. (Top panel) Box and whisker plots (median,

IQR and range) of Th17 counts (first graph), Th17 frequencies (second graph) and CCR5 expression on Th17 cells (third graph). (Bottom panel) Expression of activation markers CD38 and HLA-DR on Th17 cells, and co-expression of CCR5 together with activation markers on Th17 cells. Mann-Whitney and Kruskal-Wallis tests were used to compare groups. $P<0.05$ were considered significant. ns=not significant.

3.4.4 Relationship between *L. crispatus* (C2) and *L. iners* (C3) CTs and Th17 cell frequencies/activation status

Although both *Lactobacillus* spp. are commonly found colonizing the lower FRT in healthy women, *L. crispatus* colonization has been reported to be more beneficial than *L. iners* (Verstraelen et al., 2009). Therefore, the frequency and phenotype of Th17 cells were compared in a subset of the non-C1 adolescents (focusing on those who did not have any STIs; n=31). Of these, 42% (13/31) had an *L. crispatus*-dominated VMB (C2), and 58% (18/31) had an *L. iners*-dominated (C3) VMB. Adolescents with C2 and C3-type VMBs had similar relative counts and frequencies of cervical Th17 cells (Figure 3.4). Although there was a trend towards reduced activation of Th17 cells (co-expression of CD38 and HLA-DR) in adolescents who had an *L. iners*-dominated CT (C3) compared to those with an *L. crispatus* dominated CT (C2). Overall frequencies of activated cells and those expressing CCR5 did not differ significantly between the two groups.

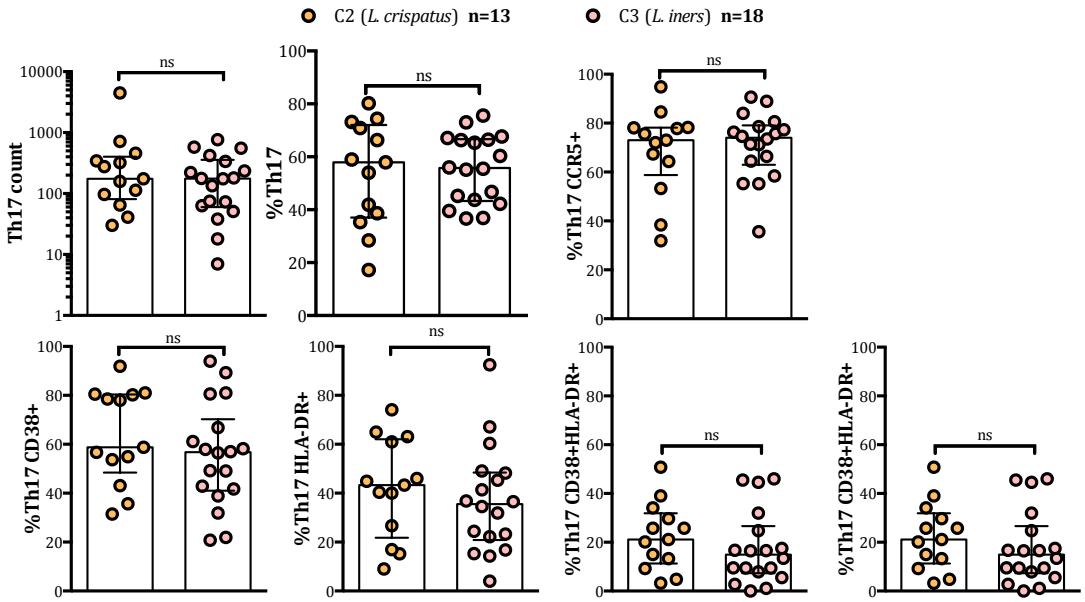

Figure 3.4. Comparison of cervical Th17 frequencies, activation status and CCR5 expression in adolescents with *L. crispatus*- (C2; orange circles) versus *L. iners*-dominated microbiota (C3; pink circles). (Top panel) Th17 counts, Th17 frequencies, and CCR5 expression on Th17 cells. (Bottom panel) Expression of activation markers CD38 and HLA-DR on Th17 cells, and co-expression of CCR5 together with activation markers on Th17 cells. The bar represent the median, the error bars represent the IQR. Mann-Whitney U test was used to compare groups. P<0.05 were considered significant. ns=not significant.

3.4.5 Impact of BV status and vaginal CT clustering on genital cytokine profiles

Th17-related cytokines, including IL-17A and IL-17F, have been described to be important in anti-bacterial immunity (Cooper, 2014). Therefore, the relationship between Th17-related cytokines and BV status and bacterial CTs was investigated. While BV status and CT clustering did not appear to influence Th17 cell frequencies or activation profiles, all 15 of the Th17-related cytokines differed significantly according to both BV status (Figure 3.5) and CT clustering (Figure 3.6). BV+ and intermediate adolescents had significantly higher genital concentrations of Th17-related cytokines compared to BV- adolescents (Figure 3.5). Interestingly, IL-17A (p=0.01, p. adj=0.01), IL-17F (p=0.04, p. adj=0.06) and

IL-10 (p=0.03, p. adj=0.04) concentrations were significantly higher in adolescents with an intermediate Nugent score compared to BV+ adolescents.

Similarly, adolescents with C2 (*L. crispatus*) and C3 microbiota (*L. iners*) had significantly lower Th17-related cytokine concentrations compared to those with a C1 microbiota (more diverse; Figure 3.6). Interestingly, the concentration of IL-1β was three times higher in adolescents with *L. iners*- (C3) compared to *L. crispatus*-dominated VMB (C2) (1.67 pg/ml vs 0.43 pg/ml, respectively, p=0.001). Genital tract IL-1β concentrations also tended to be six times higher in adolescents with intermediate Nugent scores compared to those with BV (217 pg/ml vs 35 pg/ml; Figure 3.5), although this was not significant.

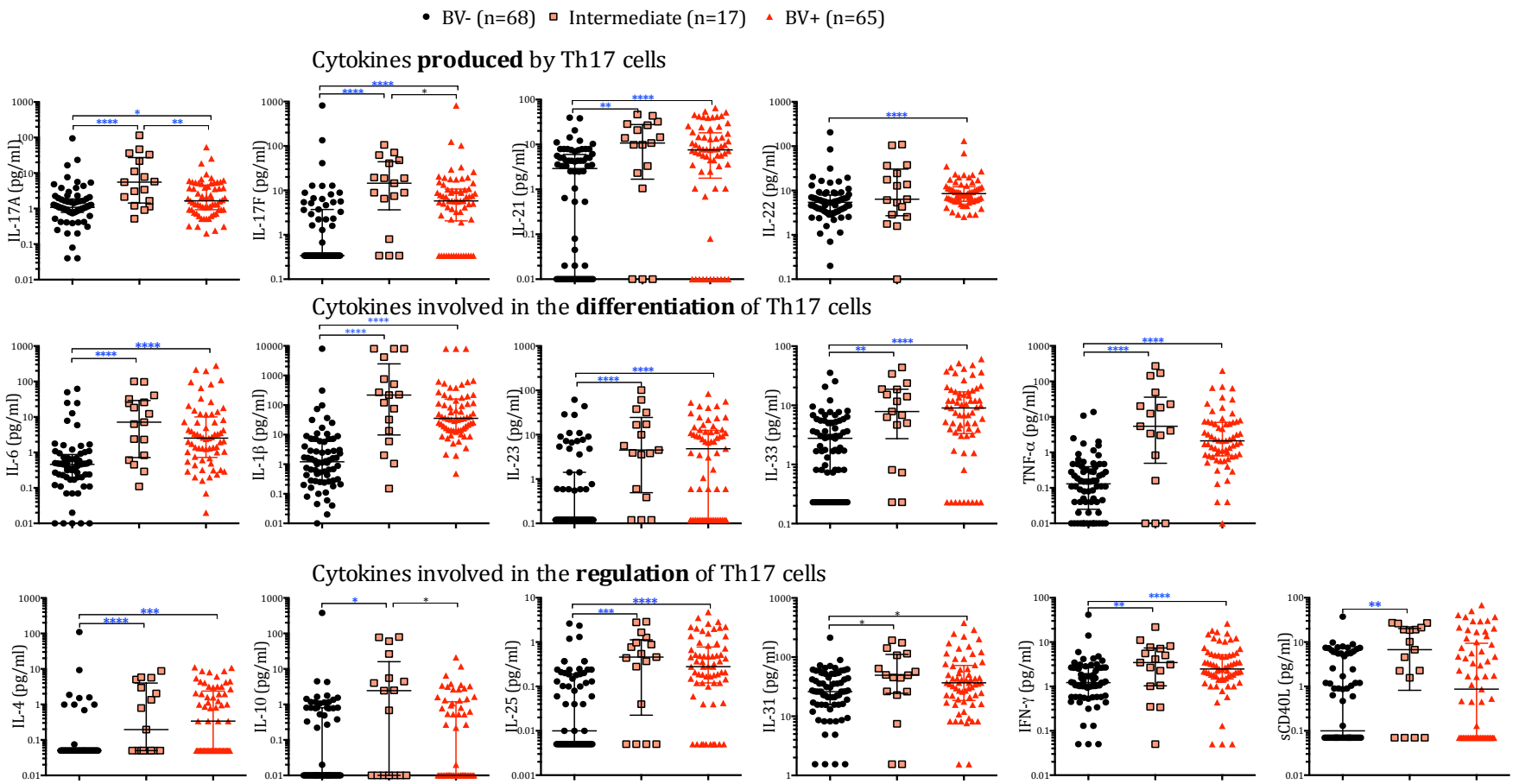

Figure 3.5. Th17-related cytokines according to BV status. Cytokines have been grouped into those produced by Th17 cells (first row), and those involved in their differentiation (second row) and regulation (third row). The middle line represents the median, and the error bars represent the IQR. Mann-Whitney U test was applied for comparisons between groups. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, and those in blue (*) remained significant after adjusting for multiple comparisons.

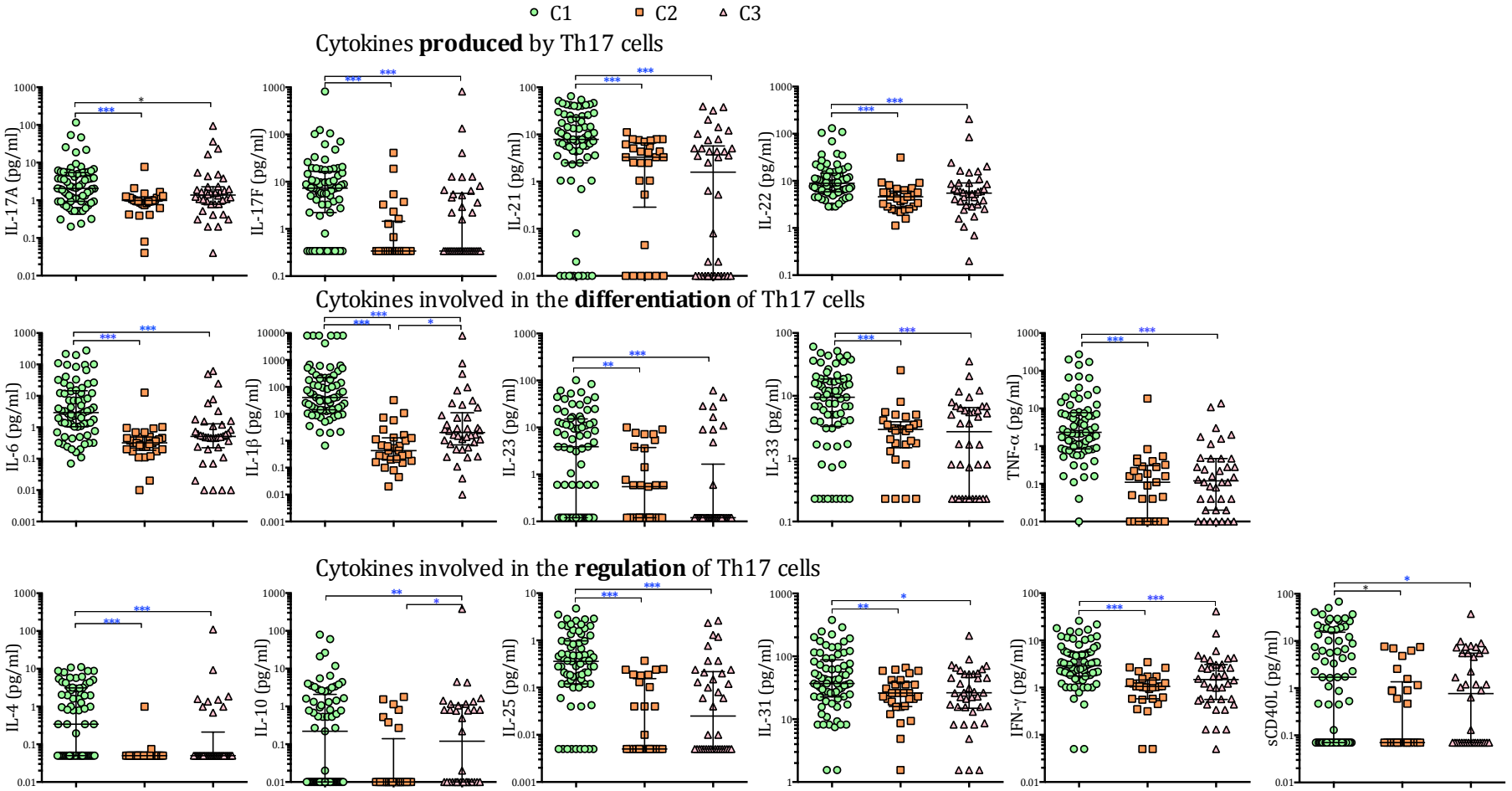

Figure 3.6. Th17-related cytokines according to the vaginal microbiome community type. Cytokines have been grouped into those produced by Th17 cells (first row), and those involved in their differentiation (second row) and regulation (third row). The middle line shows the median, and the error bars represent the IQR. Mann-Whitney U test was applied for comparisons between groups. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, and those in blue (*) remained significant after adjusting for multiple comparisons.

Because condom use and the presence of vaginal yeast differed according to BV status (see Table 3.1), the associations between Th17-related cytokines and BV were evaluated using a multivariate logistic regression in order to adjust for these potential confounders (Figure 3.7). After adjusting for STIs, IL-17A (I [intermediate]: β=2.52, CI=1.38-3.67; BV+: β=0.90, CI=0.15-1.65), IL-17F (I: β=1.67, CI=0.90-2.44; BV+: β=1.16, CI=0.64-1.68), IL-6 (I: β=1.70, CI=0.95-2.45; BV+: β=1.21, CI=0.69-1.74), IL-1β (I: β=2.22, CI=1.45-2.98; BV+: β=1.80, CI=1.21-2.39), IL-23 (I: β=1.05, CI=0.45-1.66; BV+: β=0.75, CI=0.36-1.14), IL-33 (I: β=1.14, CI=0.25-2.02; BV+: β=1.15, CI=0.58-1.72), TNF-α (I: β=1.97, CI=1.19-2.74; BV+: β=1.65, CI=0.15-1.65), IL-4 (I: β=1.14, CI=0.45-1.83; BV+: β=1.06, CI=0.54-1.58), IL-25 (I: β=1.08, CI=0.43-1.74; I: BV+: β=1.08, CI=0.65-1.51) and IFN-γ (I: β=1.17, CI=0.06-2.27; BV+: β=1.09, CI=0.39-1.79) cytokines remained positively associated with both having intermediate Nugent scores and being BV+. Further adjusting condom use and yeast infections, IL-17A (I: β=3.67, CI=0.90-2.44; BV+: β=1.32, CI=0.40-2.23), IL-17F (I: β=1.81, CI=0.77-2.85; BV+: β=1.52, CI=0.88-2.16), IL-6 (I: β=2.02, CI=1.06-2.97; BV+: β=1.65, CI=0.93-2.36), IL-1β (I: β=2.10, CI=1.23-2.98; BV+: β=1.93, CI=1.26-2.61), IL-23 (I: β=0.96, CI=0.27-1.65; BV+: β=0.85, CI=0.41-1.30), TNF-α (I: β=1.61, CI=0.77-2.45; BV+: β=1.80, CI=1.15-2.45), IL-4 (I: β=1.05, CI=0.29-1.81; BV+: β=1.18, CI=0.62-1.74), IL-25 (I: β=1.05, CI=0.30-1.80; BV+: β=1.21, CI=0.71-1.71) and sCD40L (I: β=0.83, CI=0.15-1.52; BV+: β=0.43, CI=0.04-0.83) remained significant for both BV intermediate and positive.

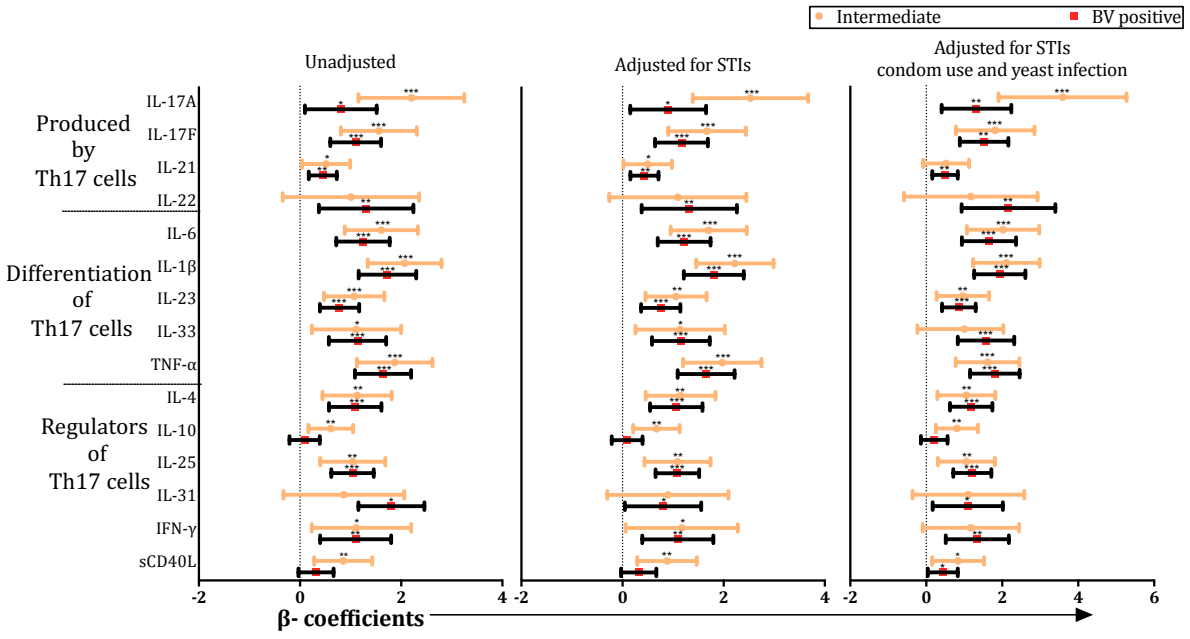

Figure 3.7. Multivariate logistic regression analysis of the relationships between Th17-related cytokines in adolescents with intermediate Nugent scores (orange) and those who were BV+ (black) adolescents (compared to BV- adolescents), adjusting for potential confounders (STIs, yeast infections and reported condom use). Multivariate logistic regression was used to determine associations between log_{10}-transformed cytokine concentrations and BV status. Th17-related cytokines in BV negative adolescents were used as the reference group. (Left panel) Unadjusted; (middle panel) Adjusted for STIs; (right panel) Adjusted for STIs, yeast and condom use. Dots/blocks indicate β-coefficients, and the error bars indicate the 95% confidence intervals (CI). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

IL-10 has a non-redundant role in anti-inflammatory responses by acting on variety of immune cells (Hedrich and Bream, 2010; Lyer and Cheng, 2012), which includes negatively regulating Th17 immune responses by inhibiting the synthesis of pro-inflammatory cytokines (Huber et al., 2011). Since IL-10, IL-17A and IL-17F were significantly elevated in BV intermediate compared to BV positive adolescents (see Figure 3.5), the relationship between IL-10 and IL-17A and F were investigated according to BV status. IL-17F correlated weakly albeit positively with IL-10 in BV negative adolescents (r=0.26, p=0.03; Figure 3.8, first row), while no significant correlation in the intermediate group was observed. In BV+ adolescents, there was a significant positive correlation of IL-10 with both IL-17A (r=0.39, p=0.001) and IL-17F (r=0.50, p<0.0001). The ratio of IL-17A:IL-10 and IL-17F:IL-10 was also investigated (Figure 3.8, second row). The ratios did not differ significantly, although the median ratio of IL-17A:IL-10 in the intermediate group tended to be lower, suggesting a lower concentration of IL-10 compared to IL-17A in this group.

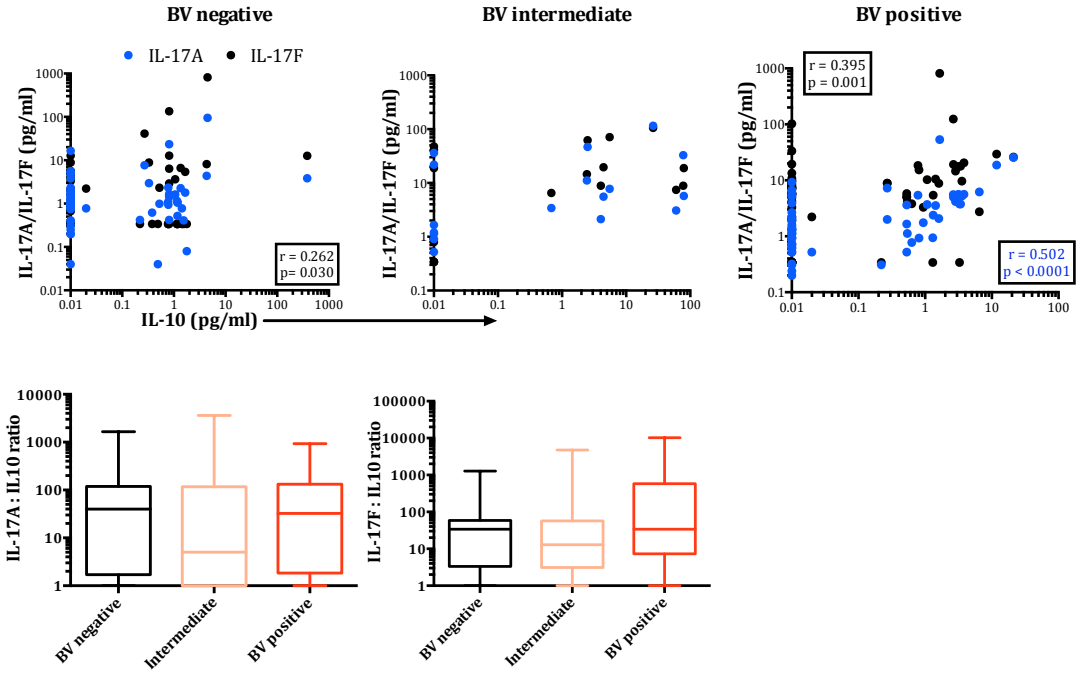

Figure 3.8. Correlation between IL-10 and IL-17A (blue dots) or IL-17F (black dots) concentrations (top panels) and IL-17:IL10 ratios (bottom panels), measured in BV negative, intermediate and BV positive adolescents. Box and whisker plots represent the median, IQR and range. Only significant p-values≤0.05 are displayed on the graphs, in blue (IL-17A) or black text (IL-17F).

3.4.6 Relationship between *L. crispatus*-(C2) and *L. iners*-dominated (C3) vaginal CTs and Th17-related cytokines

To compare the impact of *Lactobacillus* dominated microbiota on genital cytokine concentrations, Th17-related cytokines (in adolescents without STIs) were analysed by CT status. In the 38 AGYW included in this analysis, Th17-related cytokine concentrations were similar in the two *Lactobacillus*-dominated CTs (Figure 3.9), with the exception of IL-21 which tended to be higher in AGYW with *L. crispatus*-dominated microbiota (3.30 pg/ml) than those with *L. iners*-dominated microbiota (0.08 pg/ml, p=0.26; Figure 3.9A). Similarly, there was a trend towards decreased concentrations of IL-1β in adolescents with *L. crispatus*-dominated vaginal microbiota (p=0.03, p. adj=0.4; Figure 3.9B).

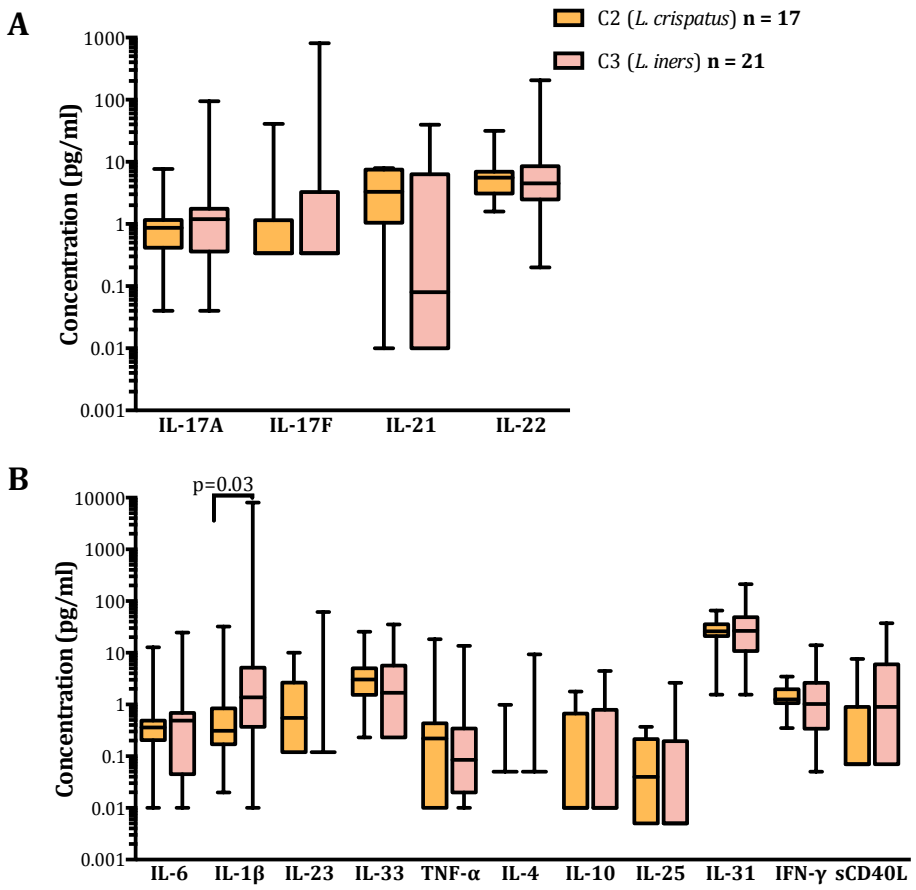

Figure 3.9. Comparisons of Th17-related cytokines in adolescents with either *L. crispatus*-(orange bars) or *L. iners*-dominated microbiota (pink bars). (A) Cytokines produced by Th17 cells. (B) Other cytokines involved in the differentiation and regulation of Th17 cells. Box and whiskers represent the median, IQR and range. Only significant p-values of ≤0.05 are displayed.

3.4.7 Relationship between Nugent-BV and Th17 cells and cytokines in the presence of chlamydia infection

Since asymptomatic chlamydia infection alone increased IL-17A genital concentrations (Chapter 2, Figure 2.11), and it was highly prevalent in this cohort (33%), the impact of BV on Th17 cells in the presence or absence of chlamydia was investigated. BV positive adolescents with chlamydia (in the absence of other STIs; BV+C+) were compared to BV positive adolescents without any STIs, including chlamydia (BV+C-), adolescents who had no BV and STIs (BV-C-), and those who only had chlamydia (BV-C+). Frequencies of

Th17 cells tended to be lower in BV+C+ girls compared to BV+C-, BV-C- and BV-C+ (47.4% vs 61.3%, 55.5% and 49.4%, respectively), however this was not significant (p=0.07, Figure 3.10A). However, the frequencies of Th17 cells expressing CCR5 and activation markers (CD38) were similar across groups (Figure 3.10B).

Next, the concentrations of Th17-related cytokines were compared between groups. Adolescents who had both BV+C+ had a significantly higher IL-17A, IL-17F, and IL-10 concentrations compared to BV+C- adolescents (p=0.02, p=0.03, and p=0.002, respectively), in addition to TNF-α (p=0.02, Figure 3.10C), suggesting an additive effect of co-infection on these Th17-related cytokines. However, only IL-10 remained significant after adjusting for multiple comparisons (adj. p=0.03). Furthermore, BV+C+ adolescents had elevated IL-17F (p=0.001), IL-21 (p=0.04), IL-22 (p=0.005), IL-6 (p=0.007), IL-1β (p<0.0001), IL-33 (p=0.002), TNF-α (p<0.0001), IL-4 (p=0.03), IL-10 (p=0.006), IL-25 (p=0.0007), IL-31 (p=0.03) and IFN-γ (p=0.001) compared to adolescents who only had chlamydia and no BV (C+BV-, Figure 3.10C).

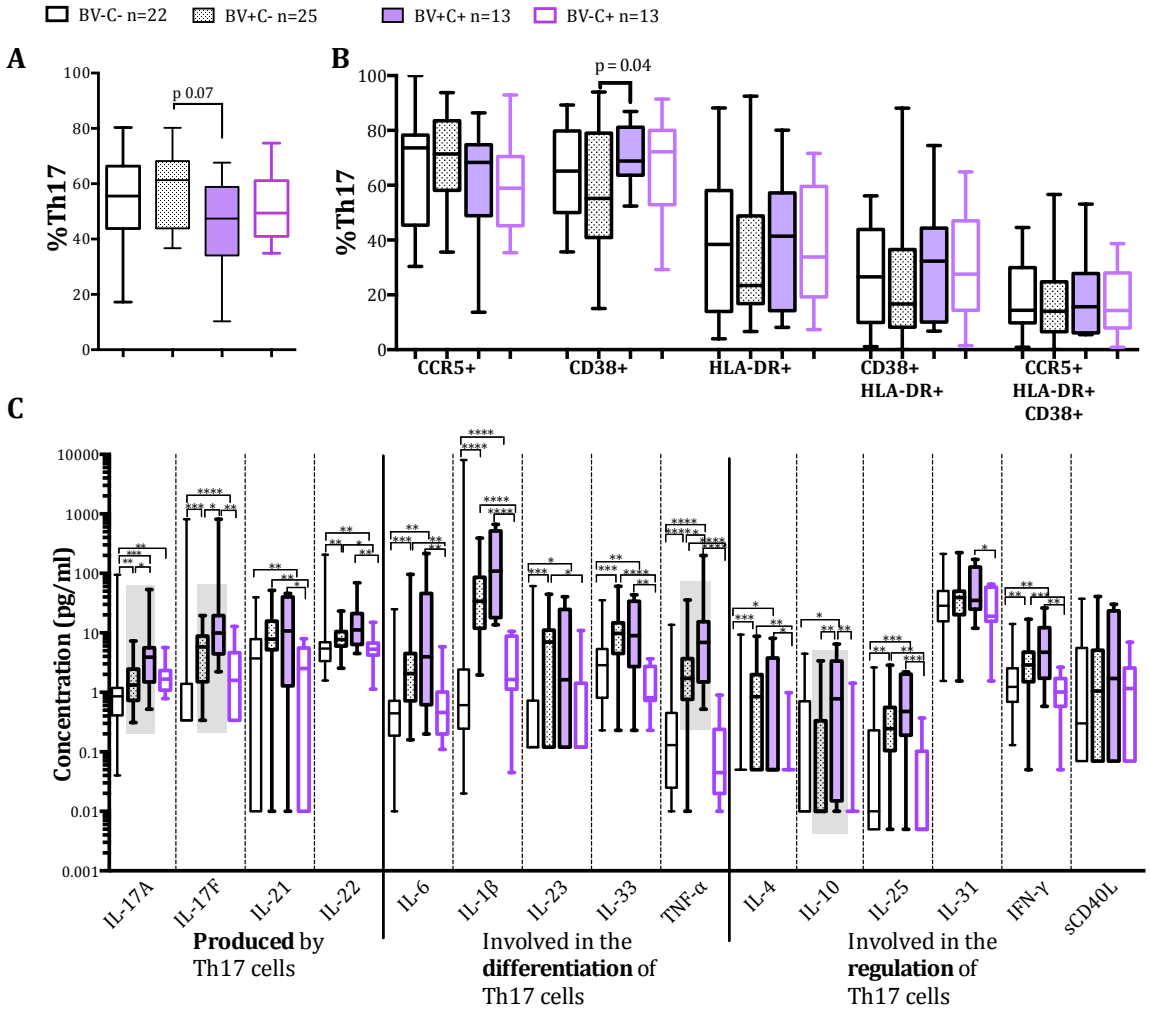

Figure 3.10. Comparison of genital Th17 cells and Th17-related cytokines, according to BV status, in the presence and absence of chlamydia co-infection. (A) Frequency of Th17 cells in BV-chlamydia- (BV-C-; clear boxes), BV+chlamydia- (BV+C-; dot filled boxes), BV+chlamydia+ (BV+C+; purple filled boxes) and BV-chlamydia+ AGYW (BV- C+; purple outlined clear boxes). (B) Activation (CD38 and HLA-DR) and expression of CCR5 on Th17 cells by BV and C group. (C) Th17-related cytokines by BV and C group. Box and whisker plot represent the median, IQR and range for each parameter. Significant differences in Th17-related cytokines between BV+C- and BV+C+ adolescents are shaded in grey. Mann-Whitney U tests were used to compare groups and $p<0.05$ were considered significant. *$p<0.05$; **$p< 0.01$; ***$p< 0.001$; ****$p<0.0001$. Only significant p-values of ≤0.05 are displayed.

3.5 Discussion

The vaginal microbiome is likely to interact with local genital tract immunity and epithelial barrier function in the FRT, and alterations in this microbiome, such as BV, have been linked to increased HIV susceptibility to HIV infection (Low et al., 2011; Cohen et al., 2012; Gosmann et al., 2017; McClelland et al., 2018). In this Chapter, the impact of BV and an altered VBM on Th17 cells, the cytokines they produce and that regulate them were explored in AGYW, at risk for HIV acquisition. BV+ adolescents as well as those who had intermediate Nugent scores (4-6) had higher vaginal pHs than BV- adolescents, in addition to having more diverse C1 CT, defined by fewer lactic acid producing lactobacilli (Lennard et al., 2017). BV+ adolescents reported less regular condom use, although they were also less likely to have yeast infections by Gram stain. Hutchinson et al. (2007) similarly reported that more consistent condom use was associated with decreased incidence of BV in women whom they followed for a total of 3 years. Moreover, van de Wijgert (2007) and colleagues reported a strong negative association between BV and vaginal yeast.

Previous studies that characterized the VMB in African women (African American women from the US and women currently living in Africa) showed that fewer of these women tended to have *Lactobacillus*-dominated microbiota, but rather had more diverse microbiota compared to women of North American and European women (Ravel et al., 2011; Anahtar et al., 2015; Bayigga et al., 2018; Lennard et al., 2017). As others from our group have previously reported in AGYW from the same community, the VMB of adolescents in this study clustered into three CTs (Lennard et al., 2017): just over half had C1-dominated VMBs in which vaginal swabs were characterized by the presence of *Gardnerella* and other anaerobic bacterial species; while the rest of the AGYW had VMBs classified as either C2 (dominated by *L. crispatus*) or C3 (dominated by *L. iners*). Furthermore, adolescents who were BV+ or who had intermediate Nugent scores had a predominantly C1 VMB, with the most abundant species being *G. vaginalis*, *BVAB1*, *Megasphaera*, *L. iners* and *Prevotella* spp., while BV- adolescents primarily had either *L. crispatus* (C2) or *L. iners* (C3) dominated microbiota.

Th17 cells are thought to play a critical role in destroying extracellular bacteria (Carey et al., 2016; Cosorich et al., 2017), although they are also highly susceptible to HIV infection (McKinnon et al., 2015; Stieh et al., 2016). In this cohort, neither the frequency nor activation status of Th17 cells (defined by being CCR6+ and CCR10- CD4+ T cells) differed by BV status or VMB CT classification. This data suggests that vaginal CCR6+CCR10- Th17 cells were not altered by BV nor the microbiome in this cohort. Although no previous studies have evaluated the interplay between the VMB and genital Th17 cells, conflicting results have emerged on the impact of BV on genital tract CD4+ T cell frequencies. While some studies found that vaginal dysbiosis was not associated with any change in genital CD4+ T cell frequencies or activation status (Lennard et al., 2017), others have reported increased frequencies and more activated CD4+ T cells in women with BV or vaginal dysbiosis (Rebbapragada et al., 2008; Thurman et al., 2015; Gosmann et al., 2017). In this study, the overall frequency of CD4+ T cells and activation of these cells were also not altered in adolescents with BV or dysbiosis.

Since several studies have reported that *L. crispatus* is more beneficial than *L. iners* (Borgdorff et al., 2015; Gautam et al., 2015; Petrova et al., 2017), the phenotype of Th17 cells in adolescents with an *L. crispatus* were compared to those with an *L. iners*-dominated VMB, in the absence of any confounding common STIs (including *C. trachomatis*, *N. gonorrhoeae, M. genitalium, or T. vaginalis*). The frequency and activation status of cervical Th17 cells did not differ significantly in AGYW with *L. crispatus*- and *L. iners*-dominated VMBs, those with *L. iners*-dominated VMBs tended to be less activated than those with *L. crispatus*-dominated VMBs.

It was interesting to note in this study that AGYW with *L. iners*-dominated microbiota (C3) tended to have higher genital concentrations of IL-1β than adolescents with *L. crispatus*-dominated VMBs (C2). None of the other cytokines produced by Th17 cells (including IL-17A, IL-17F, IL-21 and IL-22), or others involved in Th17 differentiation or regulation appeared to differ by Lactobacilli group. IL-1β is also thought to be important in driving the differentiation of Th17 cells (Lasigliè et al., 2011), although adolescents with an *L. iners*-dominate VMB had similar frequencies of Th17 cells with those with an *L. crispatus*-dominated microbiota in this cohort. Previous in vitro studies have suggested that *L. crispatus* inhibits inflammatory cytokine responses, which may be

important in the context of lowering HIV risk (Rizzo et al., 2015; Chetwin et al., 2019). A recent study from our group in adolescents from the same community in Cape Town reported that a *L. iners*-dominated VMB was not associated with an inflammatory cytokine profile compared to those with a *L. crispatus*-dominated VMB (Lennard et al., 2017). The role of *L. iners* in vaginal health is unclear, since it is detected in normal conditions as well as during vaginal dysbiosis (Petrova et al., 2017). Several previous studies have considered *L. crispatus* and *L. iners* to largely be functionally similar, and both outcompete other vaginal commensals for resources, a survival strategy needed to maintain a healthy vaginal microbiota (Yamamoto et al., 2009; France et al., 2016). Others have also argued that *L. iners* might be more adaptable to survive in a dysbiotic vaginal state compared to *L. crispatus*, and might help to facilitate recovery to a lactobacilli-dominated microbial community (Ferris et al., 2004; Jakobsson and Forsum, 2007; Srinivasan et al., 2010; Lambert et al., 2013; Petrova et al., 2017). Of all the *Lactobacillus* spp. that typically colonize the lower FRT, *L. iners* has the smallest genome size (~1.3Mbps; Macklaim et al., 2011). In their comprehensive description of the *L. iners* genome, Macklaim and co-authors (2011) reported that 766 genes predicted within *L. iners* genome were shared between *L. crispatus, L. acidophilus* and *L. gasseri*, although these "core" genes represent >60% of the *L. iners* coding sequence compared to only 37% of *L. crispatus*, and 44% of *L. acidophilus* and *L. gasseri* sequence space. Further studies are needed to investigate whether the reason *L. iners* is also found in women with vaginal dysbiosis is because of its adaptable nature and its greater ability to compete with other organisms compared to *L. crispatus*, or perhaps it has a pathogenic nature that is driving dysbiosis.

Effector cytokines are central mediators of Th17 immunity (Ouyang et al., 2012), and it was therefore important to know if alterations in the VMB influenced the production of these cytokines. It was interesting to note therefore that all of the cytokines measured were elevated in BV+ adolescents and those with intermediate Nugent scores (4-6) compared to BV- adolescents, and most of these remained significant after adjusting for multiple comparisons, STIs, reported condom use and yeast infections. Furthermore, it was surprising that adolescents with intermediate VMB had higher concentrations of genital IL-17A, IL-17F and IL-10 compared to BV+ adolescents. In BV+ AGYW, IL-10 correlated positively with both IL-17A and IL-17F. Previous studies, focusing on

inflammatory cytokines and chemokines, reported that BV (Nugent 7-10) and intermediate VMB (Nugent 4-6) induced similar magnitudes of FRT inflammation (Hedges et al., 2006; Masson et al., 2014; Guédou et al., 2014; Deese et al., 2015).

Since Amsel et al. (1983) first defined the clinical criteria for diagnosing BV, which included vaginal pH>4.5 for the detection of women with BV, vaginal pH has been evaluated as a sensitive but non-specific tool to diagnose vaginal dysbiosis (Hemalatha et al., 2013). In this cohort, a vaginal pH of 4.7 was observed in BV- (Nugent 0-3) adolescents, which was higher than the normal vaginal pH of 4.5 or less that is considered normal (Amsel et al., 1983; Ravel et al. 2011). Although BV- AGYW had vaginal pHs above what is considered normal internationally, it was interesting to note that adolescents with BV (Nugent 7-10) or intermediate VMBs (Nugent 4-6) had significantly higher vaginal pHs compared to their BV- counterparts. Previous studies have reported a positive correlation between vaginal pH and abnormal vaginal microbiota (Caillouette et al., 1997; Ravel et al., 2011; Hemalatha et al., 2013; O'Hanlon et al., 2013; Krauss-Silva et al., 2014), which was supported by the findings from this Chapter. The composition of the microbial ecosystem undoubtedly influences the vaginal pH and *vice versa*. *Lactobacillus* species have been shown to produce lactic acid, which is required to maintain this low vaginal pH (Tachedjian et al., 2017; Chetwin et al., 2019). In this adolescent cohort, vaginal pH was not associated with changes in Th17-related cytokines.

In support of the Nugent scoring analysis, adolescents in this study with more diverse vaginal microbiota (belonging to C1 CT) also had elevated Th17-related cytokines compared to those with *L. crispatus*- (C2) or *L. iners*-dominated VMB (C3). Gosmann et al. (2017) also found that Th17-related cytokines IL-17, IL-1β, and IL-23 were elevated in women with high diversity, low *Lactobacillus* abundance vaginal bacterial communities. Anahtar et al. (2015) showed that altered vaginal microbiota increase genital inflammation by pattern recognition receptors sensing of pathobionts by vaginal epithelial and mucosal antigen presenting cells (APC). Lower Th17-related cytokines in adolescents with *Lactobacillus*-dominated microbiota (C2 and C3) compared to those with C1 VMBs may also be a result of immune-regulatory properties of vaginal

lactobacilli and their metabolites like lactic acid (Spurbeck and Arvidson, 2011; Hearps et al, 2017).

C. trachomatis was the most prevalent STI in this cohort of AGYW, with 33% of adolescents being infected at baseline. Previously, BV was found to predict chlamydia risk (Wiesenfeld et al., 2003). Hence, the potential synergistic effect of chlamydia and BV on vaginal immune responses in adolescents was investigated. Although adolescents co-infected with *C. trachomatis* and BV tended to have lower frequencies of cervical Th17 cells than those who only had BV, a greater proportion of these cells were activated (CD38+). Moreover, concentrations of IL-17A, IL-17F, TNF-α and IL-10 cytokines were significantly higher in BV+ chlamydia+ adolescents compared to those who were BV+ but didn't have chlamydia.

In summary, the frequency and activation of Th17 cells was similar irrespective of BV status or vaginal CT profiles of the adolescents. However, Th17-related cytokines were elevated in adolescents who were BV+ and had intermediate Nugent scores, which reflected the diversity revealed by vaginal microbial community typing. In the presence of the other prevalent genital condition – *C. trachomatis* infection - BV appeared to have an additive effect on Th17-related cytokine production. This study adds to the body of evidence confirming the highly inflammatory nature of an altered vaginal microbiome in relation to Th17-related cytokine responses although not in frequencies of Th17 cells.

Chapter 4

Effect of hormonal contraceptives on Th17 cells in the genital tract

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4.1 Abstract

Several studies have suggested that HCs may increase susceptibility to HIV acquisition in women. AGYW in SA continue to be at a heightened risk of acquiring HIV compared to adolescent boys or young men. Biological mechanisms are likely to contribute significantly to this high risk, including the number and activation status of HIV target cells available in the lower genital tract to become infected in adolescent girls. Since Th17 cells are highly susceptible to HIV infection compared to other CD4+ T subsets, the aim of this Chapter was to evaluate whether this T cell subset and their related cytokines were altered in the FRT of adolescents randomized to various HC methods, including NuvaRing, NET-EN and COCPs. One hundred and thirty adolescent girls were enrolled and randomized to one of the three HCs. After four months, participants crossed over to another HC for another four months before exiting the study. In an intra-individual analysis at crossover, NuvaRing use was associated with increased proportions of cervical Th17 cells that expressed activation markers HLA-DR/CD38, despite decreased frequencies of Th17 cells overall compared to NET-EN and COCPs use. In addition, adolescents on NuvaRing had increased concentrations of cervical Th17-related cytokines, including IL-21, IL-1β, TNF-α and IFN-γ. Contraceptive arms at crossover did not differ by activation or chemokine receptor expression by cervical Th17 cells, although an increase in Th17-related cytokines (IL-21, IL-1β, IL-33, IL-4, TNF-α, IFN-γ and sCD40L) in participants on NuvaRing was noted. No difference in genital tract concentrations of Th17-related cytokines was noted between NET-EN and COCPs users. Together, this data suggest that the use of the inserted vaginal NuvaRing alters the frequency of Th17 cells at the genital mucosa and Th17-related cytokines to a greater extent than NET-EN and COCPs.

4.2 Introduction

It is undisputable that HCs are important in preventing unwanted pregnancies (Kallner and Danielsson, 2016). The synthetic progestins DMPA and NET-EN are widely used in SSA because of their relatively long-acting nature, compared to other contraceptives like COCPs which need to be taken daily (Heffron et al., 2012; UNDESA, 2015). NET-EN is administered bi-monthly, while DMPA is administered every third month. Because of affordability, DMPA has been a popular product offered in the public sector in developing countries, although NET-EN is increasingly being used at higher costs.

Despite the contraceptive benefits, there is public health concern that injectable contraceptives increase a woman's susceptibility to HIV (Polis et al., 2016). It has been estimated that injectable HCs may be responsible for up to 130 000 HIV-1 infections per year globally (Butler et al., 2013). The World Health Organization recently reviewed their guidelines on the medical eligibility of DMPA for contraceptive use and stated that women at high risk for HIV acquisition should be informed of the uncertainty regarding injectable contraceptives (WHO, 2015). In support of this recommendation, the first multi-center, open-label, randomized trial of DMPA versus other contraceptive methods (levonorgestrel implant and copper IUD) on HIV outcomes – called the Evidence for Contraceptive Options and HIV Outcomes (ECHO) - is under way and the eagerly awaited results, which will be announced later this year, will provide more information on HIV susceptibility with the use of HCs (http://echo-consortium.com). Some of the biological mechanisms by which DMPA use is thought to increase HIV susceptibility include suppression of local protective immunity, alteration in protective *Lactobacilli*-dominated vaginal microbiota, and elevated levels of mucosal inflammatory cytokines which could result in an influx of HIV target cells to the genital mucosa (Ghanem et al., 2005; Chandra et al., 2013).

In contrast to injectable contraceptives like DMPA, a recent systematic review found that COCPs did not similarly increase HIV risk (Polis et al., 2016). In addition, this review also suggested that the available data on NET-EN do not suggest an association with HIV risk in women, although the trend was positive. In

a population that has a high HIV prevalence, it is important to answer the question whether HCs increases HIV acquisition and provide safer options for women. With a variety of options to choose from, women should be able to make informed decisions regarding the type of contraception to choose. The use of safe and effective contraceptives in regions at the highest risk for HIV infections, particularly in AGYW is a global health priority.

NuvaRing® (containing etonogestrel and ethinyl estradiol), a hormonal vaginal ring placed in the cervix for three weeks, has been recently licensed for use in South Africa, adding another HC option for women in South Africa. This combined contraceptive vaginal ring (CCVR) has previously been found to increase "healthy" *Lactobacilli* abundance in women with a high BV prevalence (Hardy et al., 2017). There is still limited data on how CCVR affect the immune microenvironment of the genital tract.

Stieh et al. (2016) recently reported that HIV preferentially infects Th17 cells. These cells are thought to be important in maintaining the mucosal epithelial integrity, through cytokine and chemokine production (Dandekar et al., 2010). Little is currently known about how this highly susceptible CD4+ T cell population in the genital mucosa is affected by the use of HCs. The primary aim of this Chapter was therefore to compare the effects of NuvaRing, NET-EN and COCPs on Th17 cells and related cytokines in the genital tract of adolescent girls in an open-label, randomized crossover study conducted in Cape Town, South Africa. The hypothesis is that NuvaRing would offer some safety advantage over NET-EN in terms of mucosal inflammation and HIV target cell activation.

4.3 Materials and Methods

4.4.1 Study design

This cohort was a sub-study to an NIH-funded parent study called "Choices for Adolescent Prevention Methods for South Africa (CHAMPS)" which aimed to evaluate the acceptability, feasibility, and adherence to various HC choices in AGYW, with the view to extend this to preferences for HIV prevention modalities as more HIV prophylactic products are developed (PI: Prof Linda-Gail Bekker, Desmond Tutu HIV Foundation, UCT). The study design was an open-label, randomized crossover study of 130 sexually active female adolescents aged 15 – 19 years from Masiphumelele, Cape Town, South Africa. Inclusion criteria included being HIV-negative, non-pregnant, seeking hormonal contraceptives or a change in method, and willing to refrain from inserting non-study vaginal products or objects into the vagina for the duration of the study. Exclusion criteria included not being sexually active, being HIV positive, having any symptomatic STIs or an abnormal Pap smear, a positive urine pregnancy test, being contra-indicated to any study product, or if informed consent (adolescents ≥18 years) or assent with parental consent (adolescents <18 years) was not obtained.

After the screening visit, adolescents who met the inclusion and exclusion criteria returned within 40 days for enrolment into this study. After enrolment, adolescents were randomly assigned in a 1:1:1 ratio to one of the three study arms, according to the following schema (Figure 4.1):

- **Arm 1:** Adolescents received injectable NET-EN (intramuscular injection containing 200mg of progestogen norethisterone oenanthate) once every 8 weeks for 4-months.
- **Arm 2:** Adolescents received the CCVR NuvaRing® (Merck, Sharp and Dohme, containing 0.12mg etonogestrel and 0.015mg ethinyl estradiol) inserted for three weeks continuously followed by one ring free week for a 4-month period. The study clinicians carried out vaginal examination and removed the used rings (as requested by participants).

- **Arm 3:** Adolescents received COCPs (Nordette® or Triphasil®). The Triphasil® regimen included six tablets containing 30µg ethinyl estradiol/50µg levonorgestrel (phase 1), five tablets containing 40µg ethinyl estradiol/75µg levonorgestrel (phase 2), ten tablets containing 30µg ethinyl estradiol/125µg levonorgestrel (phase 3), and seven inert tablets. The Nordette® regimen included 26 tablets containing 30µg ethinyl estradiol/150µg levonorgestrel, plus seven inert tablets. For both COCPs products, participants were required to take a daily tablet for 21 days and a placebo tablet for seven days (day 22-28) each month, for a 16-week period.

After being randomized onto each of these contraceptive methods for four months, adolescents crossed over to another form of HC (Figure 4.1). Those receiving either NET-EN or the daily COCPs (Arm 1 and Arm 3, respectively) for the first 16 weeks of the study switched over to using the CCVR (Arm 2), while adolescents receiving CCVR as the first method were given the choice between either NET-EN or the daily COCPs as their second method. After a total of 32 weeks, the adolescents returned for a final visit at the clinic and exited the study.

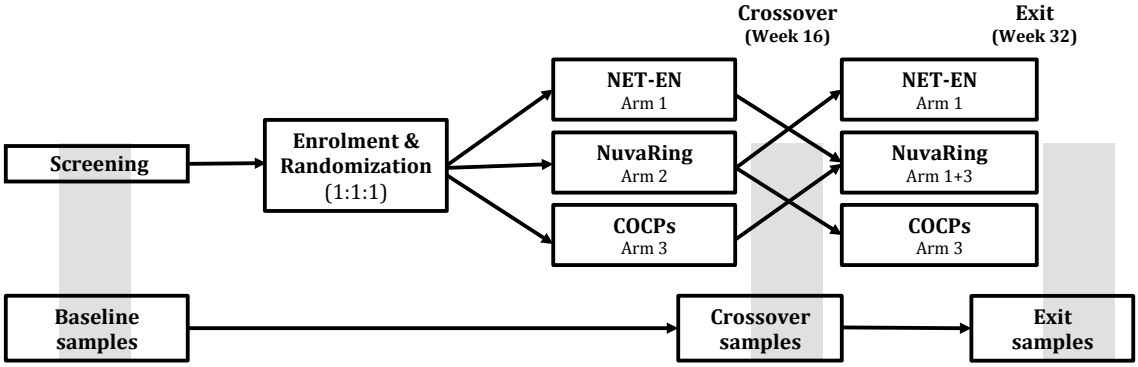

Figure 4.1. Summary of the randomized cross over study design used to investigate the effect of HCs on Th17 cells/Th17-related cytokines in adolescents. Adolescents were screened and the eligible ones randomized to NET-EN, NuvaRing or COCPs. Samples were collected at the screening visit (baseline), after four months on randomized product (crossover), and after crossover to an alternative HC for four months (exit).

4.4.2 HIV and pregnancy testing

A rapid HIV test (Alere Determine™ HIV-1/2 Ag/Ab Combo; Alere, MA, USA) and pregnancy test (U-test Pregnancy strip; Humor Diagnostica, Pretoria, SA) was performed at all study visits (screening, enrolment, crossover and exit). If HIV seroconversion occurred during the study, the participant was counselled and referred for HIV management while being allowed to continue her participation in the study.

4.4.3 Sample collection

The following genital tract samples were collected in the following order at screening, crossover and exit:

1. Menstrual cup: Cervicovaginal secretions were collected by inserting a menstrual cup (Softcup®) for 30 minutes (section 2.3.8). These were used to analyse Th17-related cytokines by Luminex (section 2.3.9).
2. STI testing: A vulvovaginal swab was collected for STI testing (section 2.3.4) using an in house validated multiplex PCR assay, which was performed at the STI Reference Laboratory at the NICD, Sandringham as previously described (Lewis et al., 2012).
3. BV Nugent scoring: Posterior fornix and lateral wall swabs were collected to prepare a wet mount slide for BV Nugent scoring by Gram staining, and for detection of yeast hyphae and spores (section 2.3.4). Nugent scoring was performed at the STI Reference Laboratory at the NICD, Sandringham, Johannesburg.
4. Vaginal microbiome: A lateral vaginal wall swab was collected for 16S rRNA and shotgun metagenomic sequencing, performed by Christina Balle (Dept Immunology, UCT). The microbiome data will not be included in this dissertation as it formed part of Christina Balle's PhD dissertation, which is currently being examined.
5. Flow cytometry: An endocervical cytobrush (Digene® Corporation, MD, USA) was collected for CMCs (section 2.3.5), which were analysed using flow cytometry (section 2.3.6).

4.4.4 BV and STI diagnosis

Collected vulvovaginal swabs were screened for *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis* and *M. genitalium,* as described in section 2.3.3. BV was diagnosed by Gram staining using Nugent's criteria, as described in section 2.3.4.

4.4.5 Hormone measurement

Blood was collected using SST tubes to measure serum hormone levels. Concentrations of E2, LH and FSH were measured in blood specimens, which was measured by BARC, as described previously in section 2.3.3.

4.4.6 Flow cytometry

An endocervical cytobrush (Digene® Corporation) was collected from each participant under speculum examination, and processed within 4 hours as previously described in section 2.3.4. CMCs were stained with the following monoclonal antibodies: CD3, CCR6, CCR5 (BD Biosciences); CD38 (eBioscience); CD4 (Invitrogen); CD8, CCR10, HLA-DR (Biolegend) as previously described in section 2.3.5. A DUMP channel consisting of CD14, CD19 (Invitrogen) and ViVid (Life Technologies) was included to remove monocytes, B-cells and dead cells. Th17-like cells were considered as those that expressed CCR6 but not CCR10, while highly activated T cells were considered as those that expressed CD38 and HLA-DR. CCR5 was included as the major HIV co-receptor involved in viral entry during sexual transmission (Lopalco, 2010). A representative figure showing the gating strategy is shown in Chapter 2, section 2.3.7.

4.4.7 Cytokine measurements

The concentrations of Th17-related cytokines, including IL-17A, IL-22, IL-21, IL-1β, IL-4, IL-6, IL-10, IL-17F, IL-23, IL-25, IL-31, IL-33, IFN-γ, sCD40L and TNF-α, were measured as described in section 2.3.9, using the Bio-Plex Pro™ Human Th17 cytokine Luminex kit (Bio-Rad Laboratories Inc).

4.4.8 Statistical analyses

Statistical analysis was conducted using two different approaches: (1) within-subject assessment, which compared intra-individual immunological phenotypes before and after initiation of contraception method, where each participant served as her own control; and (2) between study arms assessment, which compared inter-individual immunological phenotypes by different study interventions (NET-EN, COCPs and NuvaRing). A Wilcoxon matched-pairs signed rank test was used to compare individuals within each study arm before and after being on a particular contraceptive. A Mann-Whitney U test was applied to compare the three HC arms. Data were analysed as both intention to treat (ITT) and per protocol (PP). The ITT analysis does not take into account participants who deviated from the protocol, and analysis is based on the arm to which participants were initially randomized. The PP analysis considered participants who deviated from protocol, and they are analysed according to the actual arm (HC product) they used. Statistical analyses were performed using Prism version 6 (GraphPad Software, CA, USA), and STATA™ version 12 (StataCorp, TX, USA). All tests were two sided and a p-value of ≤0.05 was considered significant. Where stated, multiple comparisons were adjusted using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995).

4.4 Results

4.4.1 Characteristics of study participants

One hundred and fifty-six adolescents were screened for this study. Of these, 130/156 were enrolled. Of those that did not enroll, 21/156 (13%) were not interested in the study, and 5/156 (3%) did not meet the enrolment criteria. Reasons for not including these five adolescents included that they were younger than 15 years at the time of screening (n=1), were on TB treatment (n=1), anaemic (n=1), had elevated blood pressure (n=1), or raised liver function (n=1). The 130 adolescents who where eligible and enrolled were randomized to the three hormonal contraceptive arms, with 45/130 being randomized to NuvaRing, 45/130 to NET-EN, and 40/130 to COCPs (Figure 4.2). Of these, 108/130 completed visit 2 (83%), and 93/130 completed the final visit (72%). One participant assigned to the NET-EN arm seroconverted before the crossover visit and mucosal sampling was not performed at the crossover and exit visits, and one participant withdrew consent for mucosal sample collection at the exit visit. The final numbers was thus 107 at crossover and 92 at exit. A total of nine adolescents deviated from the protocol: In the NuvaRing arm, one participant changed to COCPs and three changed to NET-EN before the crossover visit; in the COCPs arm, one participant changed to NuvaRing and three changed to NET-EN before the crossover visit; in the NET-EN arm, one participant deviated from protocol by changing to COCPs. Some of the reasons for changing methods included poor adherence (mainly in the COCPs arm), discomfort with study product (mainly in the NuvaRing arm) and spotting (in the NET-EN arm).

The baseline characteristics of the cohort are summarized in Table 4.1. There was no difference in baseline characteristics across arms (including age, endogenous hormone level, BMI, vaginal pH, genital infections, BV, prior use of contraception, sexual risk behaviour and intra-vaginal insertion practices). Although the initial intent was to recruit adolescents who were naïve to HCs into the study, very few adolescents in this age group in this community were not currently using HCs (5/130). As a result, the protocol had to be amended to include adolescents who were otherwise eligible and considering method change, of whom none had

previously used NuvaRing prior to study initiation. Within the age group enrolled, the majority of the adolescents were not contraceptive naïve at enrolment, with the majority previously using NET-EN (56%).

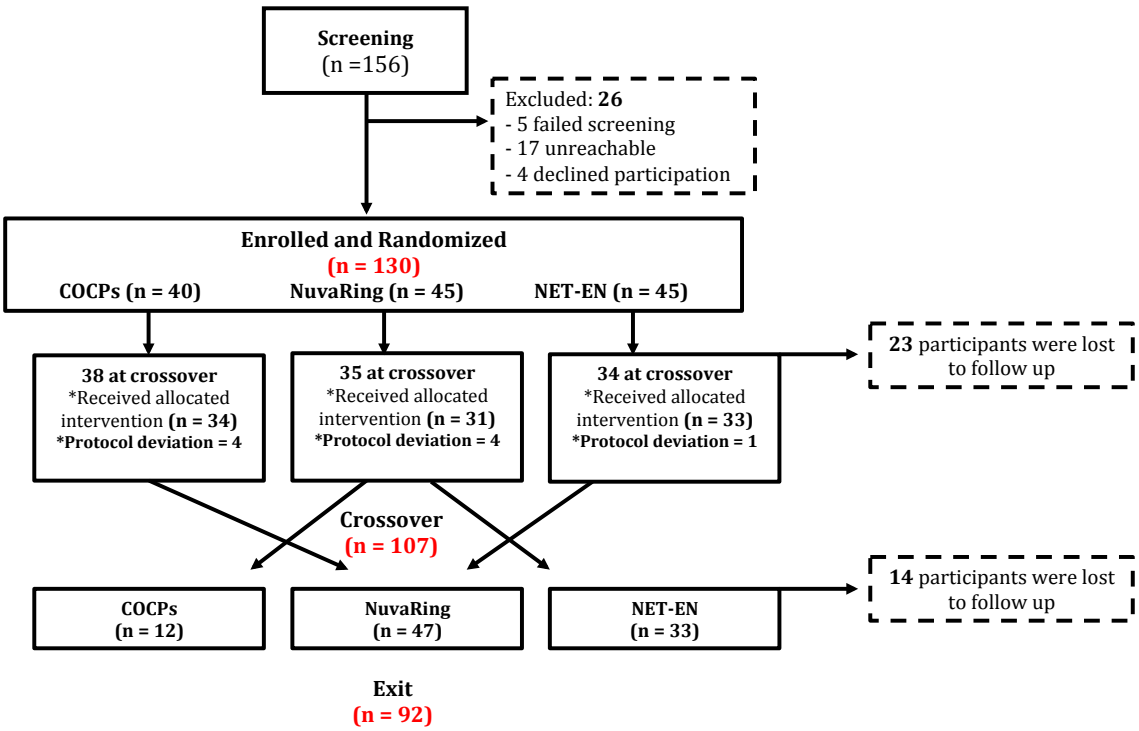

Figure 4.2. Flow diagram showing number of the adolescents screened, enrolled and randomized into each of the contraceptive arms. The numbers of participants excluded or lost to follow up are shown in the dotted rectangles. Figures in red indicate the total number of participants at each visit.

Table 4.1 Baseline characteristics of adolescents according to randomization arm

	NuvaRing n=45	**NET-EN** n=45	**COCPs** n=40	**P-value**
Median age at screening	17 (16 –18)	17 (16 –18)	17 (16 –18)	0.91
Serum hormone concentration				
E2 (pmol/l)	97.0 (63.0–138.0)	106.0 (85.2–157.8)	90.5 (75.5–137.0)	0.46
FSH (U/L)	4.7 (2.8–5.8)	4.7 (3.6–5.8)	5.5 (4.2–6.2)	0.08
LH (IU/l)	4.4 (2.0–5.9)	4.5 (2.0 –6.5)	4.0 (2.0–5.4)	0.93
BMI (kg/m^2)	25.0 (22.1–27.8)	25.0 (22.5–29.1)	25.7 (21.5–28.3)	0.75
Vaginal pH	4.7 (4.4–5.2)	5.0 (4.7–5.3)	4.7 (4.4–5.2)	0.33
Genital infections at screening				
Any STIs	19 (42.2%)	22 (48.9%)	15 (37.5%)	0.48
Chlamydia trachomatis	14 (31.1%)	17 (37.7%)	12 (30.0%)	0.70
Neisseria gonorrhoeae	5 (11.1%)	5 (11.1%)	3 (7.5%)	0.97
Trichomonas vaginalis	4 (8.8%)	5 (11.1%)	4 (10.0%)	0.75
Mycoplasma genitalium	0 (0.0%)	2 (4.4%)	1 (2.5%)	0.36
HSV-2 serology	12 (26.7%)	13 (28.8%)	14 (35.0%)	0.49
Presence of yeast hyphae	10 (22.2%)	4 (8.8%)	6 (15.0%)	0.22
BV status				0.69
BV- (Nugent 0-3)	23 (51.1%)	18 (40.0%)	20 (48.8%)	
Intermediate (Nugent 4-6)	3 (6.6%)	7 (15.6%)	4 (9.8%)	
BV+ (Nugent 7-10)	19 (43.2%)	20 (44.4%)	16 (40.0%)	
Prior contraceptive method				0.44
Naive	2 (4.4%)	2 (4.4%)	1 (2.5%)	
Not currently	6 (13.3%)	10 (22.2%)	10 (25.0%)	
DMPA	5 (11.1%)	7 (15.9%)	7 (17.5%)	
NET-EN	28 (62.2%)	21 (46.7%)	20 (50.0%)	
COCPs	3 (6.7%)	2 (4.5%)	1 (2.5%)	
Implanon	1 (2.2%)	2 (4.5%)	0 (0.0%)	
Sexual risk behaviour				
Age of sexual debut	15 (14 –16)	15 (14 –16)	15 (14 –16)	0.97
Condom use always	61.4%	61.9%	60.9%	0.99
Multiple sexual partners	0 (0.0%)	1 (2.2%)	0 (0.0%)	1.00
Number of sexual partners	1 (1-1)	1 (1-2)	1 (1-1)	0.16
Vaginal practices				
Use of any products	1 (4.6%)	4 (8.8%)	2 (2.5%)	0.50
Washing with soap	4 (8.8%)	6 (13.3%)	2 (5.1%)	0.47
Tampon use	2 (4.6%)	5 (11.1%)	1 (2.5%)	0.28
Washing with water	5 (11.1%)	6 (13.3%)	5 (12.8%)	1.00
Douching	0 (0.0%)	1 (2.2%)	0 (0.0%)	1.00

BV, bacterial vaginosis; BMI, body mass index; E2, estradiol; S-FSH, follicle stimulating hormone; LH, luitenizing hormone

4.4.2 Baseline genital cytokine profiles by randomization arm

To evaluate potential differences in cytokine profiles in adolescents randomized to the three study arms, Th17-related cytokine concentrations were measured in menstrual cup secretions at baseline. No significant differences were noted in the concentration of any of the fifteen Th17-related cytokine between study arms at baseline (Figure 4.3).

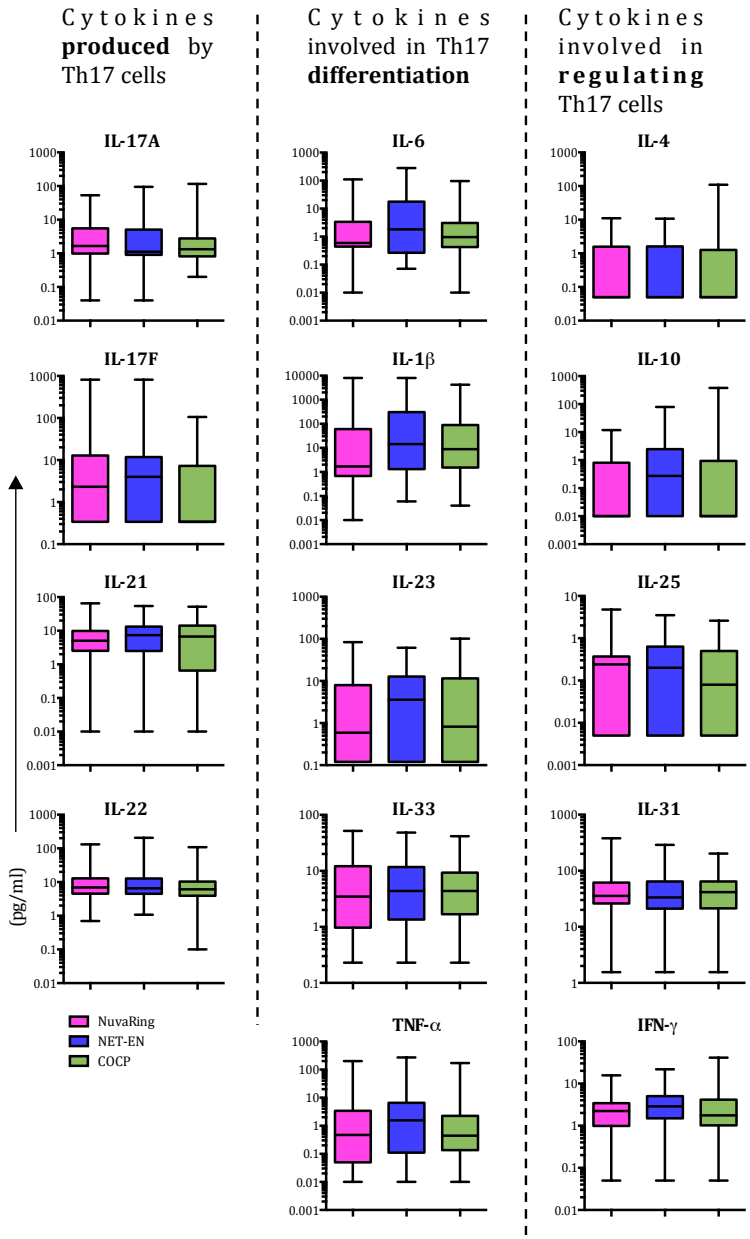

Figure 4.3. Comparison of genital Th17-related cytokines at baseline, stratified by study arm. Based on their functional relatedness to Th17 cells, cytokines have been divided into those produced by Th17 cells (first column; including IL-17A, IL-17F, IL-21, IL-22), and those involved in their differentiation (second column; including IL-6, IL-1β, IL-23, IL-33, TNF-α) and those involved in their regulation (third column; including IL-4, IL-10, IL-25, IL-31, IFN-γ). Pink boxes indicate adolescents randomized to NuvaRing, blue boxes indicate adolescents randomized to NET-EN, and green boxes indicate those randomized to COCPs. A Mann-Whitney U test was used to compare medians with p≤0.05 being considered significant. No significant differences were noted.

4.4.3 Baseline genital Th17 cell frequencies and activation did not differ by study arm prior to randomization

The phenotype and level of activation of genital mucosal Th17 cells at baseline was initially characterized to confirm that Th17 cells did not differ by study arm, prior to randomizations. The cell count (per cytobrush) and relative frequency of Th17 cells (of total CD4+ T cells) was the same across arms, with an overall median frequency of 54.0% (IQR 44.0% - 64.3%; Figure 4.4) being R6+R10+ (Th17-like). Expression of CCR5 and activation markers (CD38, HLA-DR) was also similar across arms (data not shown).

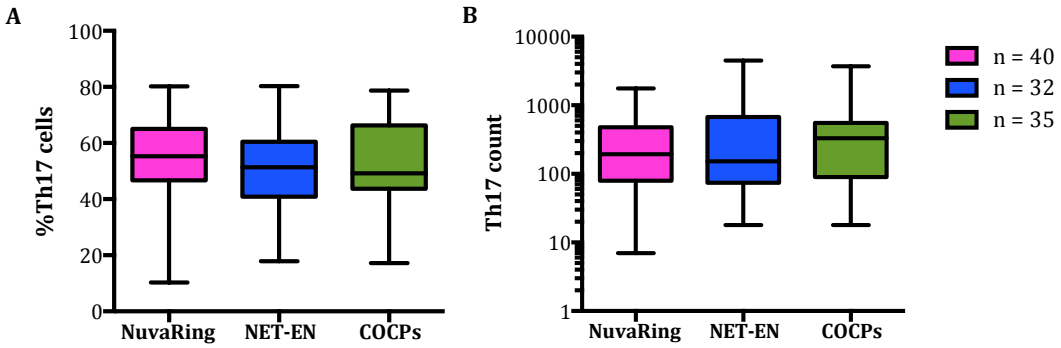

Figure 4.4. Comparison of Th17 cell frequency and number at baseline, according to contraceptive arm. (A) Frequency of Th17 cells (expressed as proportion of R6+R10- CD4+ T cells in a cytobrush). (B) Total counts of Th17 cells per cytobrush. Pink boxes indicate adolescents randomized to NuvaRing, blue boxes indicate adolescents

randomized to NET-EN, and green boxes indicate those randomized to COCPs. The box and whisker plots show the median (middle line), IQR (boxes) and whiskers extend between the minimum and maximum values. A Mann-Whitney U test was used to compare medians with a p value of ≤0.05 being considered significant. No significant differences were noted.

4.4.4 Impact of HC arm on genital Th17-related cytokine concentrations: the crossover visit

Adolescents were randomized to one of the three HC products for four months, returning to the clinic for an HC crossover visit. The following ITT and PP analyses focused on the crossover visit, where measurements were made before the adolescents crossed over to another product. In the ITT analysis, data was analysed according to randomization, while in the PP analysis, data was analysed according to the actual HC the participants were on (detailed in section 4.3.8).

Alteration of cytokines in the genital tract may have significant effect on HIV risk in women (Masson et al., 2015a; Passmore et al., 2016; McKinnon et al., 2018). Of the cytokines produced by Th17 cells (IL-17A, IL-17F, IL-21, IL-22), IL-21 was significantly elevated in genital secretions in adolescents who used NuvaRing (p=0.009; Figure 4.5A). In addition, several of the cytokines involved in differentiation and regulation of Th17 cells (including IL-1β [p=0.007], IL-33 [p=0.04], IL-4 [p=0.02], TNF-α [p=0.01], IFN-γ [p=0.01], and sCD40L [p=0.02]) were significantly elevated in genital secretions from adolescents using NuvaRing (Figure 4.5B and C). IL-21, IL-1β, IFN-γ and TNF-α remained significant after adjusting for multiple comparisons. Adjusting for protocol violations (early change in HC), the PP analysis showed similar results (data not shown).

In contrast to NuvaRing, NET-EN and COCPs use was not associated with any significant changes in Th17-related cytokine concentrations within individuals before and after randomization (Figure 4.6 and 4.7, respectively). In an ITT

analysis comparing these three HCs cross-sectionally at week 16, adolescents on NuvaRing had elevated IL-17A (p=0.009), IL-17F (p=0.03), IL-21 (p=0.03) and IL-33 (p=0.03) compared to those on NET-EN; while IL-21 (p=0.04), IL-6 (p=0.03), IL-1β (p=0.03) and TNF-α (p=0.04) were elevated in adolescents on NuvaRing compared to those on COCPs (Appendix Table A2). None of these observations remained significant after adjusting for multiple comparisons.

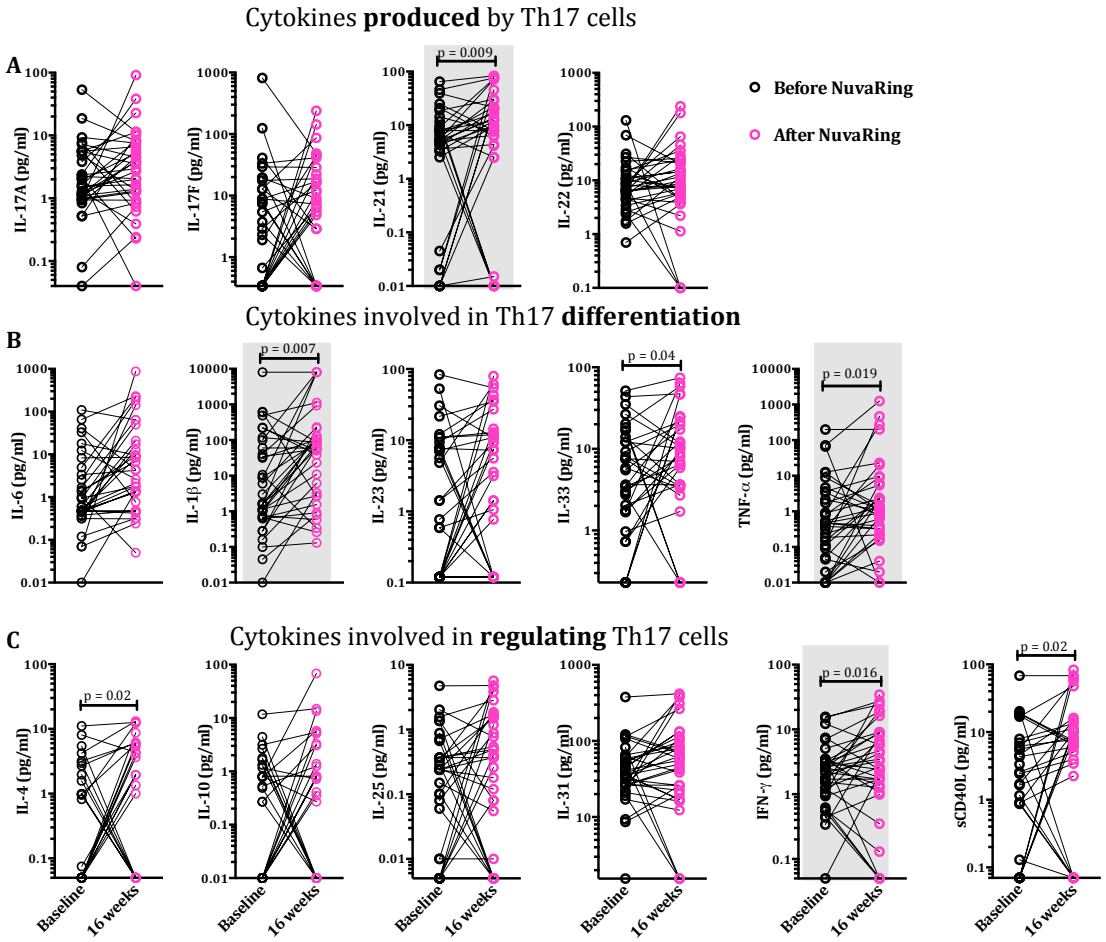

Figure 4.5. Comparing genital concentrations of Th17-related cytokines before (black dots) and after using NuvaRing for 16 weeks (pink dots). Cytokines have been divided based on their role in Th17 cell regulation or differentiation: (A) Cytokines produced by Th17 cells. (B) Cytokines involved in the differentiation of Th17 cells. (C) Cytokines involved in the regulation of Th17 cells. Black circles indicate cytokine concentrations prior to using NuvaRing while pink circles indicate concentrations after four months of NuvaRing. Black lines link paired data points in an individual adolescent before and after using NuvaRing. Cytokine concentrations were compared using a

Wilcoxon matched-pairs signed rank test, and only significant p-values are displayed (p ≤0.05). Cytokines highlighted by the grey shaded box remained significant after adjusting for multiple comparisons.

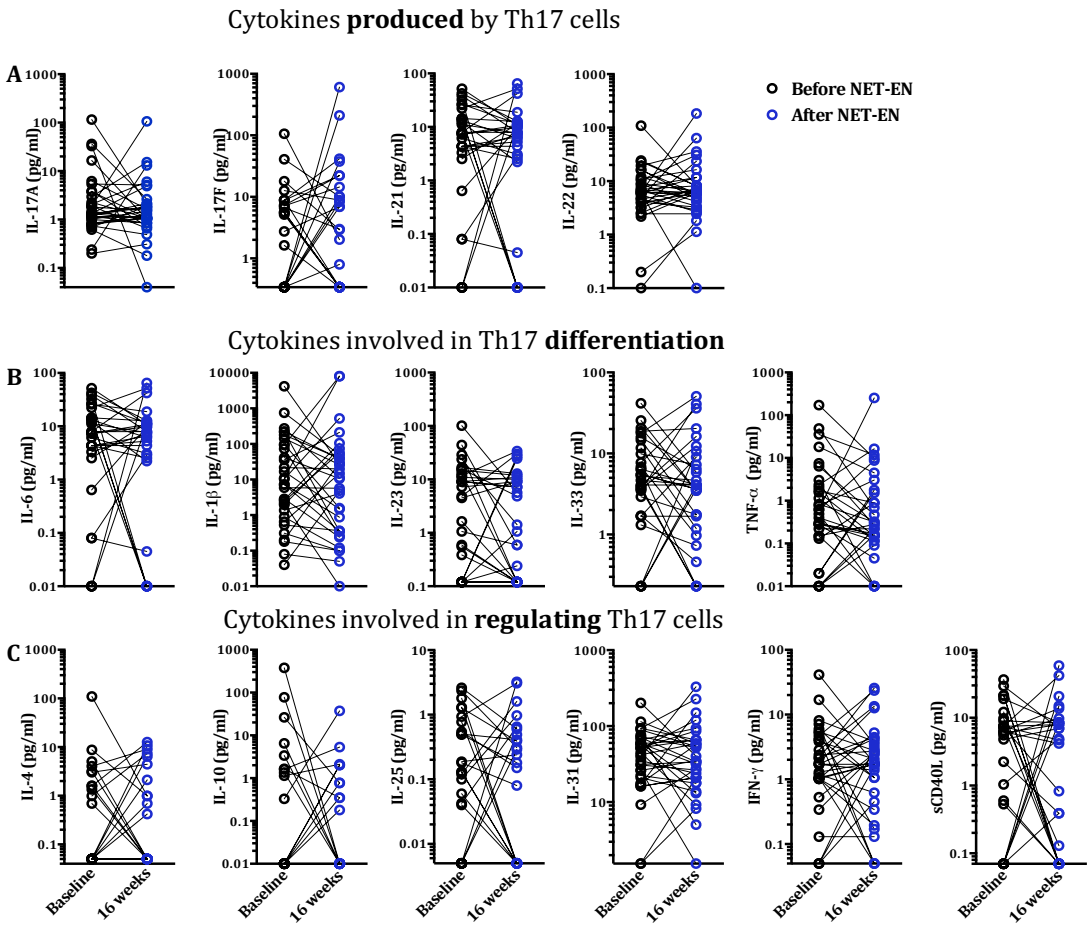

Figure 4.6. Comparing genital concentrations of Th17-related cytokines before (black dots) and after using NET-EN for 16 weeks (blue dots). Cytokines have been divided based on their role in Th17 cell regulation or differentiation: (A) Cytokines produced by Th17 cells. (B) Cytokines involved in the differentiation of Th17 cells. (C) Cytokines involved in the regulation of Th17 cells. Black circles indicate cytokine concentrations prior to using NET-EN while blue circles indicate concentrations after four months of NET-EN. Black lines link paired data points in an individual adolescent before and after using NET-EN. Cytokine concentrations were compared using a Wilcoxon matched-pairs signed rank test, and only significant p-values are displayed (p≤0.05).

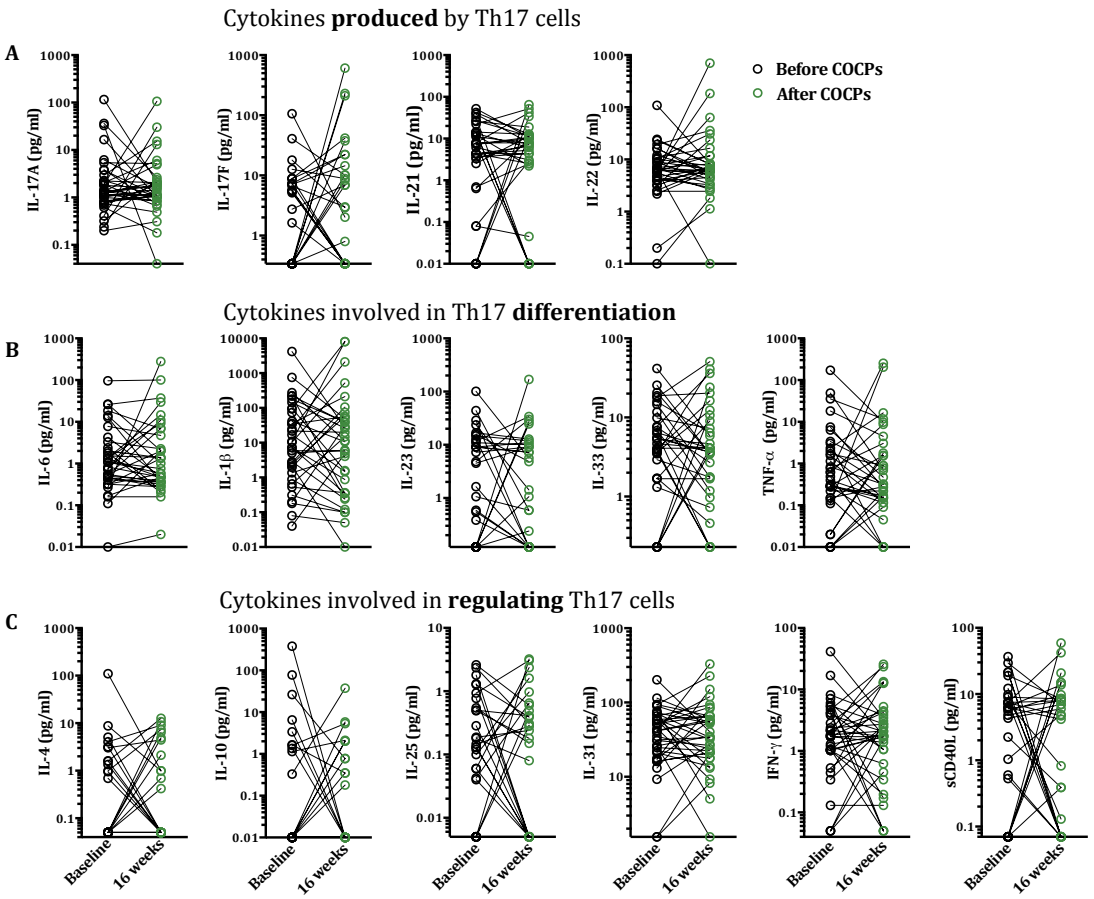

Figure 4.7. Comparing genital concentrations of Th17-related cytokines before (black dots) and after using COCPs for 16 weeks (green dots). Cytokines have been divided based on their role in Th17 cell regulation or differentiation: (A) Cytokines produced by Th17 cells. (B) Cytokines involved in the differentiation of Th17 cells. (C) Cytokines involved in the regulation of Th17 cells. Black circles indicate cytokine concentrations prior to using COCPs while green circles indicate concentrations after four months of COCPs. Black lines link paired data points in an individual adolescent before and after using COCPs. Cytokine concentrations were compared using a Wilcoxon matched-pairs signed rank test. Only significant p-values are displayed ($p \leq 0.05$).

4.4.5 Impact of HC arm on genital Th17 cell frequencies and activation status: the crossover visit

The impact of NuvaRing on genital Th17 cell frequencies and activation status within participants was investigated next, where each adolescent was her own baseline control. In the ITT analysis, a significant decrease in the frequency of genital Th17 cells was observed after four months on NuvaRing (Figure 4.8A; p=0.001; adj. p=0.008). In addition, the frequency of CD4+ T cells was also reduced within the NuvaRing arm (p=0.03; data not shown). While the frequency of Th17 cells were reduced following NuvaRing use, an increase in highly-activated cervical Th17 cell frequencies was noted (HLA-DR/CD38; p=0.02; adj. p=0.08), including those expressing the HIV co-receptor (HLA-DR/CD38/CCR5; p=0.01; adj. p=0.04). The PP analysis showed similar results.

Cervical Th17 frequencies tended to be lower after COCPs use within individuals (Figure 4.8B), although their activation status was similar. The use of NET-EN did not influence the frequency or the expression of CCR5 on Th17 cells (Figure 4.8C); however, Th17 cells tended to be more activated after being on NET-EN (HLA-DR/CD38; p=0.03; adj. p=0.1).

Although the impact of NuvaRing (Figure 4.8A) on the frequency of cervical CD4+ T cells that were Th17 cells and their level of activation (HLA-DR/CD38) appeared to be more dramatic compared to NET-EN (Figure 4.8C) or COCPs (Figure 4.8B), this was not significant across HC arms (data not shown).

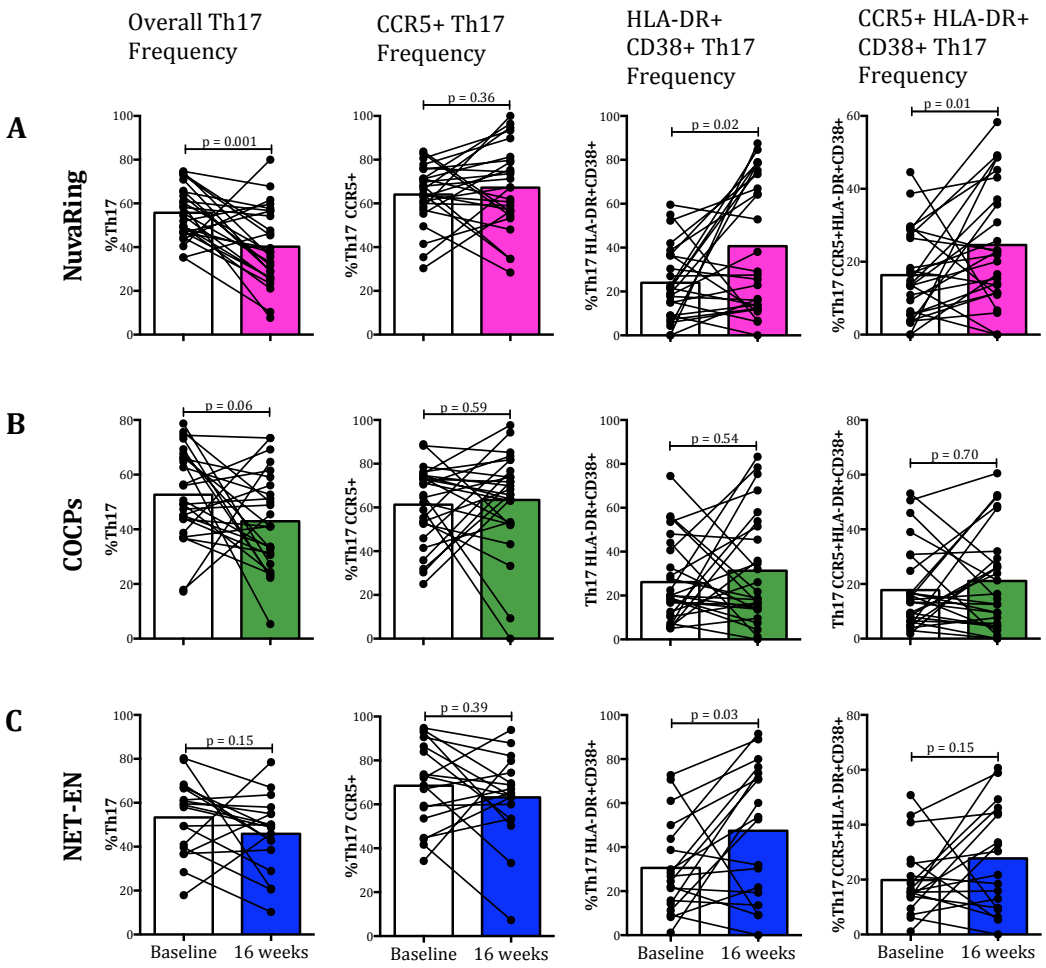

Figure 4.8. Phenotype of Th17 cells before (baseline) and after (16 weeks) being on (A) NuvaRing (B) COCPs and (C) NET-EN. The frequencies of Th17 cells and expression of CCR5, CD38 and HLA-DR on these cells were assessed before and after 16 weeks of adolescents being on the randomized HC. Pink boxes indicate adolescents randomized to NuvaRing, blue boxes indicate adolescents randomized to NET-EN, and green boxes indicate those randomized to COCPs. A Wilcoxon matched-pairs signed rank test was applied and a p-value ≤0.05 was considered significant.

4.4.6 Changes in Th17 cells and Th17-related cytokines in participants changing between HCs

Changes in Th17-related cytokines in participants changing from NuvaRing to NET-EN, NuvaRing to COCPs, NET-EN to COCPs and *vice versa* at all the three visits were investigated. After adjusting for multiple comparisons, IL-21 ($p=0.011$), IL-6 ($p=0.003$), IL-1β ($p=0.003$), IL-33 ($p=0.010$), TNF-α ($p=0.025$), IL-25 ($p=0.028$) and IFN-γ ($p=0.025$) were elevated in participants when they were on NuvaRing compared to when the same participants were on NET-EN (Figure 4.9). Except for IL-4, all other Th17-related cytokines were elevated when participants were on NuvaRing compared to when they were on COCPs (Figure 4.10). In contrast, no significant differences in the concentration of Th17-related cytokines in participants changing from NET-EN to COCPs or *vice versa* were observed (Figure 4.11).

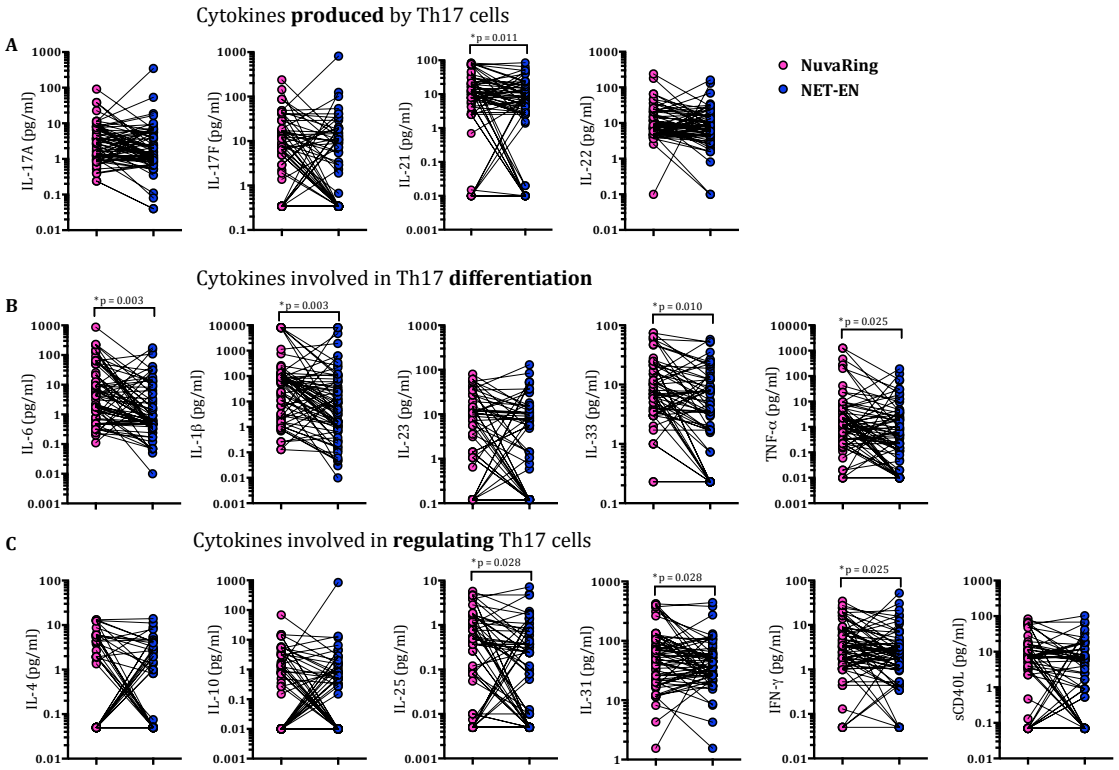

Figure 4.9. Th17-related cytokines (A) those produced by Th17 cells (B) those involved in the differentiation of Th17 cells and (C) those involved in the regulation of Th17 cells, in adolescents changing from NuvaRing to NET-EN and *vice versa*. A Wilcoxon matched-pairs signed rank test was applied and a p-value ≤0.05 was considered significant. Only significant p-values≤0.05 are displayed and those indicated by a* remained significant after adjusting for multiple comparisons.

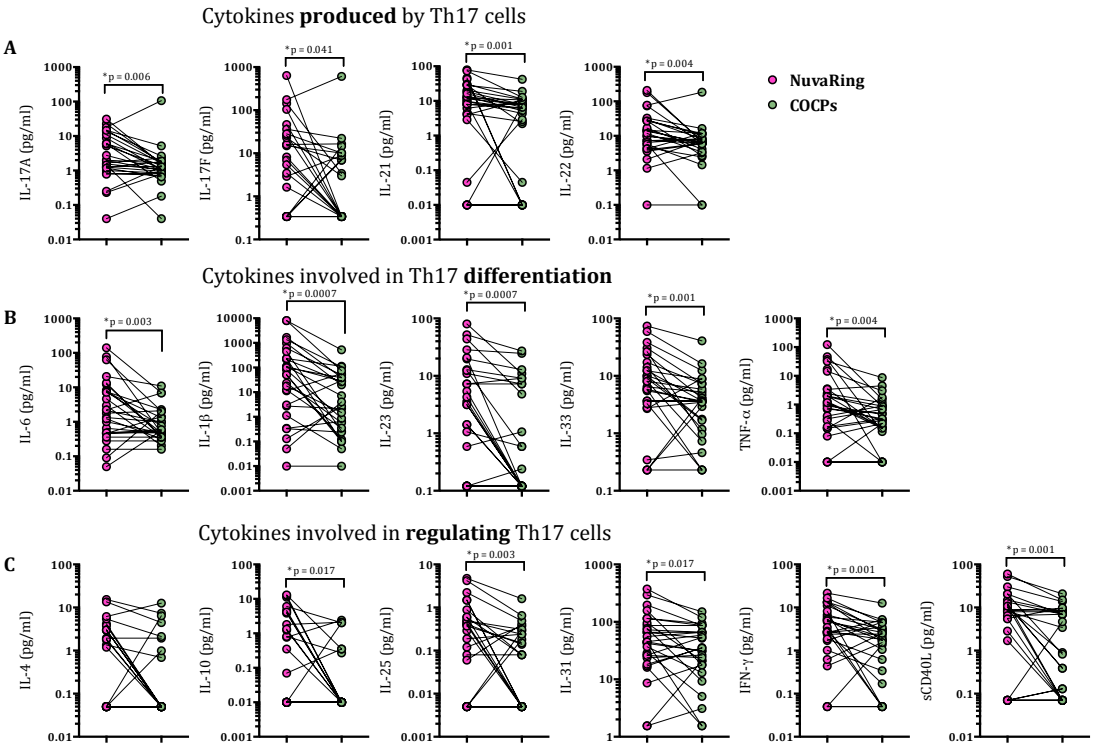

Figure 4.10. Th17-related cytokines (A) those produced by Th17 cells (B) those involved in the differentiation of Th17 cells and (C) those involved in the regulation of Th17 cells, in adolescents changing from NuvaRing to COCPs and *vice versa*. A Wilcoxon matched-pairs signed rank test was applied and a p-value ≤0.05 was considered significant. All p-values remained significant after adjusting for multiple comparisons (indicated by *).

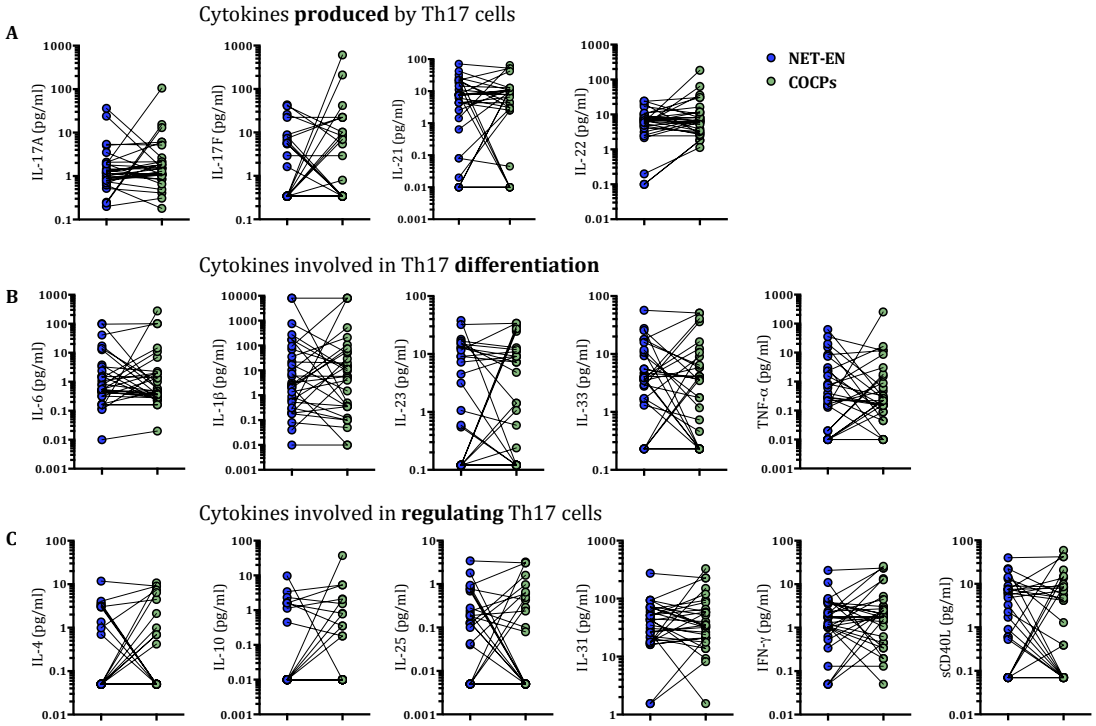

Figure 4.11. Th17-related cytokines (A) those produced by Th17 cells (B) those involved in the differentiation of Th17 cells and (C) those involved in the regulation of Th17 cells, in adolescents changing from NET-EN to COCPs and *vice versa*. A Wilcoxon matched-pairs signed rank test was applied and a p-value ≤0.05 was considered significant. Only significant p-values are displayed.

In a paired analysis, the phenotype of Th17 cells in adolescents changing from NuvaRing to COCPs, NET-EN to COCPs and NuvaRing to NET-EN or *vice versa* was assessed at all the three combined visits. Similar to the reduction in Th17 cell frequencies in adolescents initiated on NuvaRing (at crossover; Figure 4.8A), the frequency of Th17 cells was significantly reduced when participants were on NuvaRing compared to when the same participants were on NET-EN (p=0.0002, adj. p=0.001). Moreover, activated Th17 cells expressing CCR5/HLA-DR/CD38 also tended to be elevated when participants were on NuvaRing (p=0.020, adj. p=0.1; Figure 4.12A). There was no significant difference in participants changing from NuvaRing to COCPs and *vice versa* (Figure 4.12B), and those changing from NET-EN to COCPs and *vice versa* (Figure 4.12C).

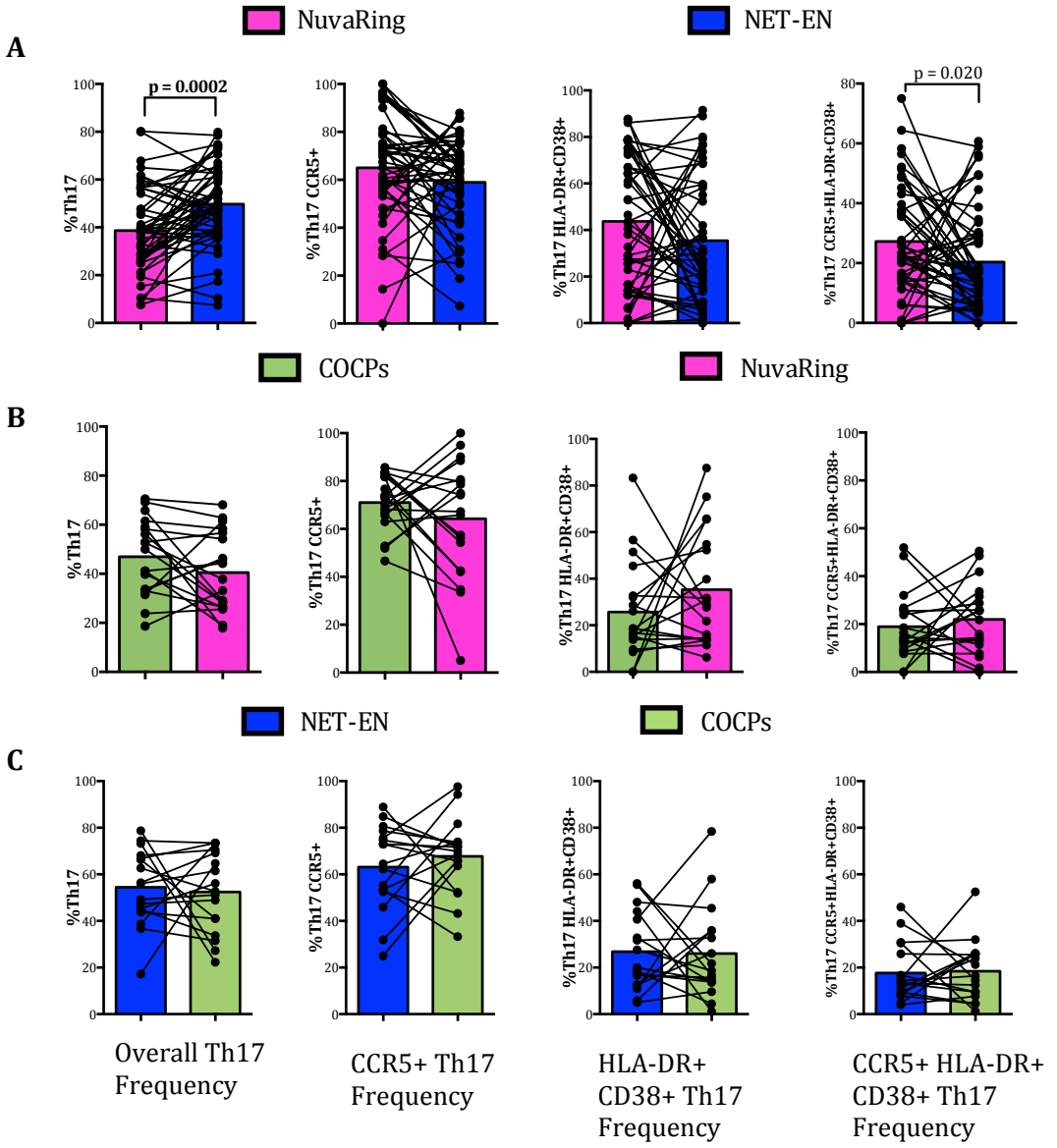

Figure 4.12. Phenotype of Th17 cells in participants who changed between different HCs. (A) Participants who changed from NuvaRing to NET-EN and *vice versa*. Data from 38 participants is included. (B) Participants who changed from COCPs to NuvaRing and *vice versa*. Data from 16 participants is included. (C) Participants who changed from NET-EN to COCPs and *vice versa*. Data from 19 participants is included. Wilcoxon matched-pairs signed rank test was applied. Only significant p-values are displayed on the graph, and those in bold remained significant after adjusting for multiple comparisons.

4.4.7 Longitudinal changes in genital Th17 cells and Th17-related cytokines across the three visits

Changes in Th17 cells and Th17-related cytokines with the use of HCs were investigated across the three visits in all the available participants, and also in matched participants from baseline to the exit visit, to evaluate whether the shift from one contraceptive method and back again influenced activation or frequencies of Th17 cells or changes in cytokine profiles. This longitudinal analysis focused on the Th17-related cytokines that changed significantly in the NuvaRing arm analyses shown in Figure 4.14 (including IL-17A, IL-21, IL-6, IL-1β, IL-23, IL-33, IL-4, IL-10, IL-25, IL-31, IFN-γ, TNF-α, and sCD40L).

4.4.7.1 NuvaRing first (16 weeks) to NET-EN or COCPs (32 weeks)

For adolescents randomized on NuvaRing and subsequently swopped to NET-EN, there was a significant difference in the frequency of Th17 cells across the three visits (p=0.0003; Figure 4.13 left panel), and increased expression of HLA-DR/CD38 and CCR5/HLA-DR/CD38 (p=0.02 and 0.01, respectively). In the matched analysis, a decrease in the frequency of Th17 cells (p=0.04; Figure 4.13 middle panel) and increased expression of HLA-DR/CD38 on these cells (p=0.04) at week 16 was observed, which did not return to baseline levels when participants swopped to NET-EN (p=0.01 and p=0.02, respectively). For adolescents randomized on NuvaRing and subsequently swopped to COCPs, due to sample size, only matched analysis was performed (Figure 4.13 right panel). For the 4 participants available, there was a trend towards a decreased frequency of Th17 cells at week 16 on NuvaRing, and this was not restored to baseline levels when participants swopped to COCPs. However, these results should be treated with caution due to small numbers.

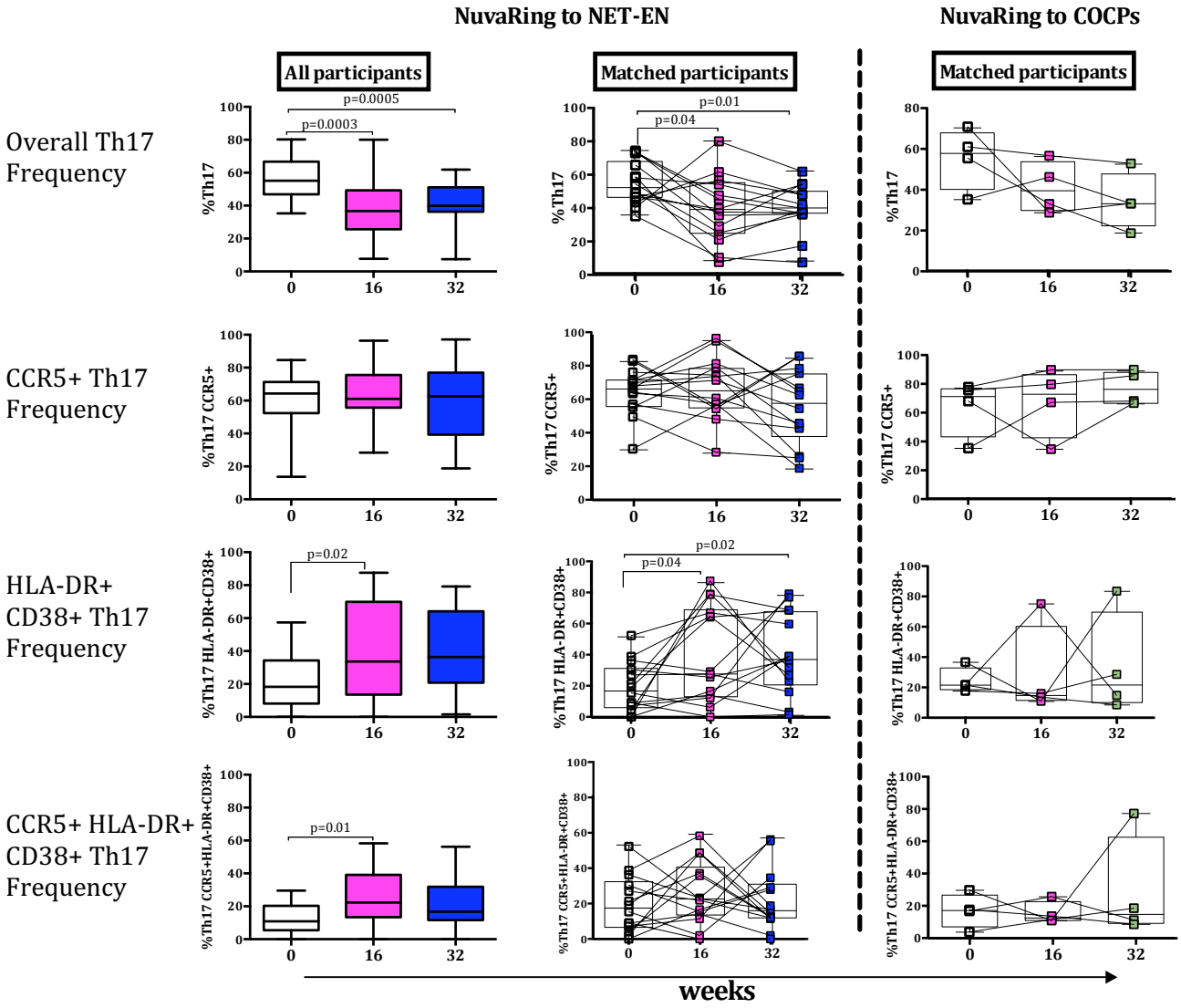

Figure 4.13. Longitudinal changes in phenotype and activation of cervical Th17 cells in adolescents randomized to use NuvaRing first and then either crossing to NET-EN (left panel) or COCPs (right panel). (A left panel) Participants that crossed over to NET-EN (baseline n=21, crossover n=18, exit n=17). Mann-Whitney U test was applied to compare the groups. (A middle panel) Paired participants for all visits (n=14). (B) Participants that crossed over to COCPs (n=4). Each line connects a single participant across all the three visits. Box and whiskers show the median, IQR and range. Wilcoxon matched-pairs signed rank test was applied to compare matched samples.

In matched samples from adolescents using NuvaRing first, IL-6, IL-1β and IL-33 were significantly elevated compared to baseline (p=0.02, p=0.04, p=0.05

respectively; Figure 4.14), and subsequently decreased when they swopped to NET-EN even after adjusting for multiple comparisons (p=0.001, adj. p=0.006; p=0.006, adj. p=0.02; p=0.001, adj. p=0.006, respectively). This same pattern was observed in the unmatched analysis for these three cytokines (IL-6, IL-1β, IL-33). Genital concentrations of INF-γ also increased at week 16 after NuvaRing use (p=0.04) and decreased at week 32 after NET-EN use (p=0.003, adj. p=0.01) in the matched analysis. Furthermore, adolescents had a significant decrease in genital concentrations of IL-21 (p=0.001), IL-23 (p=0.009), IL-4 (p=0.01), IL-10 (p=0.006), IL-25 (p=0.006), IL-31 (p=0.001), TNF-α (p=0.006) and sCD40L (p=0.05) when they swopped from NuvaRing to NET-EN in the matched analysis, and they remained significant after adjusting for multiple comparisons except for IL-4 and sCD40L.

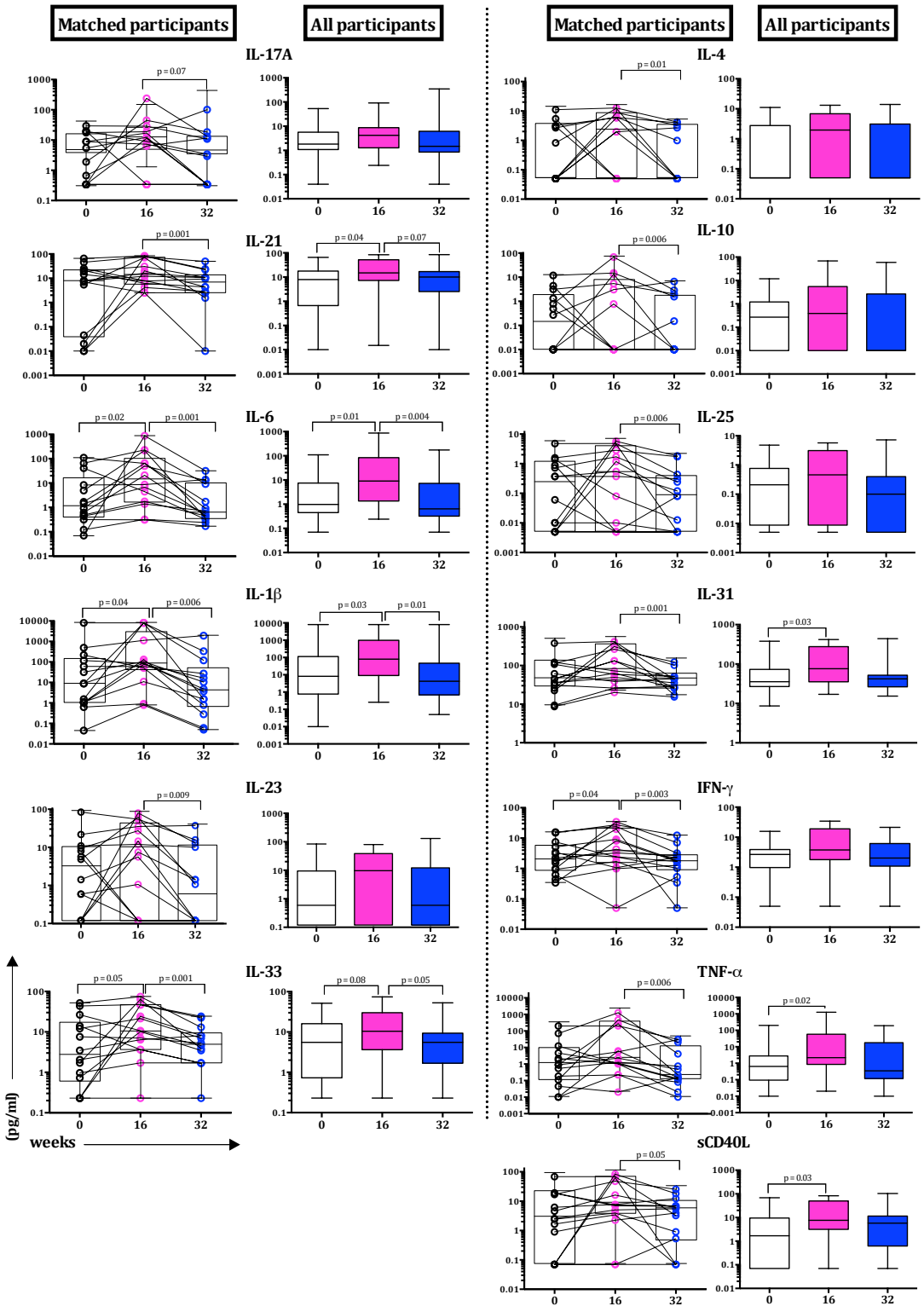

Figure 4.14. Longitudinal changes in Th17-related cytokine concentrations in adolescents initially randomized to NuvaRing and who subsequently crossed over to NET-EN. Matched samples were compared from baseline to exit (n=14) using a

Wilcoxon matched-pairs sign ranked test. Unmatched samples were compared using a Mann-Whitney U test (baseline n=24, crossover n=22, exit n=22).

In adolescents who initially used NuvaRing and subsequently swopped to COCPs, no significant changes in genital cytokine concentrations were observed (Figure 4.15). Numbers were small for this analysis and should be treated with caution (n=4 matched; n=7-8 all). However, cytokine concentrations appeared to be elevated after using NuvaRing for 16 weeks and these appeared to be lower after switching to COCPs for only a subset of cytokines (including IL-4, IL-10, IL-1β and sCD40L, Figure 4.15), although not significantly.

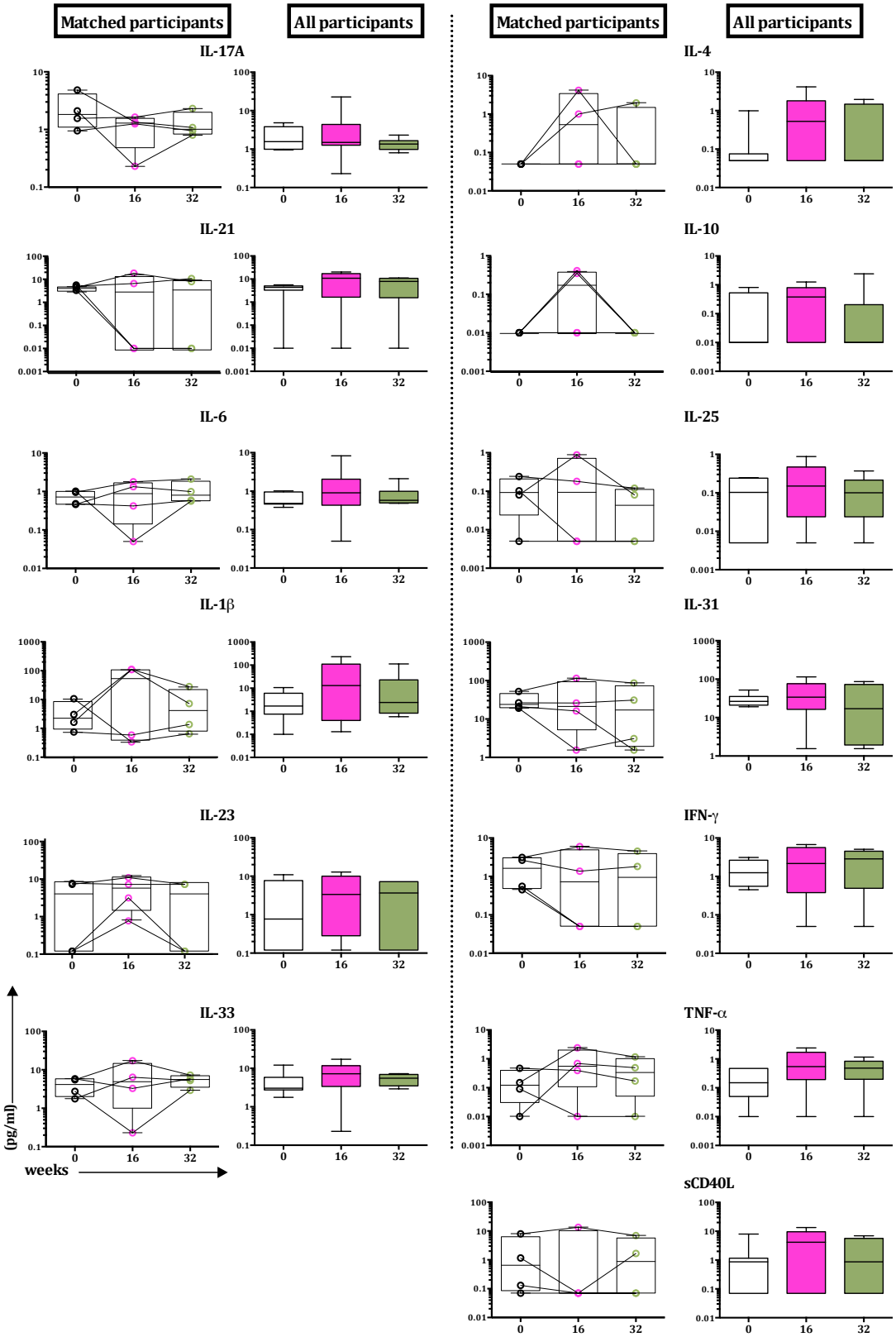

Figure 4.15. Longitudinal changes in cervical Th17-related cytokine concentrations in adolescents initially randomized to NuvaRing and who subsequently crossed over to COCPs. Matched samples were compared from baseline

to exit (n=4) using a Wilcoxon matched-pairs signed ranked test. Unmatched samples were compared using a Mann-Whitney U test (baseline n=7, crossover n=8, exit n=8).

4.4.7.2 NET-EN first (16 weeks) to NuvaRing (32 weeks)

For adolescents initially randomized to NET-EN and then swopped to NuvaRing, no significance difference in the frequency of Th17 cells was observed in NET-EN users compared to baseline, although Th17 cells were reduced in frequency at week 32 after switching to NuvaRing (p=0.02; Figure 4.16 left panel). Moreover, expression of activation markers (HLA-DR/CD38) on cervical Th17 cells were significantly higher at week 16 (p=0.01) than baseline and week 32 on NuvaRing (p=0.006). In a paired analysis, adolescents who swopped to NuvaRing at crossover (using the CCVR from 16-32 weeks) tended to have decreased Th17 frequencies at study exit and elevated co-expression of HLA-DR/CD38 and CCR5/HLA-DR/CD38 (Figure 4.16 right panel).

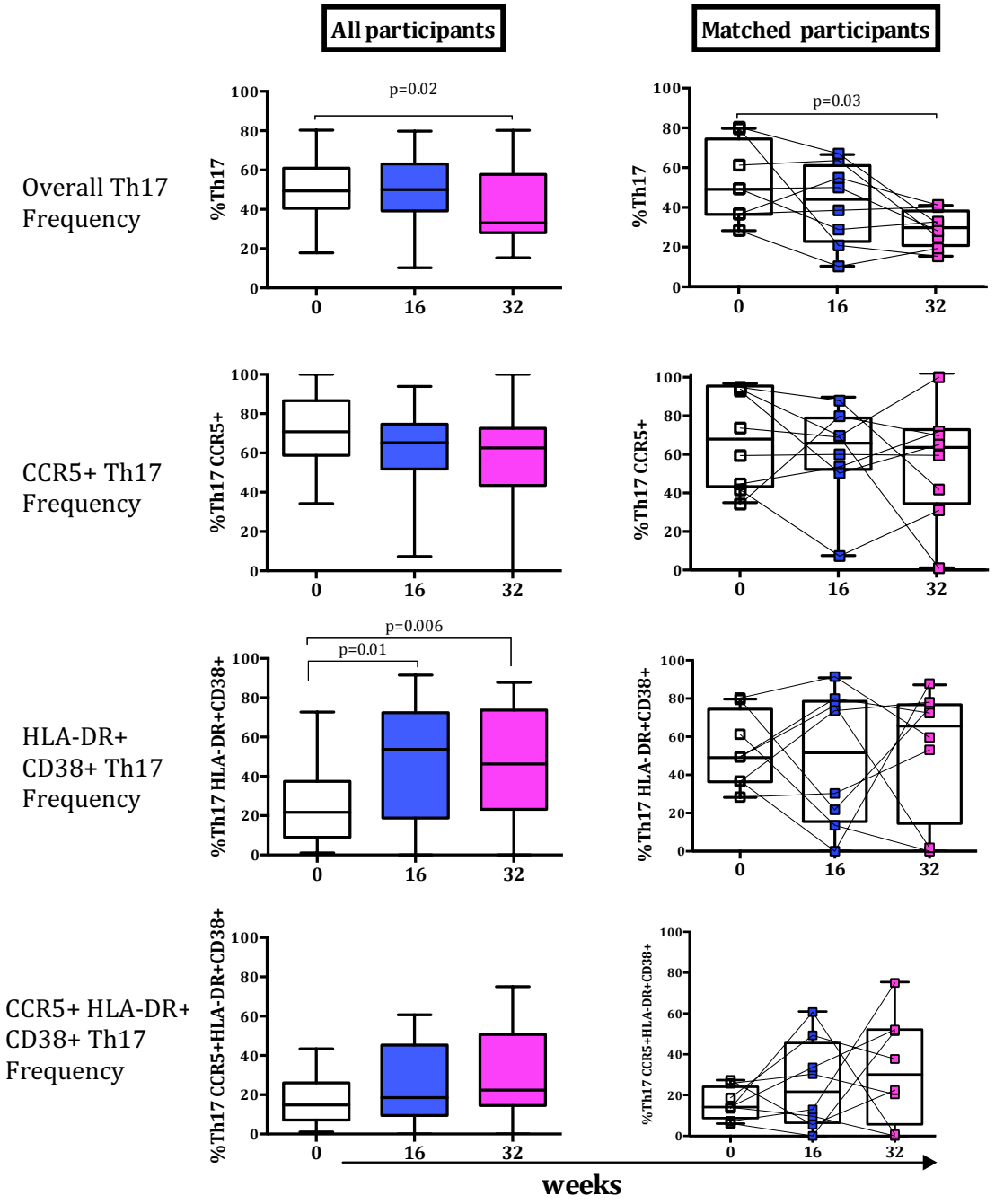

Figure 4.16. Changes in phenotype and activation of cervical Th17 cells across the three visits [baseline (0), crossover (16) and exit (32)] of adolescents randomized to NET-EN first and then crossing over to NuvaRing. Left panel shows the data for all participants from baseline to exit visit (baseline n=31, crossover n=21, exit n=21). Mann-Whitney U test was applied to compare the groups. Right panel column shows only paired participants for all visits (n=8 for each visit). Each line connects a single participant across all the three visits. Box and whiskers show the median, IQR and range. Wilcoxon matched-pairs signed rank test was applied to compare matched samples.

In adolescents randomized to NET-EN and subsequently swopped to NuvaRing, no significant changes in genital cytokine concentrations were observed compared to baseline, and when they swopped over to NuvaRing (Figure 4.17). Although IL-6 and IL-33 tended to be elevated when matched participants were on NuvaRing at week 32 compared to week 16 and baseline. Due to a small sample size, participants changing from NET-EN to COCPs were not investigated.

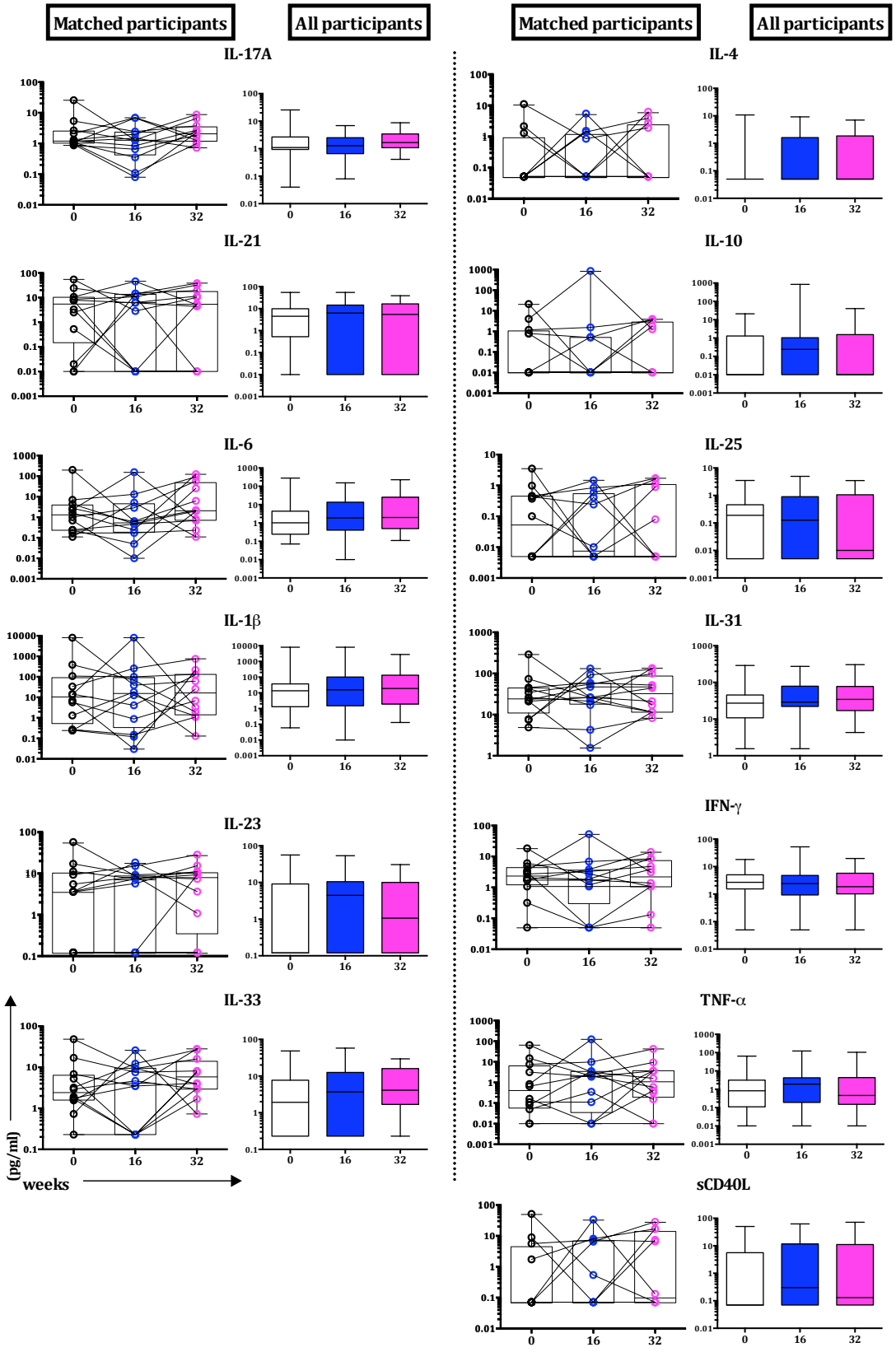

Figure 4.17. Longitudinal changes in Th17-related cytokine concentrations in adolescents initially randomized to NET-EN and who subsequently crossed over to NuvaRing. Matched samples were compared from baseline to exit (n=12) using a

Wilcoxon matched-pairs sign ranked test. Unmatched samples were compared using a Mann-Whitney U test (baseline n=23, crossover n=26, exit n=23).

4.4.7.3 COCPs first (16 weeks) to NuvaRing (32 weeks)

For participants randomized to COCPs first and who then crossed to using the NuvaRing, frequencies of cervical Th17 cells remained unchanged after 16 weeks of COCPs although these decreased after swopping to NuvaRing for the second phase of the study (to 32 weeks; p=0.01; Figure 4.18 left panel). Expression of CCR5 on Th17 cells was elevated after using COCPs for 16 weeks (p=0.006), which remained elevated to 32 weeks (after swopping to NuvaRing). While this increase in expression of CCR5 was significant in the unpaired analysis that included all participants, it was not significant in the paired analysis (including 12 adolescents for whom matched samples were available at all visits, Figure 4.17 right panel).

For Th17-related cytokines, no significant changes were observed from baseline to week 16 after participants have been on COCPs (Figure 4.19). However, a significant increase in IL-6 and IL-1β was observed in both the matched (p=0.007 and p=0.0003, respectively) and unmatched analyses (p=0.03 and p=0.04, respectively) at week 32 when adolescents have swopped and been on NuvaRing. IL-17A (p=0.01, adj. p=0.01), IL-21 (p=0.003, adj. p=0.009), IL-23 (p=0.0007, adj. p=0.002), IL-33 (p=0.02, adj. p=0.02), IL-25 (p=0.008, adj. p=0.01), IL-31 (p=0.03, adj. p=0.03), IFN-γ (p=0.0005, adj. p=0.002), TNF-α (p=0.01, adj. p=0.01) and sCD40L (p=0.003, adj. p=0.009) were elevated at week 32 on NuvaRing, and those with adjusted p-values (adj. p) remained significant. Only 3 samples were available for adolescents who swopped from COCPs to NET-EN, and therefore, no analysis was performed (data not shown).

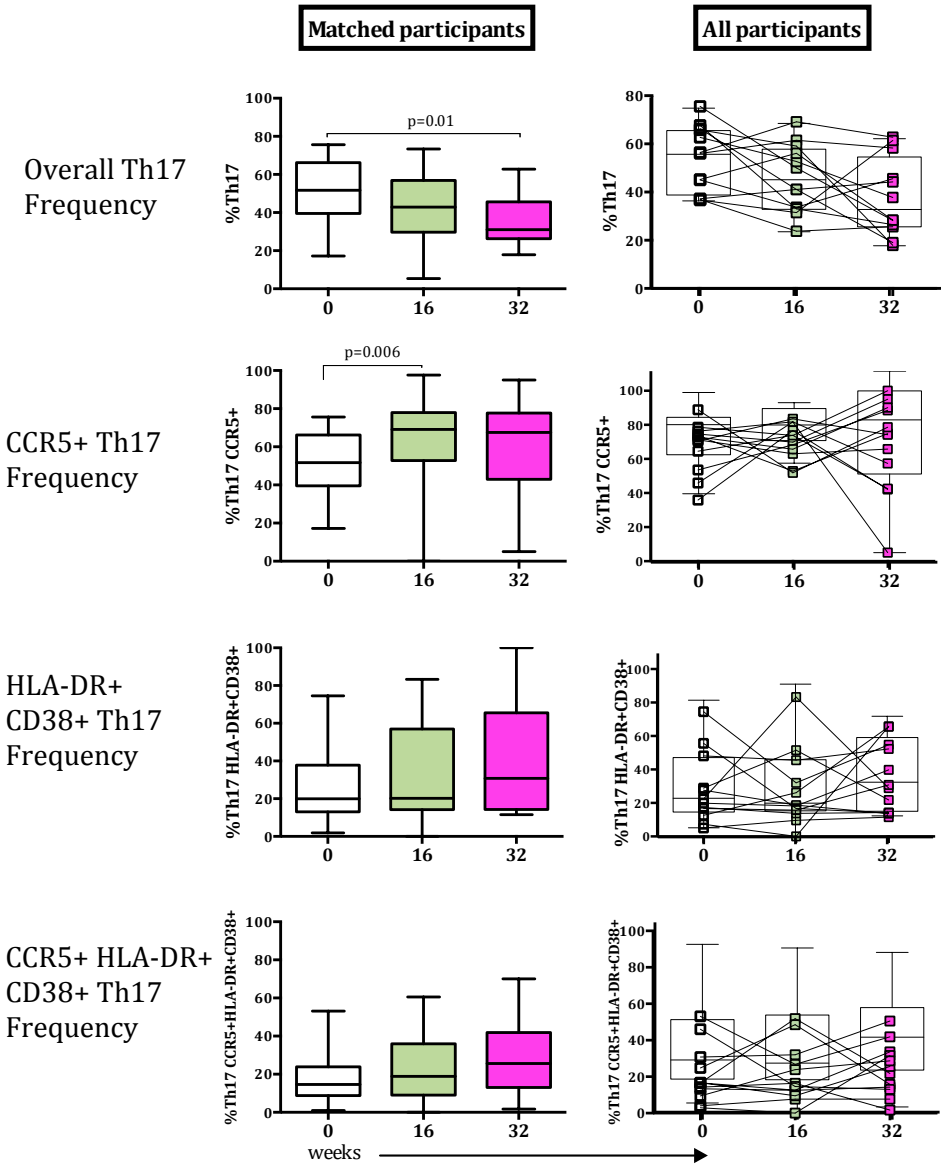

Figure 4.18. Changes in the phenotype and activation of cervical Th17 cells across the three visits [baseline (0), crossover (16) and exit (32)] in adolescents randomized initially to use COCPs and who subsequently crossed over to NuvaRing. Left panel shows the data from all participants from baseline to exit (baseline n=20, crossover n=22, exit n=16). A Mann-Whitney U test was applied to compare the groups. Right panel shows only paired participants for all visits (n=12). Each line connects a single participant across all the three visits. Box and whiskers show the median, IQR and range. A Wilcoxon matched-pairs signed rank test was applied to compare matched samples.

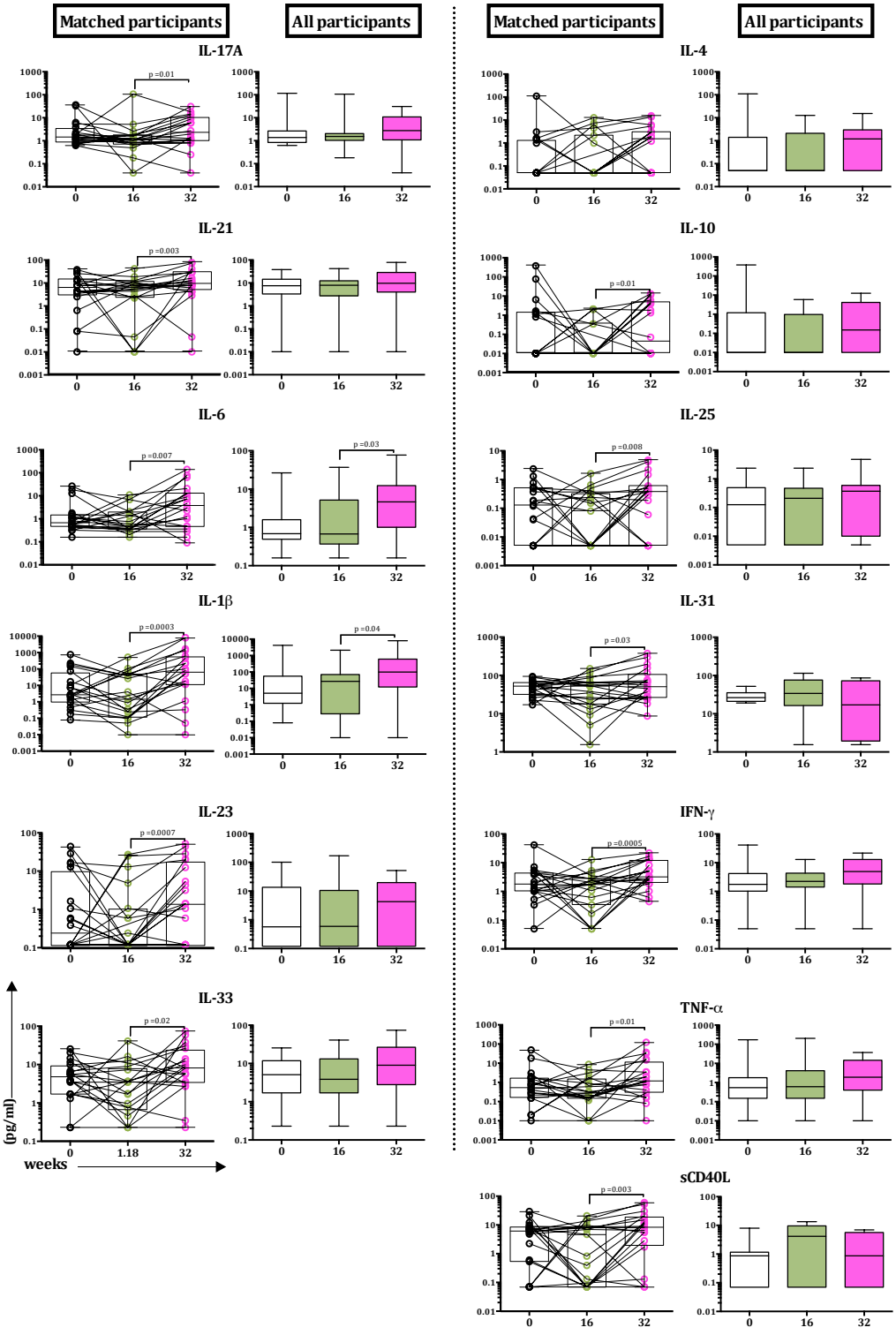

Figure 4.19. Longitudinal changes in Th17-related cytokine concentrations in adolescents initially randomized to COCPs and who subsequently crossed over to NuvaRing. Matched samples were compared from baseline to exit (n=20) using a Wilcoxon matched-pairs sign ranked test. Unmatched samples were compared using a Mann-Whitney U test (baseline n=22, crossover n=20, exit n=15).

4.5 Discussion

The aim of this Chapter was to investigate the impact of various HC products on genital tract CD4+ T cell activation and numbers in adolescents, in a randomized crossover trial comparing NuvaRing, NET-EN and COCPs, focusing on Th17 cells because they are thought to be highly susceptible to HIV infection (Brenchley et al., 2008; Kim et al., 2013; Stieh et al., 2016). This may provide valuable insight into mechanisms underlying increased HIV risk in adolescent girls. One of the major findings was that genital tract IL-21, IL-1β, TNF-α and IFN-γ concentrations were elevated in those randomized to use NuvaRing at crossover compared to baseline. In contrast, no such changes in genital tract cytokines were observed in adolescents initially randomized to NET-EN or COCPs.

One of the many functions of Th17-related cytokines, including IL-17A, IL-17F, IL-6, IL-23 and IL-1β is to recruit Th17 cells to mucosal surfaces including the genital tract (Conti et al., 2009; Sandquist and Kolls, 2018). Therefore, how HCs altered the frequency and activation of Th17 cells within individuals was investigated. Adolescents using NuvaRing had significantly elevated frequencies of highly activated cervical Th17 cells (expressing both CD38 and HLA-DR), although these were present at lower frequencies compared to baseline. Frequencies of highly activated Th17 cells also tended to be elevated in adolescents initially randomized to NET-EN, although not after adjusting for multiple comparisons. No differences in the frequency or activation status of cervical Th17 cells were observed in adolescents changing from NuvaRing to COCPs or those changing from NET-EN to COCPs at crossover and exit visits. Adolescents using COCPs tended to have a decreased frequency of Th17 cells, with a significant increase of CCR5 expression on these cells compared to baseline, although this was not confirmed in the paired analysis. Overall, this data suggests that all three HCs investigated altered Th17 cells in the genital tract compared to when these young girls came into the study.

The median age of sexual debut in this cohort was 15 years, and having had sex at or before the age of 16 has been reported to be a strong marker for future poor sexual health patterns (Cavazos-Rehg et al., 2010; Zuma et al., 2014). Unlike

in older women, the underdeveloped cervix of adolescents is going through physical changes including changes in the vaginal epithelium (Moscicki et al., 1999). In addition, adolescent girls are at a stage of hormonal changes associated with sex maturation. These changes in addition to initiating HCs might all contribute to perturbations seen in the genital tract. In this study, the range of E2 serum levels (74-142 pmol/L) were lower than the reported reference ranges in older women (161-774 pmol/L; Smritee Dabee PhD thesis). It would be interesting to see whether NuvaRing perturbs the vaginal immune microenvironment in older women at the same extent seen in adolescent girls, given the differences in their biological sexual maturity of the genital tract.

Overall, the results observed in this study show elevated genital Th17-related cytokine concentrations in adolescents using NuvaRing but not in those using NET-EN and COCPs. Amongst the cytokines produced by Th17 cells (IL-17A, IL-17F, IL-21 and IL-22), IL-17F was less affected by the use of NuvaRing. Although both cytokines play a critical role in tissue inflammation by inducing the release of proinflammatory cytokines, IL-17F has been reported to be less potent at inducing cytokines (Sueki et al., 2008; Ouyang et al., 2012; Brembilla et al., 2018; Glatt et al., 2018). Increased Th17-related cytokines observed when participants were on NuvaRing might suggest both hormonal and mechanical responses to the copolymer evatane vaginal ring (forming the NuvaRing) in the vagina. Early studies suggested local leukocytic inflammatory responses after IUD insertion in the endometrium and fallopian tubes (Moyer and Mishell, 1971). Furthermore, the presence of an LNG-IUD in the genital tract has been associated with inflammatory cytokines including IL-1β, TNF-α, IFN-γ, GM-CSF, and IFN-α in the endocervical canal (Shanmugasundaram et al., 2016; Sharma et al., 2018), and insertion of the copper IUD was associated with increase in IL-1α, IL-1β, IL-6 and TNF-α (Sharma et al., 2018). This shows that changes in cytokines can be induced both mechanically and hormonally.

With elevated inflammatory cytokines including IL-1β noted in adolescents using NuvaRing, one would expect increased numbers and/or frequencies of Th17 cells or total CD4+ T cells in these participants. It was therefore surprising that

the overall frequency of total genital CD4+ and Th17-like T cells were reduced in adolescents assigned to the NuvaRing arm. Alteration in Th17 cells was observed in all HCs compared to baseline, indicating that exogenous hormones partly caused these perturbations. Highly activated genital tract T cells are more likely to die by apoptosis than resting T cells (Nkwanyana et al., 2009), and one could hypothesize that the reduction in frequencies of cervical Th17 cell in adolescents using NuvaRing and NET-EN might reflect cell death of these highly activated cells. IL-10, IL-25, IL-4 and IFN-γ negatively regulate Th17 cells, and these cytokines might also contribute to the dramatic decrease in Th17 cell frequencies seen in the NuvaRing arm, in addition to other immune cells including Tregs not measured in this study, but are known to be important in suppressing Th17 responses (Lee et al., 2015b). In one study, NuvaRing and COCPs users were found to have reduced frequencies of CD207+ Langerhans cells in the vagina (Mitchell et al., 2014). Langerhans cells are antigen-presenting cells that are known to activate cutaneous Th17 responses in response to bacterial infections (Deckers et al., 2018). Perhaps participants on NuvaRing also had a reduction in these antigen-presenting cells thereby decreasing the presence of Th17 cells at the genital tract. Furthermore, the vaginal ring is recognized as a foreign object in the lower reproductive tract, thereby inducing microbial and immune changes. The localised hormones delivered directly into the vagina by NuvaRing might also contribute to this effect.

Hardy et al. (2017) recently reported that the NuvaRing acquires a complex bacterial biomass after insertion, including several BV-associated bacterial strains. They postulated that even though the delivered hormones from NuvaRing increased the concentration and relative abundance of vaginal *Lactobacillus* spp., metabolites and bacteriocins produced by *Lactobacillus* were not effective against a poly microbial biofilm. While the microbial composition of the biofilms associated with CCVRs in this study was not determined, it is possible that these bound microbial products on the ring bind to TLRs expressed on different epithelial and immune cells including T cells and neutrophils, thereby initiating cytokine and chemokine production. Although neutrophil infiltration was not measured, Th17 cells are known to recruit neutrophils by

producing IL-17A and IL-17F (Roussel et al., 2010; Schofield et al., 2016). Activation of Th17 cells has been reported to result in a large amount of inflammatory cytokine production including IL-17A, IL-17F, IL-21 and IL-22 and this partially explains why NuvaRing had increased Th17-related cytokine concentrations due to highly activated Th17 cells observed (Tesmer et al., 2008; Ouyang et al., 2012). The use of NuvaRing had been reported to increase vaginal wetness, and some have linked this to inflammation due to a slight increase in vaginal white blood cells in CCVR users (Schwan et al., 1983; Veres et al., 2004). Vaginal wetness, however, was not determined in this study.

Aside from CCVRs, vaginal rings containing HIV drugs have also been assessed as pre-exposure prophylaxis (PrEP) modalities, although genital tract immune cells are typically not measured in these clinical studies (Johnson et al., 2012; Smith et al., 2015; Baeten et al., 2016). However, insertion of a silicon intravaginal ring into female macaques was not associated with inflammatory cytokines IL-6 and IL-8. Both tenofovir-medicated and unmedicated rings in macaques have been shown to form biofilms (Gunawardana et al., 2011).

This study has several limitations. Although the initial plan was to recruit adolescents who are contraceptive naïve into the study, the age range of this young cohort (15-19 years) biased recruitment to those who were already sexually active and had initiated HCs. Moreover, these participants were recruited from a family planning clinic rather than from a broader community. For participants <18 years of age, the recruitment process was difficult, as they had to get a parental consent and expose that they were sexually active in a culture where it is not typically widely accepted. Importantly, there was no washout period due to the nature of the study assuring that participants were protected from unwanted pregnancies. Sexual risk behaviour and vaginal practices was self-reported, however, these were similar across HC arms thereby excluding possible confounders. Another important limitation is the small sample size, decreased further by sub-analyses, and hence underpowered to detect some changes.

In conclusion, results in this part of the study show a perturbation of Th17 cell frequencies and increased concentrations of genital Th17-related cytokines in the lower reproductive tract of adolescents who were using NuvaRing. To my knowledge, this is the first study to compare NuvaRing, NET-EN and COCPs in a randomized crossover designed trial in adolescents around sexual debut. Further investigation to understand the changes seen in Th17 cells in response to the various HCs is urgently needed. It was concerning that Th17-related cytokine changes localised primarily to adolescents in the NuvaRing arm.

Chapter 5

Effect of hormonal contraceptives on genital tract CD8+ T cells

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5.1 Abstract

Cytotoxic CD8+ T cells are the primary adaptive immune mechanism responsible for controlling HIV. Although it is widely accepted that Th17 cells (CD4+) are more susceptible to HIV infection, a subset of CD8+ T cells are also capable of producing IL-17 and therefore considered T cytotoxic IL-17 cells (Tc17 cells). IL-17 is associated with inflammation and Th17 cells are susceptible to HIV infection, nonetheless, the relative role of Tc17 cells in HIV infection and risk is less well understood. Here, the impact of HCs on CD8+ T cells generally and on the frequencies of Tc17 cells (CD8+CCR6+) was investigated in adolescents. In a paired-analysis comparing baseline to the crossover visit, adolescents using NuvaRing had lower frequencies of cervical CD8+ T cells, which expressed lower levels of HLA-DR, but had higher frequencies of CD38 and CCR5/CD38. Moreover, frequencies of Tc17 cells were decreased in these adolescents. Similarly to NuvaRing, adolescents using NET-EN had reduced frequencies of CD8+ with elevated frequencies expressing CD38 and CCR5/CD38, and reduced HLA-DR, in addition to reduced Tc17 frequencies. The use of COCPs was associated with reduced frequencies of Tc17 cells, and increased expression of CCR5/CD38 and reduced expression of HLA-DR on cervical CD8+ T cells. In adolescents changing between HCs, those using NuvaRing had decreased frequencies of Tc17 cells compared to when the same participants were on NET-EN or COCPs. This data suggests that HCs reduced the frequencies of Tc17 cells, while altering activation markers on CD8+ T cells, which may have implications for mucosal immune control in relation to HIV infection.

5.2 Introduction

Alteration of immune cells in the FRT may have a significant effect of HIV acquisition and transmission. Like CD4+ T cells, CD8+ T cells continue to play a central role in mucosal immunity (Luckheeram et al., 2012). CD8+ T cells are needed in the FRT to protect against infections including protozoal, bacterial and viral pathogens (Levy et al., 1996; Brinza et al., 2016). Mouse studies have shown that intravaginal HSV-2 infections induced high-affinity memory CD8+ T cells that were needed to control epithelial pathogens infecting the genital tract (Tang and Rosenthal, 2010; Anjuère et al., 2012). These memory CD8+ T cells are also critical in controlling HSV-2 reactivation and maintaining latency. Similar to CD4+ T cells, CD8+ T cells are classified into various memory subsets based on expression of different receptors and cytokine production (Golubovskaya and Wu, 2016), and expression of CCR6 was predominantly associated with memory CD8+ T cells (Kondo et al., 2007). Based on their ability to produce IL-17, these CD8+ T cells expressing CCR6 are known as Tc17 cells (Kondo et al., 2009). CCR6 and its ligand CCL20 act as chemotactic immune-modulatory envoy that attract lymphocytes and other immune cells to the mucosal tissues including the genital tract (Ranasinghe and Eri, 2018).

Given the importance of CD8+ T cells in FRT protective immunity, it is important to understand how the use of HCs altered them. HCs containing the synthetic progestin MPA have been shown to inhibit protective CD8+ T cell responses by promoting HSV-1 reactivation (Cherpes et al., 2008). Although most studies on HCs have focused on CD4+ T cells, it is also important to investigate their potential effects on CD8+ T cells. Alteration in CD8+ T cells may compromise immune responses to pathogens in the genital tract. However, there have been few studies focusing on how HCs affect CD8+ T cells in the genital tract, particularly in adolescent girls. The aim of this Chapter was therefore to investigate the effect of NuvaRing, NET-EN and COCPs use on CD8+ and Tc17 cell frequencies in adolescents in the uCHOOSE cohort.

5.3 Materials and Methods

5.3.1 Study design

As described in Chapter 4 (section 4.3.1), the study design was an open-label, randomized crossover study of 130 sexually active female adolescents aged 15–19 years from Masiphumelele, Cape Town, South Africa. Inclusion and exclusion criteria are described in Section 4.3.1. Adolescents were randomized 1:1:1 to three arms: (1) injectable NET-EN; (2) the CCVR NuvaRing®; or (3) COCPs (as described in Chapter 4). After being randomized onto each of these contraceptive methods for four months, participants crossed over to another form of HC (as shown in Figure 4.1). Participants receiving either NET-EN or the daily COCPs (Arm 1 and Arm 3, respectively) for the first 16 weeks of the study switched over to using NuvaRing (Arm 2), while participants receiving NuvaRing as the first method were given the choice between either NET-EN or the daily COCPs as their second method. After a total of 32 weeks, the participants returned for a final visit at the clinic and exited the study.

5.3.2 HIV and pregnancy testing

As described in Chapter 4 (section 4.3.2), a rapid HIV test (Alere Determine™ HIV-1/2 Ag/Ab Combo; Alere, MA, USA) and pregnancy test (U-test Pregnancy strip; Humor Diagnostica, Pretoria, SA) was performed at all study visits (screening, enrolment, crossover and exit).

5.3.3 Sample collection

An endocervical cytobrush (Digene Corporation, MD, USA) was collected for CMCs, which were analysed using flow cytometry (section 2.3.5 and 2.3.6).

5.3.4 Flow cytometry

Endocervical cytobrushes (Digene® Corporation) were collected from each adolescent under speculum examination, and processed within 4 hours as previously described in section 2.3.5. CMCs were stained with the following monoclonal antibodies: CD3, CCR6, CCR5 (BD Biosciences); CD38 (eBioscience); CD4 (Invitrogen); CD8, CCR10, HLA-DR (Biolegend) as previously described in section 2.3.5. A DUMP channel consisting of CD14, CD19 (Invitrogen) and ViVid (Life Technologies) was included to remove monocytes, B-cells and dead cells. The gating strategy is described in Chapter 2, section 2.3.6.

5.3.5 Statistical analyses

A Wilcoxon matched-pairs signed rank test was used to compare individuals within each study arm at baseline and after being on the randomized contraceptive. A Mann-Whitney U test was used for unmatched comparisons. For comparison between more than two groups, the Kruskal-Wallis one-way analysis of variance was used. Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, CA, USA), and STATA™ version 12 (StataCorp, TX, USA). All tests were two sided and a p-value of ≤0.05 was considered significant. Where stated, the Benjamini-Hochberg method was used to adjust for multiple comparisons.

5.4 Results

5.4.1 Effect of HCs on CD8+ T cells after HC use

Cervical CD8+ T cell frequencies and activation status were compared within each contraceptive arm, using each adolescents baseline and crossover visit time points, to determine whether they were altered by use of HCs. For NuvaRing, 29/35 genital samples were available for paired analysis. Intra-individual analysis within the NuvaRing arm showed a reduction in the frequencies of Tc17 ($p<0.0001$) and CD8+ T cells ($p=0.03$), with decreased expression of HLA-DR ($p=0.02$) and increased expression of CD38 ($p=0.01$) and CCR5/CD38 ($p<0.0001$) on CD8+ T cells after NuvaRing use (Figure 5.1A). All phenotypic changes on CD8+ T cells remained significant after adjusting for multiple comparisons.

In the NET-EN arm, 22/32 adolescents had paired genital samples available for analysis. Unlike NuvaRing, the use of NET-EN was not associated with a reduction in frequencies of total CD8+ T cells although the Tc17 cell subset was significantly decreased ($p<0.0001$; Figure 5.1B). Increased frequencies of cervical CD8+ T cells expressed CD38 ($p=0.01$) and CCR5/CD38 ($p=0.0001$) after NET-EN use, while lower frequencies of these cells expressed HLA-DR ($p=0.002$).

In the COCPs arm, 27/37 adolescents had paired genital samples available for analysis. While cervical CD8+ T cell frequencies remained unchanged after using COCPs for 4 months, the frequency of Tc17 cells was significantly reduced ($p<0.0001$; Figure 5.1C). However, the frequency of cervical CD8+ T cells expressing CCR5/CD38 was significantly higher post-COCP use ($p<0.0001$). The frequency of CD8+ T cells expressing HLA-DR decreased significantly compared to baseline ($p<0.0001$).

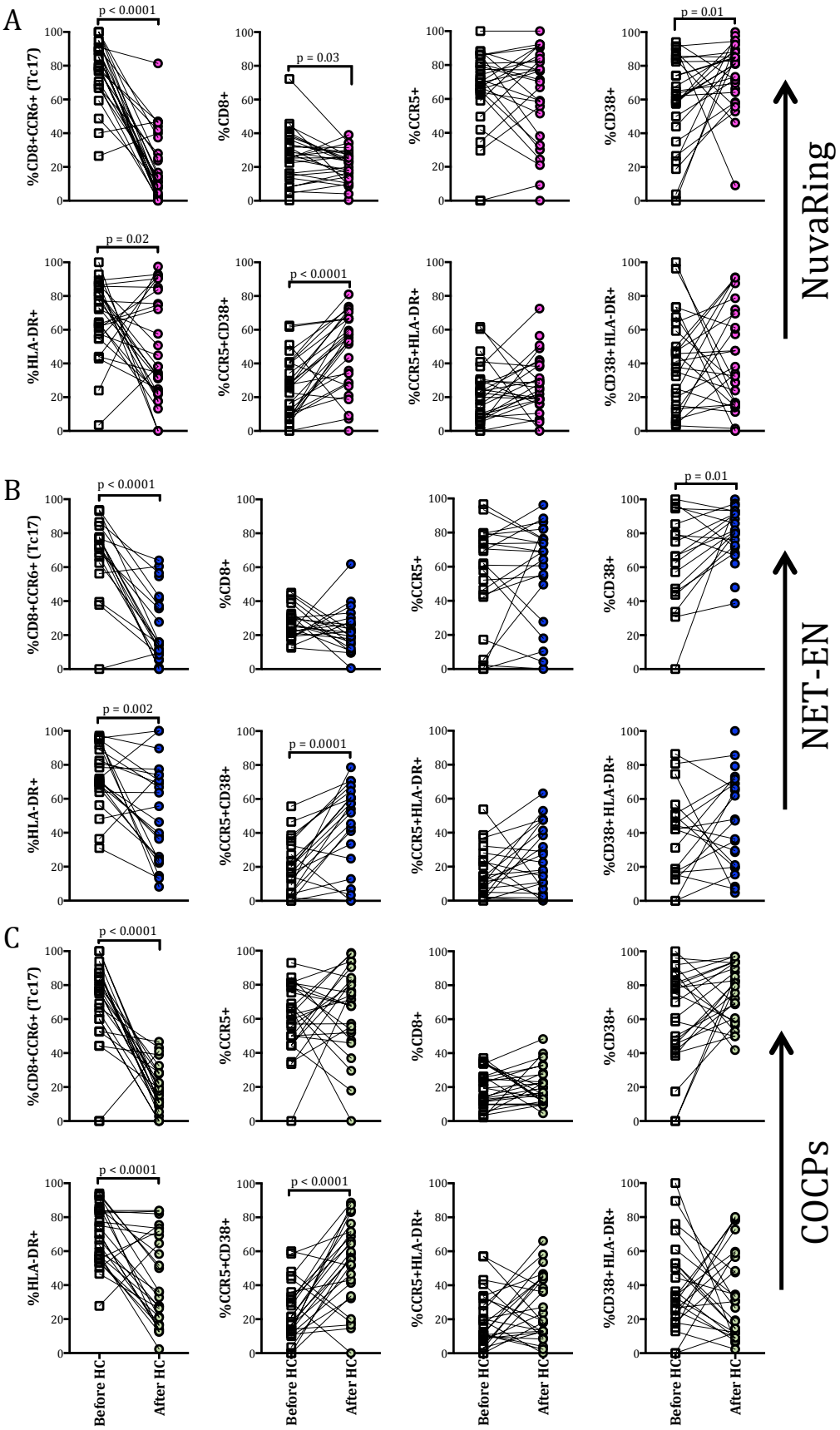

Figure 5.1. Impact of (A) NuvaRing, (B) NET-EN, and (C) COCPs on cervical Tc17 cells and CD8+ frequencies and activation state. CCR5 and activation markers (CD38 and HLA-DR)

were analysed within CD8+ T cell populations. The clear squares show participants at baseline and the circles show participants at crossover. Each black line link paired data points in an individual participant. Samples were compared using the Wilcoxon matched-pair signed rank test, and a p-value≤0.05 was considered significant.

Next, the activation and co-receptor expression characteristics of cervical CD8+ T cells were compared across arms at crossover. In an ITT analysis, there was no significant difference in the frequencies of cervical Tc17 and CD8+ T cells, according to randomization arm (Figure 5.2). Expression of CCR5 and activation markers on CD8+ T cells was also similar across the HC arms. At the exit visit, the frequency and phenotype of CD8+ T cells was also similar across the three arms (data not shown).

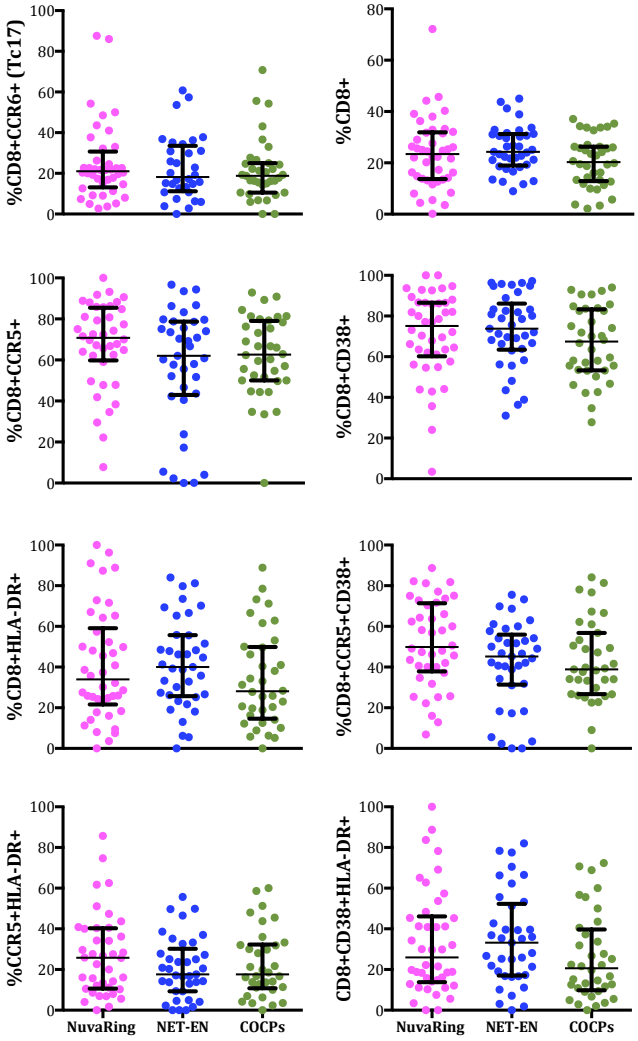

Figure 5.2. Comparison of the frequency and activation status of cervical CD8+ T cells from adolescents using NuvaRing (pink circles), NET-EN (blue circles) and COCPs (green circles). Tc17 cells, CCR5 and activation markers were analysed within the CD8+ T cell population. The middle line represents the median, and the error bars represent the IQR. Groups were compared using the Kruskal-Wallis test.

5.4.2 Analysis of CD8+ T cell frequencies and activation after changing between NuvaRing to NET-EN

The changes in CD8+ T cells in adolescents changing between NET-EN and NuvaRing were evaluated. Swopping from NET-EN to NuvaRing resulted in increased frequencies of cervical CD8+ T cells expressing CCR5 (p=0.04), CD38 (p=0.003) or both CCR5/CD38 together (p<0.0001), although Tc17 cell frequencies were lower (p=0.0004; Figure 5.3).

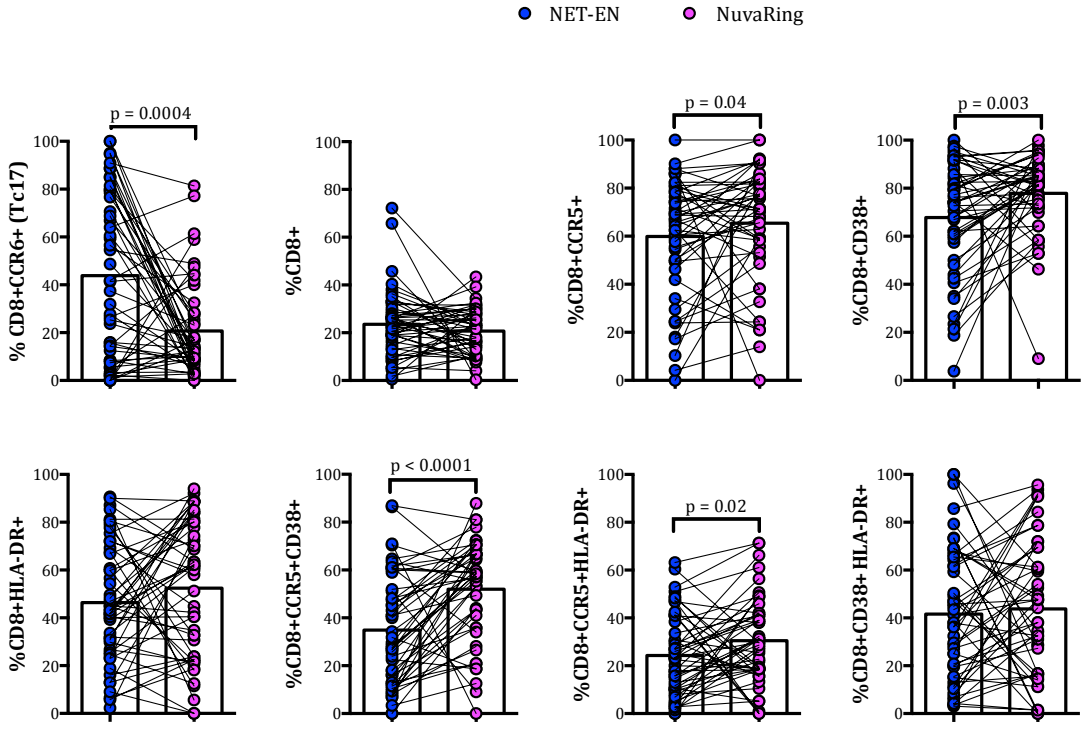

Figure 5.3. Comparison of cervical CD8+ T cells in participants changing between NET-EN and NuvaRing. Tc17 cells, CCR5 and activation markers were analysed within the CD8+ T cell population. A blue circle denotes the matched time point when adolescents were using NET-EN,

while a purple circle denotes the NuvaRing time point. The bar shows the median frequency for each subset analysed. Samples were compared using the Wilcoxon matched-pair signed rank test. P-value≤0.05 was considered significant and those that were significant are shown.

5.4.3 Analysis of CD8+ T cell frequencies and activation after changing between COCPs and NuvaRing

The phenotype of CD8+ T cells in adolescents changing between COCPs and NuvaRing was investigated (Figure 5.4). The frequency of CD8+ T cells expressing HLA-DR was significantly lower when adolescents used NuvaRing compared to when they were using COCPs, in a matched analysis (p=0.02). In contrast, the frequency of CD8+ T cells expressing both CCR5/CD38 was significantly elevated (p=0.003) when participants were using NuvaRing compared to COCPs. In addition, Tc17 cells were significantly reduced after NuvaRing use (p=0.0003).

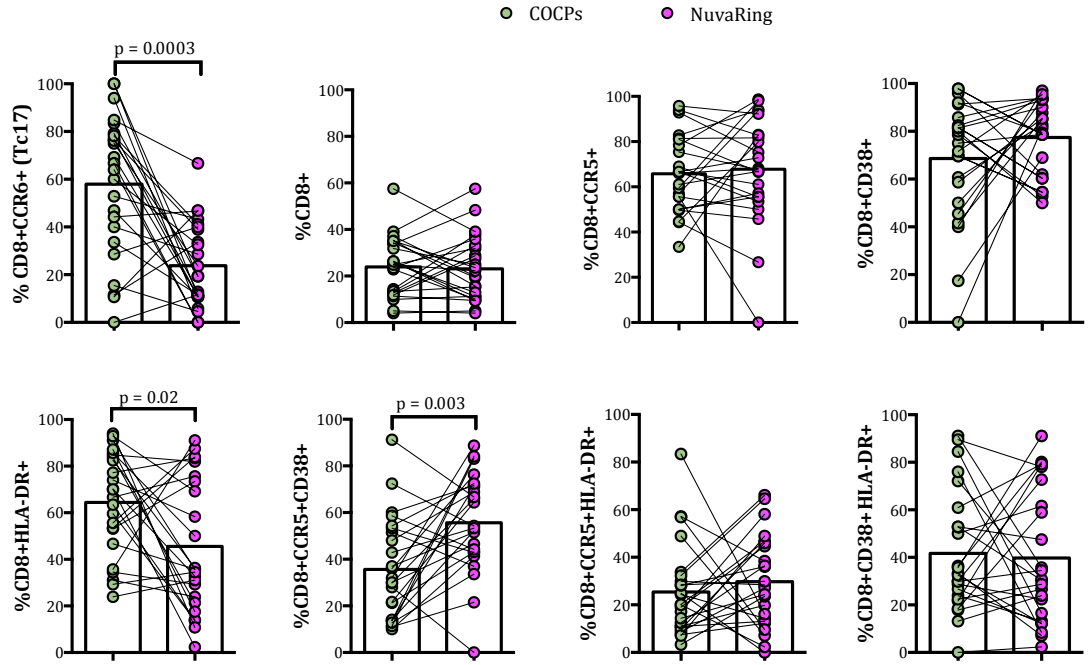

Figure 5.4. Comparison of cervical CD8+ T cell phenotype and activation status in adolescents changing between COCPs and NuvaRing. Tc17 cells, CCR5 and activation markers were analysed within the CD8+ T cell populations. A green circle denotes the matched time point when adolescents were using COCPs, while a purple circle denotes the NuvaRing

time point. The bar shows the median frequency for each subset analysed. Samples were compared using the Wilcoxon matched-pair signed rank test. P≤0.05 were considered significant and those that were significant are shown.

5.4.4 Analysis of CD8+ T cell frequencies and activation after changing between NET-EN and COCPs

While the frequencies of cervical CD8+ T cells expressing HLA-DR was significantly higher when adolescents were using NET-EN compared to when the same adolescent were using COCPs (p=0.004), the frequency of CD8+ T cells expressing CCR5/CD38 together was significantly lower (p=0.0008). Moreover, Tc17 cells were increased when the same participants used NET-EN (p=0.001; Figure 5.5).

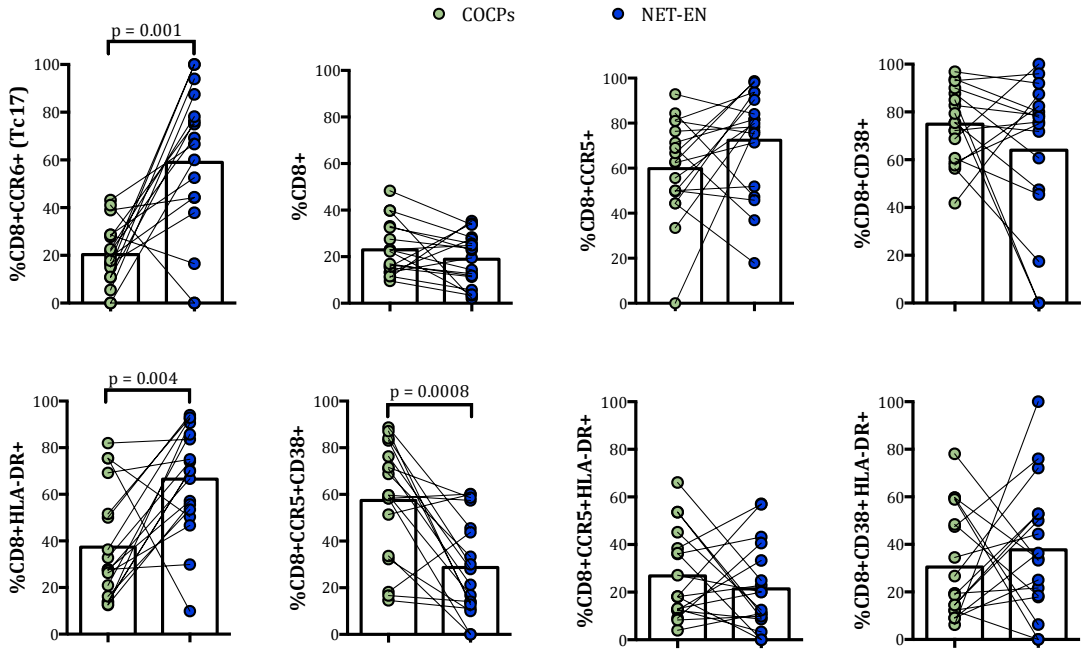

Figure 5.5. Comparison of cervical CD8+ T cell frequencies in adolescents changing between COCPs and NET-EN. Tc17 cells, CCR5 and activation markers were analysed within the CD8+ T cell population. A green circle denotes the matched time point when adolescents were using COCPs, while a blue circle denotes the NET-EN time point. The bar shows the median frequency for each subset analysed. Samples were compared using the Wilcoxon

matched-pair signed rank test. P≤0.05 were considered significant and those that were significant are shown.

5.4.5 Longitudinal changes in CD8+ T cells across all three study visits

Finally, changes in CD8+ T cell frequencies and activation between baseline, crossover and exit were assessed to determine whether profiles that were changed in response to a particular HC reverted to pre-HC phenotypes after that particular HC was discontinued. As shown in the previous analyses (Figure 5.1A), NuvaRing use appeared to decrease both Tc17 and total CD8+ T cell frequencies in adolescents compared to pre-NuvaRing (0 week) baseline. It was therefore interesting to assess whether these returned to baseline levels after the adolescents swopped to NET-EN or COCPs.

NuvaRing use decreased Tc17 cell frequencies (visible at crossover visit). These Tc17 cell frequencies did not return to baseline frequencies after adolescents changed to NET-EN ($p<0.0001$ compared to baseline; Figure 5.6A), and Tc17 cells at the NET-EN use time point (week 32) were comparable to frequencies at the NuvaRing time point (week 16). Similarly, frequencies of Tc17 cells were not restored in those swopping from NuvaRing to COCPs ($p=0.03$ compared to baseline; Figure 5.6B), and Tc17 cells at the COCPs use time point (week 32) were comparable to frequencies at the NuvaRing time point (week 16). However, it should be noted that only four participants on COCPs at week 32 were available for this comparison.

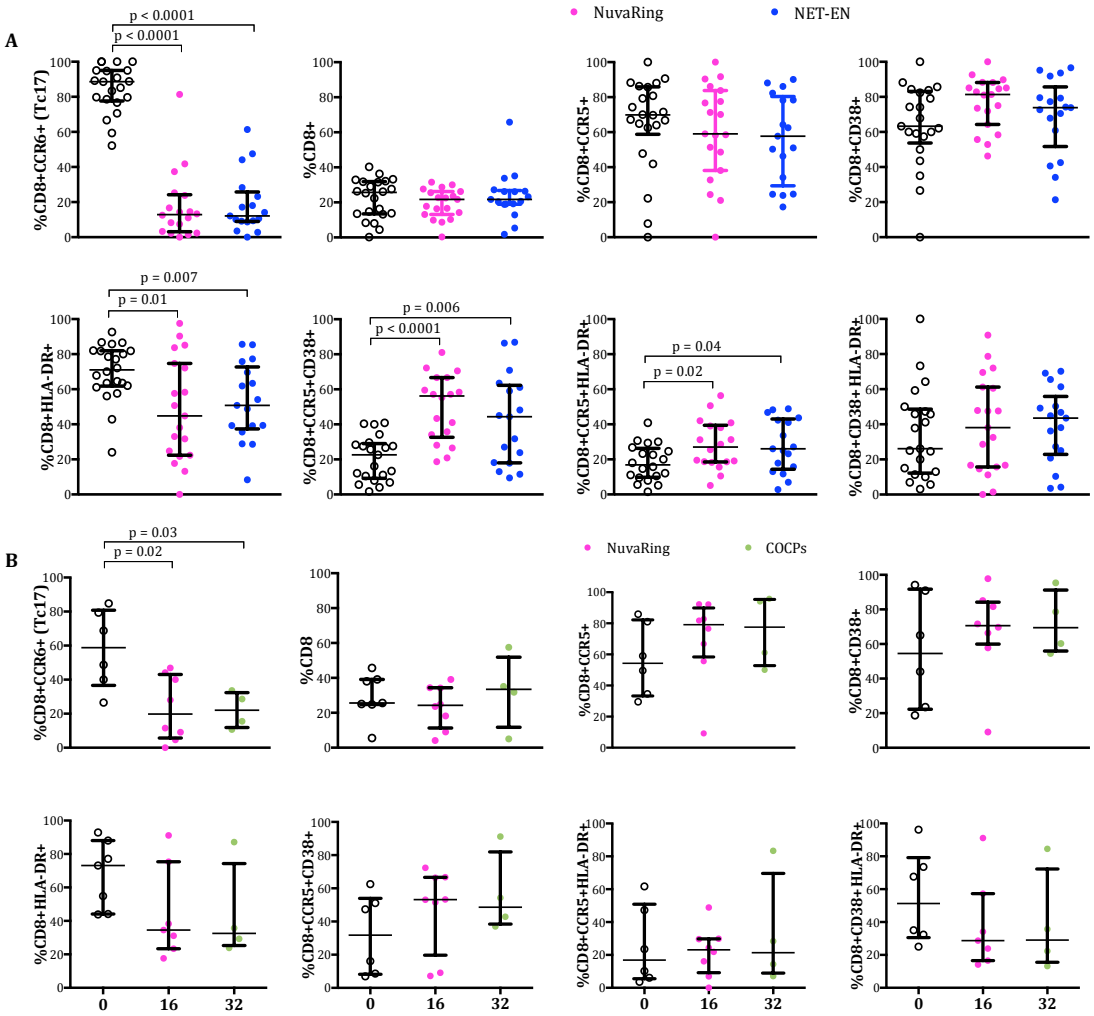

Figure 5.6. Comparison of cervical CD8+ T cells at baseline (0 weeks), crossover (16 weeks) and exit visit (32 weeks) for adolescents initially randomized to NuvaRing (until 16 weeks) and who subsequently crossed over to either NET-EN (panel A; baseline n=22, week 16 n=19, week 32 n=17) or COCPs (panel B; baseline n=7, week 16 n=8, week 32 n=4) until 32 weeks. The baseline time points are shown as clear dots, the NuvaRing time point as pink dots, and either the NET-EN or COCPs time points as blue or green dots, respectively. Tc17 cells, CCR5 and activation markers were analysed within the CD8+ T cell population. The mid line represents the median, and the error bars represent the IQR. Mann-Whitney U tests were used to compare groups. P≤0.05 were considered significant and only significant differences are shown.

Adolescents randomized to NET-EN similarly had higher frequencies of Tc17 cells at baseline that significantly declined at week 16 after NET-EN use, and further decreased after adolescent's swopped to NuvaRing (p<0.0001; Figure 5.7). Expression of

CCR5/CD38, CCR5/HLA-DR and CD38/HLA-DR on CD8+ T cells were also increased at week 16 after NET-EN use, and more so at week 32 post-NuvaRing ($p<0.0001$, $p<0.0001$ and $p=0.04$, respectively). Due to a small sample size of 3, participants randomized to NET-EN and crossed over to COCPs were not compared.

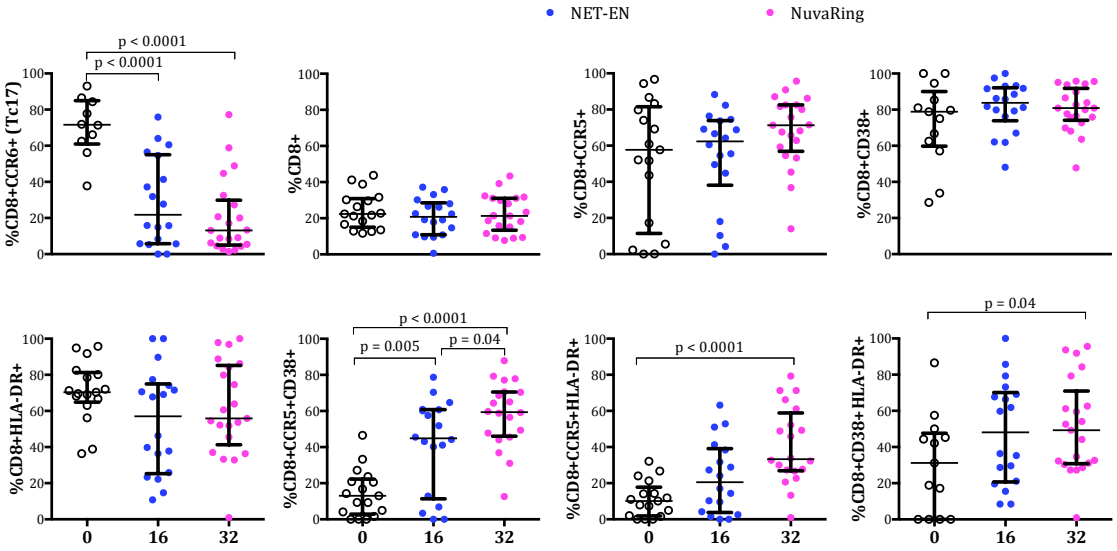

Figure 5.7. Comparison of cervical CD8+ T cells at baseline (0 weeks, n=17), crossover (16 weeks, n=18) and exit visit (32 weeks, n=21) for adolescents initially randomized to NET-EN (until 16 weeks) and who subsequently crossed over to NuvaRing (until 32 weeks). The baseline time points are shown as clear dots, the NET-EN time point as blue dots, and the NuvaRing time point as pink dots. Tc17 cells, CCR5 and activation markers were analysed within the CD8+ T cell population. The mid line represents the median, and the error bars represent the IQR. Mann-Whitney U tests were used to compare groups. P≤0.05 were considered significant and only significant differences are shown.

For adolescent randomized to COCPs, baseline frequencies of Tc17 cells declined post-COCPs, and declined further post-NuvaRing ($p<0.0001$; Figure 5.8). While the expression of HLA-DR on CD8+ T cells decreased at week 16 post-COCPs, this slightly increased post-NuvaRing although not restored to the baseline levels. Expression of CCR5/CD38 and CCR5/HLA-DR on CD8+ T cells tended to be higher at week 16 post-COCPs ($p<0.0001$ for CCR5/CD38) and at week 32 post-NuvaRing ($p=0.003$). For

adolescents randomized onto COCPs and crossed over to NET-EN, only 3 samples were available and therefore no comparisons were performed.

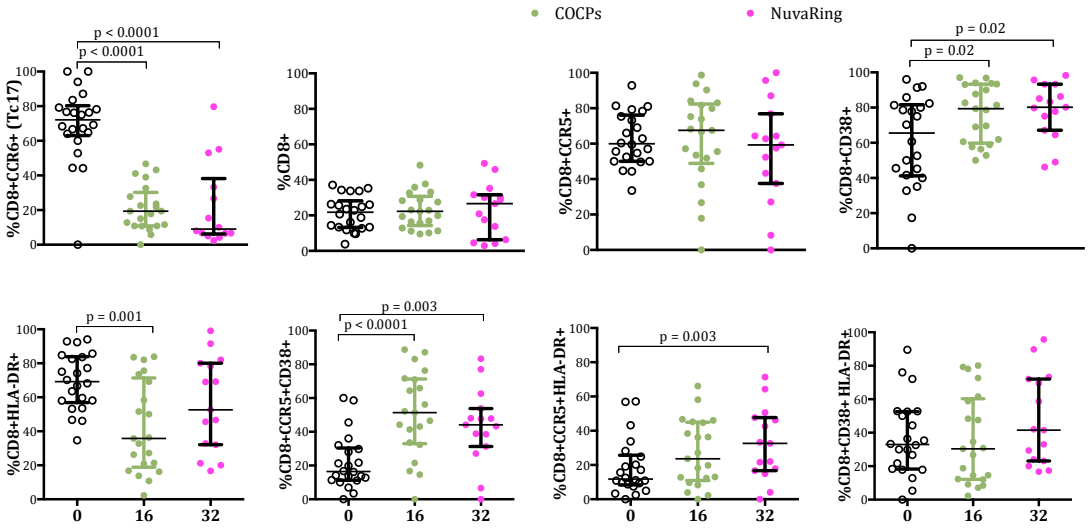

Figure 5.8. Comparison of cervical CD8+ T cells at baseline (0 weeks, n=22), crossover (16 weeks, n=21) and exit visit (32 weeks, n=15) for adolescents initially randomized to COCPs (until 16 weeks) and who subsequently crossed over to NuvaRing (32 weeks). The baseline time points are shown as clear dots, the COCPs time points as green dots, and the NuvaRing time point as pink dots. Tc17 cells, CCR5 and activation markers were analysed within the CD8+ T cell population. The mid line represents the median, and the error bars represent the IQR. Mann-Whitney U tests were used to compare groups. P≤0.05 were considered significant and only significant differences are shown.

5.5 Discussion

In this chapter, the effect of NuvaRing, NET-EN and COCPs on overall cervical CD8+ T cells and Tc17 cell subsets in the reproductive tracts of adolescent females was evaluated. Baseline frequencies and activation of CD8+ T cells were similar across all arms, confirming that randomization was effective in excluding possible confounders. Overall frequencies of Tc17 and CD8+ T cells were reduced in NuvaRing, while only the frequency of Tc17 cells in NET-EN and COCPs users were reduced by 16 weeks of HC use. While the expression of CD38 on CD8+ T cells was only reduced within the NuvaRing arm, all HCs had a significant reduction in HLA-DR and an increased expression of CCR5/CD38 on their cervical CD8+ T cells after 16 weeks. Therefore, intra-individual analysis (where each young woman was her own control) suggested that all three HCs altered cervical CD8+ T cells, although neither the frequency nor the activation of these cells differed between arms after 16 to 32 weeks of HC randomization.

Furthermore, extending the intra-individual analysis of those adolescents who subsequently changed from one HC form to another, they tended to have lower frequencies of Tc17 cells, and higher expression of CCR5, CD38, CCR5/CD38 and CCR5/HLA-DR on their CD8+ T cells when they were using NuvaRing compared to when they swopped to NET-EN, although NET-EN use did not restore expression levels to baseline. Similarly, those that changed from NuvaRing to COCPs also had lower frequencies of Tc17 cells reduced expression of HLA-DR and increased expression of CCR5/CD38 on their CD8+ T cells when they were on NuvaRing, although swopping to COCPs did not appear to restore their CD8+ T cell frequencies to baseline. In participants changing from NET-EN to COCPs, there was a decrease in Tc17 cells, reduced expression of HLA-DR and increased expression of CCR5/CD38 on CD8+ T cells when participants were on COCPs. Although NET-EN and COCPs use tended to have less impact on the frequency of Tc17 cells than NuvaRing, activated CD8+ T cells did not return to baseline levels after adolescent's swopped to NET-EN or COCPs. This suggests that all progestin-based contraceptives increase activated CD8+ T cells. Expression of activation markers during HIV infection has differential significance, such that single expression of HLA-DR on CD8+ T cells is associated with a better survival of these cells, that are polyfunctional and have higher cytolytic and proliferative ability compared to

HLA-DR+CD38+ CD8+ T cells (Saez-Cirion et al., 2007; Hua et al., 2014; Gonzalez et al., 2016). On the contrary, CD38+ CD8+ T cells are prone to apoptosis, and have been associated with residual HIV viral replication in patients on ART (Chun et al., 2004; Miguel Benito et al., 2004). Whether these characteristics apply in HIV-uninfected individuals and at the genital mucosa is yet to be investigated.

A consistent trend that was observed in this cohort was the reduction in Tc17 cells frequencies observed from baseline to week 16 and 32 of using HCs, irrespective of the HC that was being used. Implications of decreased frequencies of Tc17 cells in women are not yet known. Tc17 cells (CD8+ T cells that express CCR6, which is involved in lymphocyte mucosal homing) have been shown not to be cytotoxic upon antigen recognition so they do not kill target cells; rather they produce several inflammatory cytokines including IL-17, IFN-γ, TNF-α, and IL-2, and largely have a similar cytokine profile to Th17 cells (Kondo et al., 2009; Srenathan et al., 2016).

Several studies have shown that the presence of CD8+ T cells in the vagina is required for protection against various viral pathogens such as HIV and HSV, through the release of cytotoxic granules containing perforin and granzymes (Musey et al., 1997; Nelson et al., 2011). Anti-HIV specific CD8+ T cells are crucial in controlling viremia by increasing responses to on-going viral replication (Keoshkerian et al., 2003). MPA have been reported to reduce the capacity of CD8+ T cells to release lytic granules and produce IFN-γ (Cherpes et al., 2008). In support of this, increased endogenous progesterone levels in pregnant women have been associated with hypermethylation of the IFN-γ gene, an underlying mechanism reported to increase susceptibility of pregnant women to intracellular pathogens during pregnancy (Yao et al., 2017). Since the Tc17 cell subset appear to be more effective at cytokine production than cytolytic capacity, their reduction in the cervix might suggest lower production of cytokines by these cells, including IFN-γ which is thought to be indispensable to host defense against pathogens (Tajima et al., 2011). Although Tc17 cells cytokine production was not assessed in this study.

Tc17 cells have been defined for the purposes of this Chapter as CD8+ T cells expressing CCR6, as have several previous studies (Kondo et al., 2007, 2009). However, others have

characterized them functionally by their ability to produce IL-17 directly (Zhuang et al., 2012; Perdomo-Celis et al., 2018). As IL-17 production by these cervical Tc17 cells was not evaluated, it is not known whether the Tc17 cells detected here are producing IL-17. Since an overall decrease of CCR6 on CD4+ and CD8+ T cells was observed in the NuvaRing arm, the downregulation might be part of a regulation process caused by the excessive production of Th17-related cytokines. CCR6 deficient mice have impaired mucosal but not systemic humoral responses to rotavirus infection in the intestinal mucosa, elucidating the importance of this chemokine receptor in lymphocyte homeostasis at mucosal surfaces (Cook et al., 2000). Thus, reduced expression of CCR6 cells on CD8+ T cells may contribute to increased susceptibility to pathogens on the one hand, although decreased expression of CCR6 at the genital mucosa may blunt HIV target cell recruitment and lower the inflammatory state of the mucosa on the other hand. Furthermore, these CCR6+ CD8+ T cells would need to be further characterized and compared to CCR6- CD8+ T cells to differentiate the two subsets. Perdomo-Celis and colleagues (2018) recently reported higher IL-17 production by activated CD8+ T cells (co-expressing CD38 and HLA-DR) from HIV-negative individuals, and that CD8+ T cells from non-viremic HIV positive individuals tended to produce less IL-17 after polyclonal stimulation. Moreover, their study reported activated CD8+ T cells to be the main source of IL-17 among the CD8+ T cell population. Matched analysis in Chapter 4 showed an increase in genital concentrations of IL-17A when adolescents were on NuvaRing compared to when they were on NET-EN or COCPS. Increased activated CD8+ T cells might be one of the many reasons why adolescents had increased Th17-related cytokines when they were on NuvaRing.

A limitation of this study was that blood was not stored for measuring circulating CD8+ T cell frequencies, to determine whether this was a localised effect of HCs at the genital mucosa, or more a general effect that could be detected in the blood. One study found women using COCPs to have elevated blood CD8+ T cells compared to those not using HCs (Auerbach et al., 2002). It was also not possible to determine whether the changes in total CD8+ T cells or Tc17 cells that were observed in adolescent girls in this Chapter related to their young age, since older women were not part of this study.

In summary, HCs use was associated with reduced Tc17 frequencies, irrespective of the type of HC being used, although these changes were more dramatic with NuvaRing use. The importance of these cells in the genital tract should be studied further to understand their implication in HIV risk, and for rational designs of effective HIV vaccines.

Chapter 6

Discussion and Conclusion

6.1 Discussion

Adolescence is a time of significant endocrine and biomedical change in females, associated with increased production of female sex hormones (estrogen, progesterone, Luitenizing and follicle stimulating hormones), development of secondary sexual characteristics, menarche, and sexual debut (Sehested et al., 2000). Adolescent girls, particularly in SSA, face double vulnerabilities of unintended pregnancies and HIV infection. Despite the enormous benefits offered by HCs, public health concerns have been raised because of the heightened risk to HIV seen in women using long-acting injectable HCs (Polis et al., 2016; Hapgood et al., 2018), especially in SSA, where injectable HCs are a popular choice (Kleinschmidt et al., 2007, UNDESA, 2015) and HIV risk is highest (UNAIDS, 2017). While HCs are particularly necessary in adolescence, to prevent unwanted pregnancies, it is also critical to provide advice and HC options that do not increase risk for HIV. In light of these related mandates, the aim of this dissertation was to first characterize the immune environment in the adolescent genital tract focusing on highly HIV susceptible Th17 cells and their related cytokines, and then to investigate whether the use of injectable NET-EN, COCPs or NuvaRing (newly introduced into SA) influenced either the frequency or activation status of these highly susceptible CD4+ T cells. Through Chapters 2 to 5, this dissertation revealed four major findings: (i) that Th17 cells are a major subset in the genital tract of adolescent girls; (ii) that the presence of BV and vaginal dysbiosis did not alter cervical Th17 cell frequencies, however BV appeared to significantly increase Th17-related cytokines, by favouring a pro-inflammatory immune microenvironment; (iii) that the use of HCs tended to decrease cervical Th17 frequencies, however, NuvaRing also increased activation markers (HLA-DR+CD38+) on Th17 cells, and also increased Th17-related cytokines; and (iv) All HCs reduced the frequencies of Tc17 cells, and they all affected expression of activation markers on CD8+ T cells.

6.1.1 Activated Th17 cells are a major subset in the genital tract of adolescent girls

Susceptibility to HIV infection is influenced by many factors, including the availability of target cells at the genital mucosa (Trifonova et al., 2014). Chapter 2 focused on characterizing the highly HIV susceptible Th17 cells and Th17-related cytokines in the lower reproductive tract of adolescent girls. Th17 cells (defined by expression of CCR6 and absence of CCR10; McKinnon et al., 2015) were found to be a major CD4+ T cell subset in cervical cytobrushes, followed by CCR6-CCR10- CD4+ T cells (which are thought to be a mixture of Th1 and Th2 cells; Zhong et al., 2017). Moreover, Th17 cells found in the genital tract were highly activated (expressing CD38 and HLA-DR), and expressed high levels of the CCR5 receptor compared to CCR6-CCR10- CD4+ T cells. Although Th22 cells also express CCR6 like Th17 cells, CCR10 had been reported to be preferentially expressed by these cells, allowing one to differentiate them from Th17 cells (CCR6+CCR10-; Duhen et al., 2009; McKinnon et al., 2015). *In vitro* studies confirmed that majority of CCR6+ CD4+ T cells produced IL-17, although it was not stained for in this study. Moreover, other chemokines receptors such as CCR4 and CXCR3 not measured in this study allows for sub-classification into Th17 and Th1/Th17 subsets, respectively (Liu and Rohowsky-Kochan, 2008; Alvarez et al., 2013; Rodriguez-Garcia et al., 2014).

Different cytokines, including those produced by Th17 cells, are involved in their differentiation and regulation, such as IL-21 and IL-22 (Guglani and Khader, 2010). All Th17-related cytokines in the different classes were detected in the genital tract including IL-17A and IL-17F. Although overall frequencies of cervical Th17 cells (as a proportion of CD4+ T cells) did not correlate with concentrations of Th17-related cytokines measured in genital secretions, highly activated cervical Th17 cells (expressing CD38) correlated positively with several cytokines, including IL-17A, IL-17F, IL-6, IL-10 and sCD40L. Activation of Th17 cells is known to induce them to produce cytokines, which could partly explain the correlation observed in this study. *In vitro* studies have reported endogenous hormones to alter immune functions (Correale et al., 1998; Kanda and Watanabe, 2003; Fahey et al., 2008; Seillet et al., 2011). In this study, genital Th17-related cytokines did not correlate with serum E2 and LH, however,

activated Th17 cells were negatively associated with these hormones. Interestingly, the presence of STIs (including *C. trachomatis, N. gonorhoeae, M. genitalium* and *T. vaginalis*) did not alter Th17 cell frequencies or activation status, although IL-17A concentrations were elevated. In contrast, yeast infections were associated with lower frequencies of cervical Th17 cells.

6.1.2 BV and an altered vaginal microbiota are associated with increased concentrations of Th17-related cytokines

In Chapter 3, the relationship between BV and cervical Th17 cell frequencies and activation status was investigated. The prevalence of BV was high in this cohort of AGYW from Cape Town, with >40% of adolescents classified as having BV by Nugent scoring. Others from our group similarly found high BV rates in adolescent girls from this community, suggesting that this is a persistent problem (Barnabas et al., 2017). In this same community of adolescents from Cape Town, Lennard et al. (2017) described three major VMB clusters in the vagina, with majority of adolescents falling in the non-Lactobacilli-dominant C1 diverse microbiome cluster. Despite previous studies suggesting a relationship between Th17 cells and certain segmented filamentous commensal microbes in the gut from mouse studies (Ivanov et a., 2009), the findings from Chapter 3 show that the presence of BV or the C1 non-*Lactobacillus* dominated VMB did not influence the frequency of Th17 cells in the FRT of adolescents. However, BV as well as intermediate or C1 non-*Lactobacillus*-dominated VMB was associated with elevated concentrations of Th17-related cytokines. This data suggests that vaginal dysbiosis alters Th17-related cytokine production in the adolescent genital tract, with an overall increase in the cytokines measured, including the regulatory cytokines IL-10, IL-4 and IL-25. Although no significant difference was observed in the frequency and activation of Th17 cells according to BV status or microbiome community type, vaginal dysbiosis undoubtedly favoured the production of Th17-related cytokines.

6.1.3 All HCs altered cervical Th17 cells frequencies

The WHO called for more randomization trials to better understand how HCs, particularly the long-acting progestin-only injectable products (like DMPA), increase women's susceptibility to HIV (WHO, 2015). The aim of Chapter 4 was to investigate the impact of the injectable NET-EN, COCPs and NuvaRing (newly licensed in South Africa) on the immune environment of the adolescent FRT, focusing on Th17 cells defined in Chapters 2 and 3. NuvaRing use was accompanied by a marked decrease in frequencies of cervical Th17 cells, although these were highly activated compared to when the same individuals joined the study. Although adolescents on NET-EN and COCPs also tended to have reduced Th17 cell frequencies after 16 weeks of HC use, the changes seen in the NuvaRing arm were more substantial. The highly activated genital tract Th17 cells seen in the NuvaRing are more likely to die by apoptosis than resting T cells (Nkwanyana et al., 2009), which could explain their reduced frequencies. Little is known of how CCR6 is regulated on T cells; however, a stable expression of CCR6 on human T cells is associated with promoter demethylation (Steinfelder et al., 2011). Molecular studies are needed to investigate whether the changes seen in NuvaRing might also be related to DNA methylation. In summary, the use of NuvaRing promoted highly activated Th17 cells, although the frequencies were reduced. These findings are summarized in Figure 6.1 below.

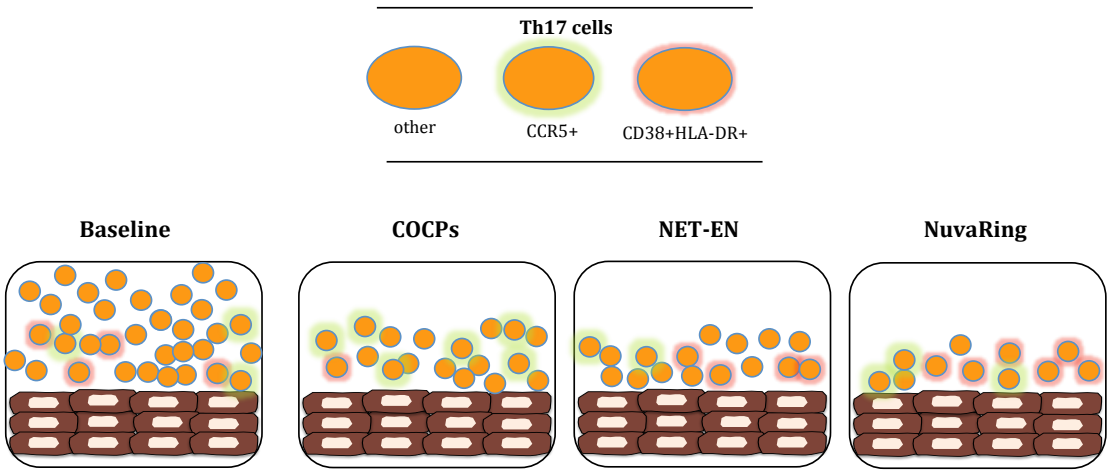

Figure 6.1. Summary of cervical Th17 cells changes observed in the genital tract of adolescents using COCPs, NET-EN and NuvaRing compared to baseline. There was an overall trend in the reduction of Th17 cell frequencies, significantly so for NET-EN and

NuvaRing, although these cells were highly activated compared to baseline. Expression of CCR5 tended to be elevated in participants on COCPs compared to baseline, but not across HC arms.

6.1.4 Only NuvaRing altered Th17-related cytokines

Th17-related cytokines were upregulated in the NuvaRing arm compared to NET-EN and COCPs arms. Pro-inflammatory cytokines including IL-17A, IL-β, IL-6 and TNF-α were among the elevated Th17-related cytokines in adolescents using NuvaRing (Figure 6.2). These cytokines are involved in activation, differentiation and recruitment of immune cells including T cells, neutrophils, DCs and macrophages to the genital tract (Dubin and Kolls, 2008; Arnold et al., 2016; Revu et al., 2018). Other important cytokines and chemokines involved in the Th17 pathway such as TGF-β, and the CCR6 ligand MIP-3a were not measured, and this would have provided more insight on the effect of NuvaRing on these cytokines. Interesting, IL-1β that positively regulate MIP-1a was elevated in adolescents using NuvaRing. Therefore, elevated cytokines in adolescents on NuvaRing might increase adolescent's susceptibility to HIV acquisition.

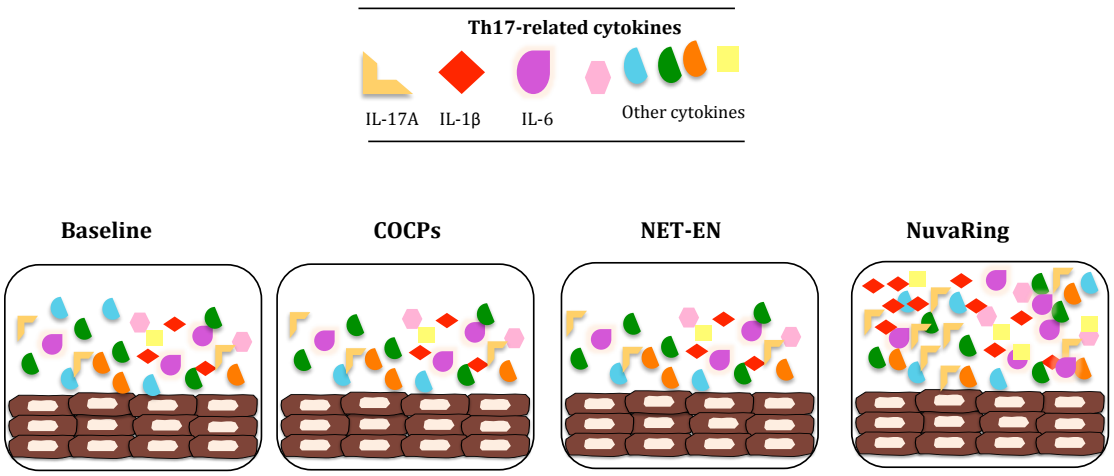

Figure 6.2. Summary of changes seen in the concentration of Th17-related cytokines in the genital tract of adolescent girls. Cytokines including IL-17A, IL-1β and IL-6 were elevated after NuvaRing use compared to NET-EN and COCPs.

6.1.5 CD8+ T cells were altered by the use of HCs

In Chapter 5, the effect of NuvaRing, NET-EN and COCPs on vaginal CD8+ T cells was investigated. The frequency of Tc17 cells, a subset of CD8+ T cells known to promote inflammation was also included in the analysis. Notably in humans, the clinical relevance of Tc17 cells is a new field, and not much is known about these cells. In mice, Tc17 cells displayed antitumor immunity through the production of IL-17, IFN- γ and TNF-α (Garcia-Hernandez et al., 2010). Although no significant differences in the overall frequency of CD8+ T cells were observed across arms at the crossover and exit visits, a significant decreased in the frequency of Tc17 cells was observed within each HC arm (Figure 6.3). Moreover, expression of activation markers on CD8+ T cells were generally affected by the use of HCs, but NuvaRing appeared to be associated with more dramatic alterations compared to either NET-EN or COCPs.

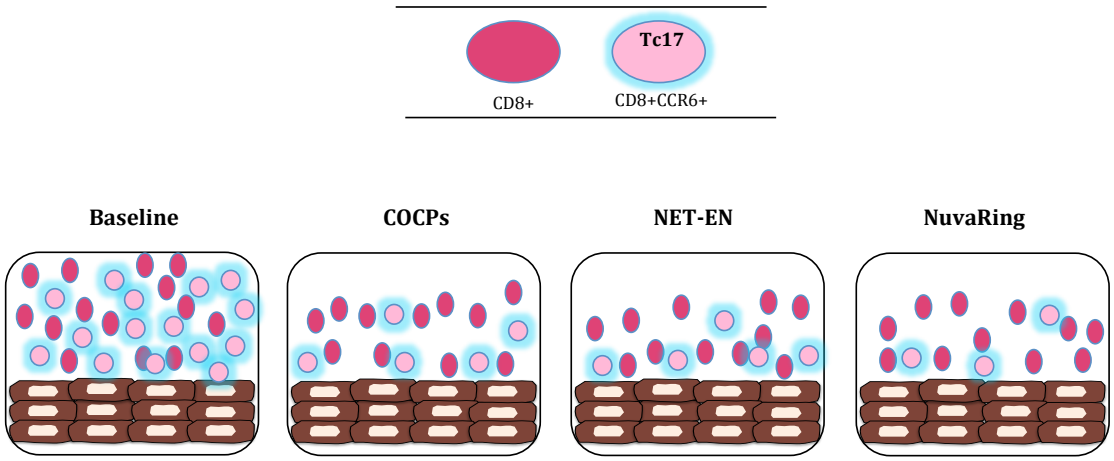

Figure 6.3. Summary of CD8+ T cells changes in the genital tract of adolescents on COCPs, NET-EN and NuvaRing compared to baseline. There was an overall trend in the reduction of Tc17 cell frequencies, although the overall frequencies of CD8+ T cells were the same compared to baseline.

6.2 Limitations and future directions

This study defined Th17 cells by chemokine receptor expression (CCR6+CCR10-) as opposed to cytokine production. It could be that CCR6+CCR10- Th17 cells measured here are not all able to produce IL-17, therefore overestimating these cells. However, identifying Th17 cells using cytokine production represent technical challenges because of the low cell yield and fragility of cervical cells, and the plasticity of Th17 cells (Boily-Larouche et al., 2017). Although this study aimed to recruit contraceptive naïve adolescents, most of the study participants were on NET-EN at baseline, and this may have biased the study. In addition, most of these young adolescents had asymptomatic BV and STIs. However, given the randomized nature of this study, there was equal distribution between arms on previously used methods of contraception, BV and STIs. The vaginal microbiome forms part of another PhD study, and was therefore not presented in Chapter 4 and 5, which could have explained some of the differences seen between contraceptive arms. Self-reported behavioural data was limited, as some adolescents anecdotally reported to have tempered with their rings, and this might have contributed to the high inflammation observed with the use of NuvaRing.

The main disadvantage of a crossover study is the potential for carry over between the HC interventions. Since this was a study in young girls seeking to prevent pregnancy, there was no washout period as the risk for unintended pregnancies during wash out was high. However, intraindividual analysis looking within-person provided participants to be their own control minimizing natural variation, and allowing evaluation of longitudinal changes from baseline, which further confirmed some of the alterations seen with the use of NuvaRing compared to NET-EN and COCPs. Although these sexually active adolescent girls were past menarche and not in their early tanner stages, having older women as a reference would have helped explain some immune changes observed in this study. Other limitations included a small sample size and adolescents being only on two out of the three HCs. Future studies should aim to recruit contraceptive naïve women, and have each participant on all the HC options. Recruiting HC naïve adolescent girls would help to link changes seen to the use of HCs.

6.3 Conclusion

In summary, the presented data suggests that HCs appear to alter cervical Th17 cells and CD8+ T cells, while only NuvaRing had elevated Th17-related cytokines. This adds to the body of evidence on the biological effect of progestins on vaginal immune cells, more importantly in adolescent girls who are one of the HIV key populations. The dramatic changes of Th17 and Tc17 cells in addition to Th17-related cytokines seen with the use of NuvaRing warrants further investigation of potential mechanisms. Using a placebo copolymer evatane vaginal ring and another silicon vaginal ring may help delineate how the hormonal and mechanical components of the CCVR impact the vaginal immune microenvironment.

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Appendix 1

Table A1. Percentage of Th17-related cytokines detected in the vaginal secretions

Cytokine	Percentage detected	Concentration (pg/ml) Median (IQR)
IL-1β	98	10.81 (1.23 – 73.99)
IL-4	37	0.05 (0.05 – 0.99)
IL-6	98	0.98 (0.32 – 5.17)
IL-10	42	0.01 (0.01 – 1.08)
IL-17A	98	1.33 (0.87 – 3.81)
IL-17F	56	3.46 (0.34 – 8.89)
IL-21	78	4.78 (0.02 – 10.85)
IL-22	97	6.44 (4.44 – 10.93)
IL-23	56	0.60 (0.12 – 9.16)
IL-25	63	0.16 (0.005 – 0.46)
IL-31	94	31.83 (16.84 – 57.44)
IL-33	80	5.01 (1.22 – 11.76)
IFN-γ	92	1.93 (1.01 – 3.50)
sCD40L	59	0.87 (0.07 – 6.54)
TNF-α	87	0.64 (0.11 – 3.26)

Table A2. Concentrations of Th17-related cytokines according to randomization arm at crossover

	NuvaRing (n=34)	**NET-EN (n=34)**	**COCP (n=37)**	Kruskal Wallis		**NuvaRing vs NET-EN**		**NuvaRing vs COCP**		**NET-EN vs COCP**	
		Median and IQR				*P-value*					
Produced by Th17 cells					Adj.		Adj.		Adj.		Adj.
IL-17A	3.24 (0.91 - 7.69)	1.05 (0.62 - 2.50)	1.48 (1.01 - 2.24)	**0.029**	0.185	**0.009**	0.135	0.110	0.196	0.225	0.970
IL-17F	6.83 (0.34 - 23.86)	0.34 (0.34 - 10.80)	0.80 (0.34 - 10.14)	0.090	0.192	**0.033**	0.142	0.118	0.196	0.658	0.970
IL-21	12.12 (3.75 - 23.59)	4.51 (0.01 - 16.10)	7.22 (2.35 - 11.43)	0.060	0.185	**0.039**	0.142	**0.049**	0.147	0.519	0.970
IL-22	9.22 (4.57 - 21.83)	7.00 (4.05 -10.81)	6.43 (4.32 - 11.73)	0.363	0.161	0.173	0.196	0.285	0.328	0.852	0.970
Differentiation of Th17 cells											
IL-6	3.43 (0.69 - 28.10)	1.01 (0.17 - 13.56)	1.01 (0.36 - 6.51)	0.074	0.185	0.076	0.483	**0.030**	0.147	0.943	0.970
IL-1β	61.12 (2.70 - 152.0)	10.68 (0.25 - 76.34)	11.32 (0.36 - 56.51)	0.067	0.185	0.061	0.142	**0.032**	0.147	0.970	0.970
IL-23	4.56 (0.12 - 13.27)	3.99 (0.12 - 11.43)	0.59 (0.12-9.54)	0.408	0.408	0.387	0.142	0.184	0.243	0.735	0.970
IL-33	8.51 (3.43 - 22.19)	3.50 (0.23 - 10.13)	3.65 (1.08 - 9.84)	0.066	0.185	**0.034**	0.142	0.063	0.156	0.605	0.970
TNF-α	1.21 (0.29 - 6.69)	1.38 (0.03 - 3.62)	0.32 (0.13 - 2.41)	0.177	0.279	0.344	0.396	**0.045**	0.147	0.583	0.970
Regulators of Th17 cells											
IL-4	0.52 (0.05 - 5.42)	0.05 (0.05 - 1.38)	0.05 (0.05 - 1.51)	0.170	0.279	0.074	0.142	0.168	0.243	0.777	0.970
IL-10	0.31 (0.01 - 1.81)	0.01 (0.01 - 1.17)	0.01 (0.01 - 0.56)	0.186	0.279	0.483	0.483	0.073	0.156	0.248	0.970
IL-25	0.37 (0.01 - 1.51)	0.01 (0.01 - 0.62)	0.01 (0.01 - 0.45)	0.067	0.185	0.059	0.142	**0.037**	0.147	0.817	0.970
IL-31	54.09 (19.30 - 108.10)	26.63 (17.14 - 61.97)	34.99 (21.03 - 65.77)	0.272	0.358	0.131	0.196	0.360	0.370	0.373	0.970
IFN-γ	2.95 (1.21 - 8.73)	2.25 (0.51 - 3.99)	1.81 (0.84 - 4.03)	0.347	0.388	0.226	0.282	0.195	0.243	0.947	0.970
sCD40L	6.70 (0.07-11.93)	0.07 (0.07 - 8.90)	0.32 (0.13 - 2.41)	0.286	0.358	0.121	0.196	0.370	0.370	0.447	0.970

Intention to treat analysis (ITT). P-value adjusted (Adj.) using the Benjamini-Hochberg.