



INVESTIGATING THE EFFECT OF SEX HORMONES ON THE IMMUNE RESPONSE TO TB AND HIV

by

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Submitted in fulfillment of the requirements for the degree of Master of Medical Science in the School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu Natal.

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DECLARATION

I, Amanda N. Mabhula, declare as follows:

1. That the work described in this thesis has not been submitted to UKZN or other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party (with the exception of the collaborative work conducted on the FRESH cohort. Measurements of DMPA in the FRESH cohort were done by myself, Amanda Mabhula, and analysis of effects of DMPA in association with risk of HIV in the same cohort was done by our collaborators stated in the attached manuscript author section. This particular section of the work was also submitted by Melis Nuray Anahtar, for the degree of Doctor of Philosophy in Immunology at Harvard University, submitted in 2015).

2. That my contribution to the project was as follows:

Design and optimization of the experiments for measurement of sex hormones, in the FRESH cohort, CAPRISA CAP004 cohort and Cryptococcal Study cohort, as well as flow cytometry assays for measuring immune responses in the Cryptococcal cohort. I, also, conducted all the laboratory work and data analysis, and wrote this thesis under supervision of Dr. Al Leslie.

3. That the contributions of others to the project were as follows:

Dr. Paul Drain, provided the Cryptococcal Study cohort plasma and PBMC samples. Dr. Lyle McKinnon, provided plasma samples from the CAPRISA CAP004 study, and all matched cervicovaginal lavage proteomic work and data analysis was done by our collaborators, Dr. Lyle McKinnon, Adam Burgener, Kenzie Birse, Laura Dunphy, and Kelly Arnold. Refilwe Molatlhegi and Liane Arcinas, helped with processing the CAPRISA CAP004 cohort plasma samples for DMPA measurements. Professor Thumbi Ndung'u and Doug Kwon provided the samples for DMPA measurement in the FRESH study and all contributors to this study are mentioned in the manuscript.



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PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

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The effect of progestin-only injectable contraceptives on HIV acquisition and the immune environment of the female genital tract: a prospective cohort study

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LIST OF ABBREVIATIONS

ART	antiretroviral therapy
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
ANOVA	analysis of variance
BCG	bacillus Calmette-Guerin
CD	cluster of differentiation
CVL	cervicovaginal lavage
CMV	cytomegalovirus
DC	dendritic cell
DTH	delayed-type hypersensitivity
DMPA	depot medroxyprogesterone acetate
DHEA	dehydroepiandrosterone
DMABC	dimethylaminobenzoyl chloride
ELISA	enzyme linked immunosorbent assay
ESI	electrospray ionization
E2	17- β estradiol
EDTA	ethylenediaminetetraacetic acid
FRT	female reproductive tract
HIV	human immunodeficiency virus
HBCs	high burden countries
HDT	host directed therapy
HAART	highly active antiretroviral therapy
HPLC	high performance liquid chromatography

IL	interleukin
iNOS	inducible nitric oxide synthase
IFN- γ	interferon gamma
IRIS	immune reconstitution inflammatory syndrome
IS	internal standard
IPC	injectable progestin-based contraceptive
ICS	intracellular cytokine staining
LTBI	latent <i>Mycobacterium tuberculosis</i> infection
LDLN	lung draining lymph node
LPS	lipopolysaccharide
LXA4	lipoxin A4
LC-MS	liquid chromatography mass spectrometry
LC-MS/MS	liquid chromatography tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
LH	luteinizing hormone
m/z	mass-to-charge
<i>M.tb</i>	<i>Mycobacterium tuberculosis</i>
<i>M. avium</i>	<i>Mycobacterium avium</i>
<i>M. marinum</i>	<i>Mycobacterium marinum</i>
MDR-TB	multi-drug resistant tuberculosis
MRM	multiple reaction monitoring
MSMD	Mendelian susceptibility to mycobacterial disease
ng/ml	nanogram per milliliter

NK	natural killer
NO	nitric oxide
NF- κ B	nuclear factor kappa B
Net-En	norethisterone enanthate or Nuristerate
OC	oral contraceptive
pg/ml	picogram per milliliter
PRR	pathogen recognition receptor
PAMPs	pathogen associated molecular patterns
PGE2	prostaglandin E2
PAR	peak area ratio
PPD	purified protein derivative
PBMCs	peripheral blood mononuclear cells
QC	quality control
QqQ	triple quadrupole
SEB	staphylococcal enterotoxin B
S/N	signal-to-noise
SRM	selected reaction monitoring
SHIV	simian-human immunodeficiency virus
SIV	simian immunodeficiency virus
SNPs	single nucleotide polymorphisms
SOCS1	suppressor of cytokine signaling 1
TB	tuberculosis
TLR	toll-like receptor
TNF α	tumor necrosis factor alpha

Th-1

Type-1 T helper

WHO

World Health Organization

XDR-TB

extensive drug resistant tuberculosis

ABSTRACT

Background:

Global incidence rates for tuberculosis (TB) indicate a gender-bias to the disease, with almost twice as many men actively infected than women (male/female ratio of 1.9). Sex hormones are known to regulate immune function and their role in tuberculosis has been suggested experimentally in animal models. However the role of sex hormones in human TB infection and whether there is any synergistic effect in HIV and TB co-infection is unknown. In addition, there is data to suggest that manipulation of sex hormones with progestin-based injectable contraceptives, particularly DMPA, increases susceptibility to both HIV and TB. Quantitation of sex hormones has relied on antibody based immunoassays, such as ELISA, which suffer both from a lack of specificity and sensitivity.

Aims:

Consequently, the purpose of this study was to develop a method for quantitation of sex hormones and their contraceptive analogues utilizing a targeted LC-MS/MS approach that has been demonstrated to increase the accuracy of hormone quantitation and to determine the effect of sex hormones and hormonal contraceptives on the immune response to TB and HIV.

Method:

Sex hormone levels were measured in plasma samples from three separate cohorts, using MRM run in positive ESI mode, on a ABSciEx Q-TRAP 5500 mass spectrometer. The method was validated and DMPA levels were measured in the FRESH cohort (n= 62) and CAPRISA CAP004 study (n= 38) to determine effects on DMPA on increased risk in HIV acquisition. Testosterone, progesterone and DMPA levels were measured in the HIV chronic patients from the Cryptococcal cohort (n = 271) and sex hormone associated changes in phenotypic expression and immunological responses using intracellular cytokine staining were determined by flow cytometry analysis.

Results:

We validated our assays and were able to identify and quantitate levels of DMPA in two blinded studies. In the FRESH cohort, we were able to correctly identify and quantitate DMPA levels in Depo-Provera users and injectable progestin-based (IPC) contraceptive use was associated with high risk of HIV acquisition ($p = 0.0142$). IPC users were found to have significant increase in CCR5+CD4⁺ T cells in the cervix as well as increased CCR5 expression. Preliminary data in the CAP004 study, showed differential expression of mucosal proteins in the cervicovaginal lavage associated with DMPA use.

In the Cryptococcal study, we found, as expected, significant differences in testosterone and progesterone levels between male and female patients, $p < 0.0001$ and $p = 0.0001$ respectively. Given that only 3 female patients reported to be using the contraceptive DMPA, we identified 42 of the total number of 172 females to have significant levels of DMPA (greater than LOQ = 0.064ng/ml) and these females had significantly lower progesterone levels than females not using DMPA ($p < 0.0001$). This large under-reporting of contraceptive use indicates the value in direct measurement as opposed to self-reporting. As expected negative correlation was observed between progesterone and DMPA levels ($p = 0.0016$, $r = -0.2445$). However, individual response profiles are highly variable and decay rates of DMPA in longitudinal samples vary greatly between individuals. We hypothesize this will impact the immunomodulatory effect of DMPA, again suggesting the need for direct measurement.

In this small sample we find no significant differences in activation and exhaustion of CD4 and CD8 T cells (determined using HLA-DR, CD38 and PD1 expression), as well as T regulatory cells (FoxP3 expression) when comparing female patients with high progesterone, low progesterone, and injectable contraceptive DMPA users, as well as males with high testosterone and low testosterone levels. However, females with high progesterone levels generally had higher CD38 expression, though non-significant. Also, we observe no significant differences in cytokine expression (TNF α , IFN γ , and IL-2) as well as markers, CD107a and Mip-1 β , upon stimulation with SEB, PPD, pp65 CMV and HIV peptides.

Conclusion:

We successfully optimized and validated a method for quantitation of sex hormones using LC-MS/MS and were able to detect and quantify levels of testosterone, progesterone and DMPA. This method has the potential in clinical studies, to eliminate the need to rely on self-reported information, as exogenous hormones or contraceptive analogues can be detected with high sensitivity and specificity. Changes in the female genital tract which may be associated with increased risk of HIV were found in injectable progestin-based contraceptive users, particularly DMPA. However, no significant immunological effects of hormone levels on immune response and phenotype expression were found in blood.

1. TUBERCULOSIS INTRODUCTION

1.1 TB EPIDEMIOLOGY

Tuberculosis (TB) is ranked alongside human immunodeficiency virus (HIV), as a leading cause of death due to an infectious disease. Despite all the various strategies being implemented to reduce and eliminate this serious threat to global human health, the World Health Organizations' Report of 2015 estimates that in 2014, 9.6 million people developed TB, and that approximately 1.5 million died from the disease (Figure 1.1) [1].

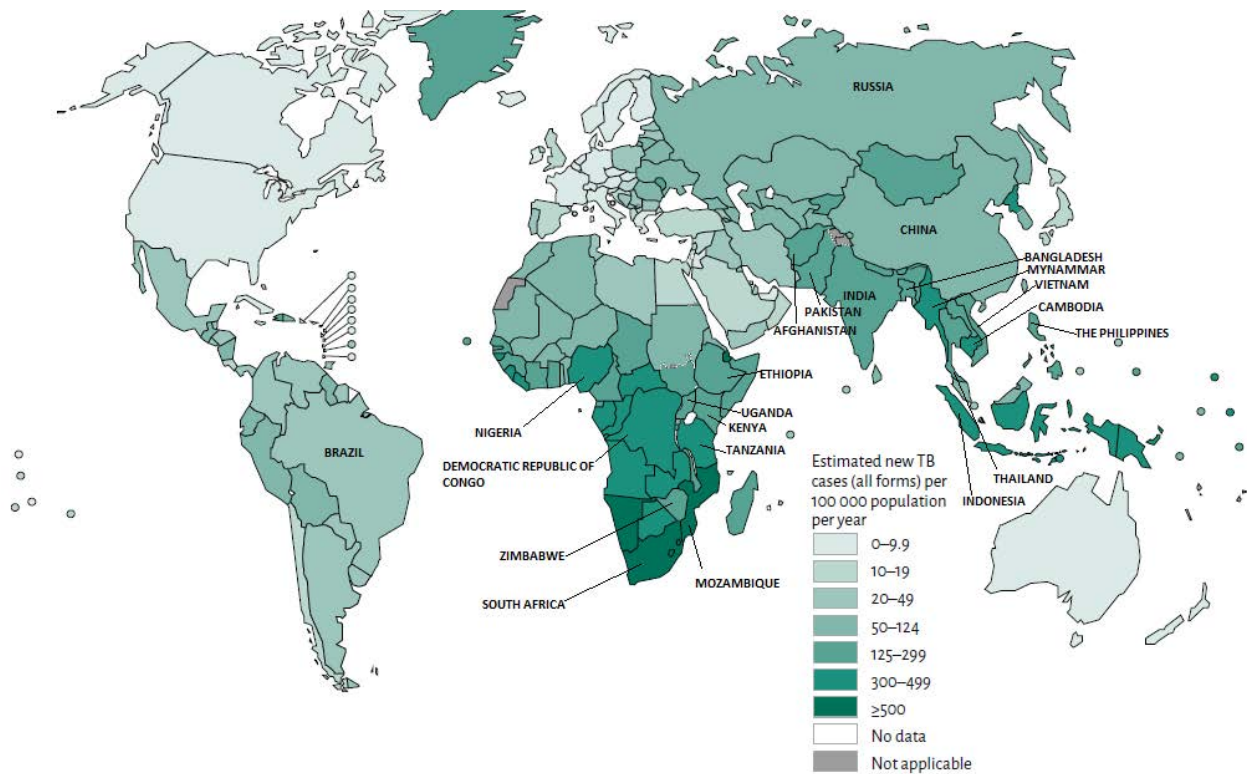


Figure 1.1. Estimated TB incidence rates per country in 2014. The map shows incident cases of TB per 100 000 population, with emphasis on the labeled countries as the ‘high burden countries’ (HBCs). Adapted from *Global Tuberculosis Report 2015*, WHO, 2015.

Of the estimated cases of people who developed TB, a quarter of the cases occur in the African region, which has the highest rates of incidence and deaths relative to population [1]. TB disease control is complicated by co-infection with HIV, which exacerbates disease incidence and severity. The sub-Saharan African region in particular, has highest incident rates due, in part, to high prevalence of HIV. HIV infected patients are 26 times more likely to present with TB and approximately 1.15 (12%) million cases of active TB in 2014 were HIV positive [1].

Although millions of incident cases of TB are reported annually, a large proportion of infected people effectively contain the bacteria and are said to have “latent” *M. tuberculosis* infection (LTBI) [2]. These people remain asymptomatic and do not show any clinical evidence of the disease. Approximately a third of the world population is estimated to have LTBI, and, in the absence of co-morbidities such as HIV, this group carries a 5-10% lifetime risk of reactivating the infection to active TB. Reactivation rate in the presence of HIV co-infection is increased by 20 to 37-fold, depending on severity of the epidemic [3]. Therefore, this means that nearly 2 billion LTBI individuals have a huge potential to develop active disease [2] (Figure 1.2).

1.2 TB PATHOLOGY

Tuberculosis, caused by the tubercle bacillus *Mycobacterium tuberculosis*, is predominantly an infection of the lung, although *M. tuberculosis* can disseminate to other organs including the lymph nodes, bone and meninges causing extra pulmonary disease [4]. Transmission of the bacillus is airborne, spread by individuals with active pulmonary tuberculosis mainly through inhalation of aerosolized bacilli.

The ability to develop an infection depends on susceptibility of host, environmental conditions, number of bacilli and size of droplets, degree of ventilation, frequency and duration of exposure, amongst other factors [5]. Only a small fraction, about five percent of the primary infections develop into clinically apparent disease (Figure 1.2).

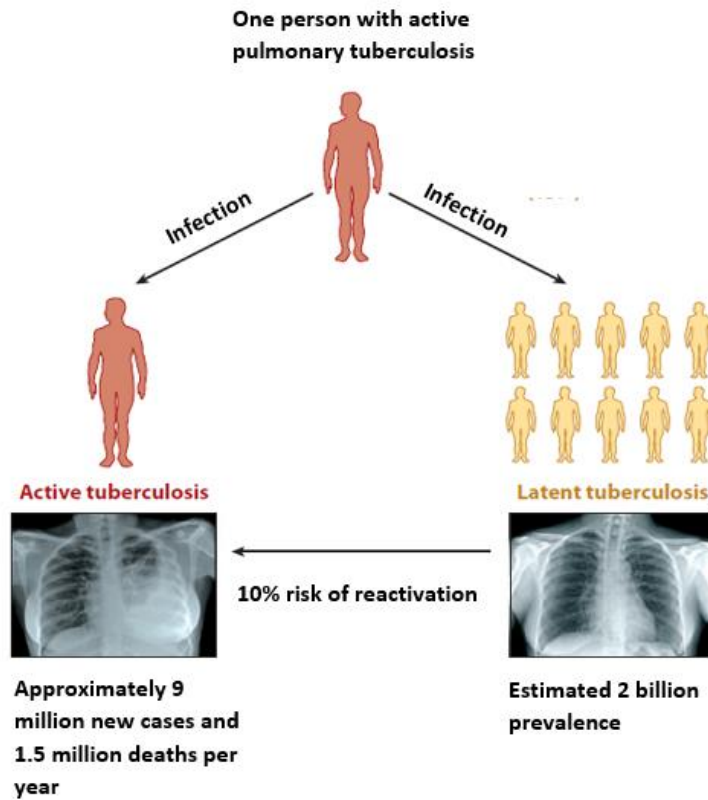


Figure 1.2. The epidemiology of *M. tuberculosis* infection—active and latent TB. Adapted from O’Garra et al. 2013 [4].

Host factors that lead to containment of infection (LTBI) or progression to active disease are not fully understood. However it is thought that a balance between the pro-inflammatory and anti-inflammatory immune responses is crucial to controlling infection.

Early after inhalation of *M.tuberculosis*, bacilli are phagocytosed by alveolar macrophages and dendritic cells (DCs) in the alveolar space. This process is mediated by several host cell receptors expressed on phagocytic cells, which include pathogen recognition receptors (PRRs) such as macrophage mannose receptors and some members of the toll-like receptor (TLR) family. These PRRs recognize pathogen-associated molecular patterns (PAMPs) present on bacterial surface [2, 5]. Macrophages then migrate to the lung parenchyma, where both innate and adaptive immune responses are initiated. Intracellular *M.tuberculosis* induces production of high levels of pro-inflammatory cytokines and chemokines such as TNF- α , IL-12, IL-6 and IL-1 β by macrophages [2].

This process drives the recruitment of uninfected macrophages and other cells such as neutrophils, monocytes and DCs from circulation into the lung where they begin to phagocytose the bacteria. Bacterial growth is arrested in infected macrophages due to delayed-type hypersensitivity (DTH) and the recruited innate immune cells begin formation of an early granuloma [4, 6]. Infected DCs in the lung migrate into the lung-draining lymph nodes (LDLN), where they present mycobacterial antigens to lymphocytes.

This process primes the adaptive immune system for a systemic response, and does not occur until about 12-21 days post-infection in TB, significantly delayed compared with other infections [7] (Figure 1.3). Mice aerosol infection studies suggest that the delayed priming of effector CD4+ and CD8+ T cells may be due to the fact that *M.tb* antigens must be transported to the LDLN for antigen presentation to occur. This delay could allow dissemination of bacteria before *M.tb*-specific T cells are activated [8, 9].

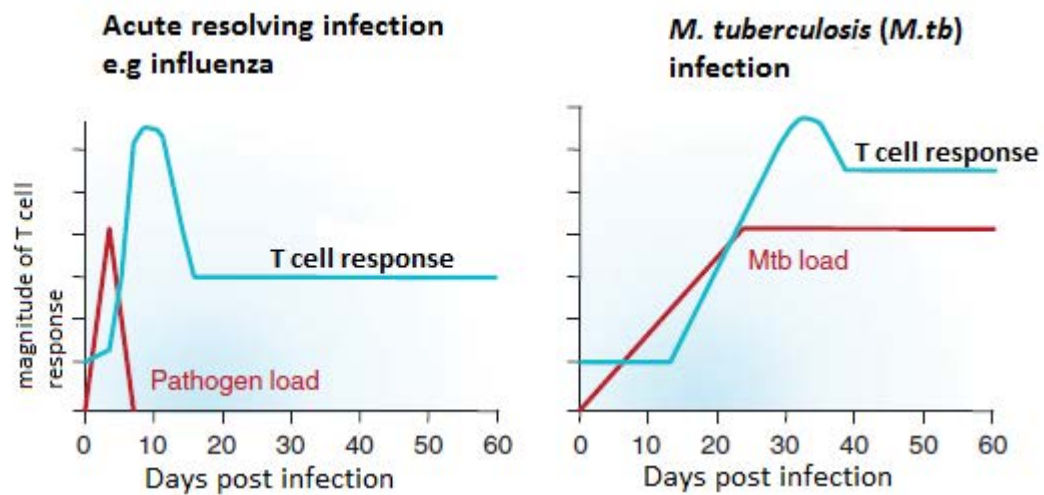


Figure 1.3. Adaptive immune response in *Mycobacterium tuberculosis* infection is significantly delayed compared with other infections. In acute, resolving infections, initiation of T-cell responses occurs 3 – 5 days after initial infection whereas aerosol infection with *M. tuberculosis*, only 9 – 11 days after infection and peak more than a few weeks after infection, without elimination of *M.tb* bacilli. Adapted from *Urdahl et al, 2011* [7].

The most common outcome of TB infection is the formation of granuloma – a hallmark pathology structure of TB. Onset of cell-mediated immunity results in a well-developed granuloma or stabilized caseous lesion, which is mainly composed of central, infected macrophages, surrounded by epithelial macrophages, dendritic cells, neutrophils, foam cells, giant Langhan type cells, peripheral recruited T and B lymphocytes and sometimes fibroblasts [10].

The granuloma functions as both a niche in which the bacillus can grow or persist and an immunologic environment in which immune host cells interact to control and prevent dissemination of infection [2]. In LTBI cases, the full extent of what latency means is not yet understood; the microbiologic state of the bacilli is not clear, since they may be in a truly latent state or in a state of persistent anaerobic metabolic non latent state. Alternatively, they may be in a state of intermittent dormancy and replication [11]. Most hosts usually have one or few granulomas and are capable of limiting the growth and spread of the bacteria. Failure to control the infection, as is the case with active TB, is associated with development of caseating granulomas leading to spread of *M.tuberculosis* throughout the lung or dissemination to other organs and initiating new granuloma formation [2].

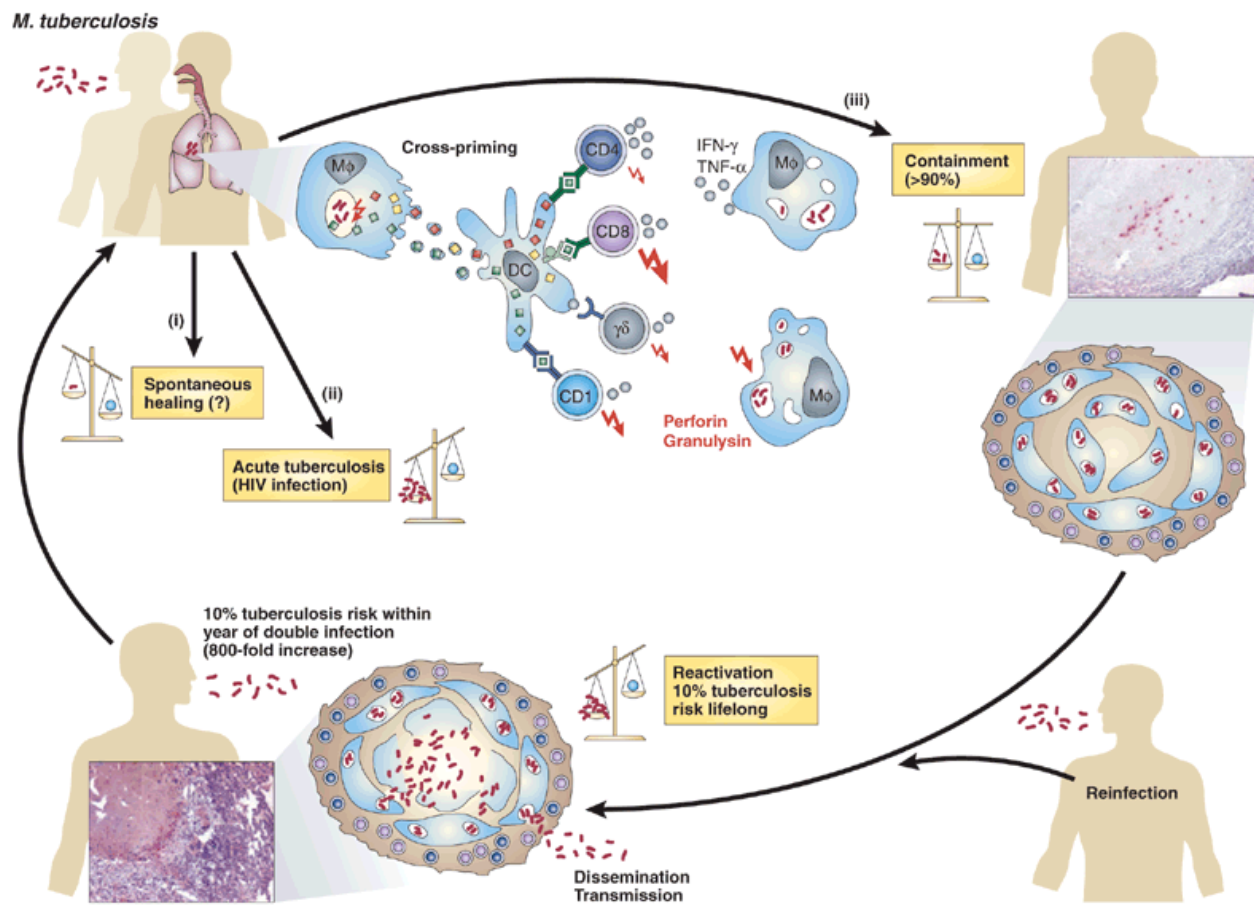


Figure 1.4. TB pathogenesis. Balance in pathogen-host interactions during *M.tb* infection can lead to (i) infection elimination, (ii) failure of host to control infection, leading to active TB, and (iii) containment of *M.tb* in LTBI individuals, with 5-10% risk of reactivation to active TB. Adapted from Kauffman & McMichael, 2005 [12].

For infection of a new host to occur, *M.tb* must exit the granuloma into the airway where they can be aerosolized into cough droplets. Studies suggest that cavity formation in the granuloma results from necrotic death of infected macrophages, leading to extracellular replication of the bacteria. Granuloma dynamics that favour necrosis are not fully understood but it is thought that *M.tb* manipulates host response factors, such as TNF, to exit granuloma for transmission [13]. Most important in cavity formation, is liquefaction of the solid caseum which then provides a permissive environment in which *M.tb* replicates. [14].The semi-liquid necrotic material spreads across the bronchi, where it can be easily coughed up, leading to transmission, or infect new macrophages to create more lesions.

1.3 TB VACCINES, TREATMENT AND OUTCOMES

1.3.1 TB vaccines

For nearly a century, an attenuated *Mycobacterium bovis*, bacillus Calmette-Guerin (BCG) vaccine has been used as a preventive measure for TB. However, there have been questions raised on the effectiveness of the vaccine due to variations in the protective effect of BCG in different populations. Though it provides some protection in infants, particularly from miliary TB, the efficacy, however, diminishes over a period of 10-15 years and does not provide protection against pulmonary TB in adults [15, 16].

A systematic review and meta-analysis study by Abubakar et al, concluded that latitude, age at which vaccine is given and the pre-vaccination tuberculin sensitivity status were some of the factors considered to contribute to the observed variation in BCG induced protection [15]. In fact, in one mouse study, sensitization with mycobacterial isolates from soil and sputum impaired the development of protective immunity to TB upon subsequent vaccination with BCG, suggesting that preexisting immunity to environmental mycobacteria could prevent multiplication of vaccine BCG and probably contributes to the limited induction of protective responses in some regions [17]. Given the very high mortality and morbidity rates in countries where most infants are vaccinated with BCG, an alternate vaccine is needed, that will widely protect against infection, and early progression as well as late progression to disease.

1.3.2 TB drug therapy

The current standard TB treatment for drug susceptible TB is a 6-month regimen that comprises of an initial intensive phase therapy of four drugs namely isoniazid, rifampicin, ethambutol and pyrazinamide given for 2 months. This is followed by a continuation phase of isoniazid and rifampicin, usually lasting 4-6 months [1]. There may be variations to this treatment depending on diagnostic category of each patient. For example, new smear positive patients, previously treated smear positive or relapse patients, treatment interrupted or treatment failure patients, may be treated differently [18].

WHO reports treatment success rate of approximately 85% or more in newly reported cases of TB. However treatment for multi-drug resistant TB (MDR-TB), defined by resistance to the two first-line TB drugs, isoniazid and rifampicin, has lower success rates and therapy lasts longer, approximately 20 months (1). This necessitates the need for effective interventions that are able to either prevent infections (e.g. effective vaccines) or shorten treatment as well as target MDR-TB and XDR-TB strains effectively.

1.3.3 Host-directed TB therapy

Recently, there has been a new focus investigating alternate interventions that target host immune responses to TB infection, rather than targeting the bacteria themselves, a concept known as host-directed therapies (HDT) [19, 20]. This concept could be used to improve treatment efficacy and outcomes, for better control of drug-resistant strains and reduce severity of infection, as well as mortality. The host provides a wide range of molecular pathways and inhibitory small molecules that could be potential targets for HDT, from mechanisms involved in macrophage function such as lipid metabolism, eicosanoids and cell death, phagosome acidification, to cytokine modulation and tissue homeostasis, to mention a few. [21] For example, a proof of concept study by Mayer-Barber et al 2014, showed that induction of eicosanoid, prostaglandin E2 (PGE2) through interleukin-1 (IL-1), limited production of excessive type-1 interferon (IFN), known to promote disease exacerbation [22].

These strategies demonstrate that manipulation of host mechanisms represent feasible therapies that could be used in conjunction with, or alternative to conventional chemotherapy. Understanding the function of immune responses, and how these could be exploited with HDT, could inform more target situations such as sex-based differences in immune responses and susceptibility to TB observed in men, compared to women, which will be described later, in chapter 3.

2.0 TUBERCULOSIS AND HIV CO-INFECTION

2.1 TB/HIV EPIDEMIOLOGY

Emergence of HIV/AIDS pandemic in the 1980s led to a major upsurge in TB cases and TB mortality. Today, HIV is the most prevailing known risk factor predisposing for *M.tuberculosis* infection and progression to active disease. WHO estimates that 12% of people who developed TB worldwide were HIV positive and 78% of which were in the African region [1]. The greatest burden of TB and HIV co-infection is observed in the sub-Saharan Africa, where the enormous size of the problem is tragically, inversely proportionate to the paucity of resources available for its control [23]. The risk for LTBI reactivation is approximately 20-fold more in HIV-infected people [24].

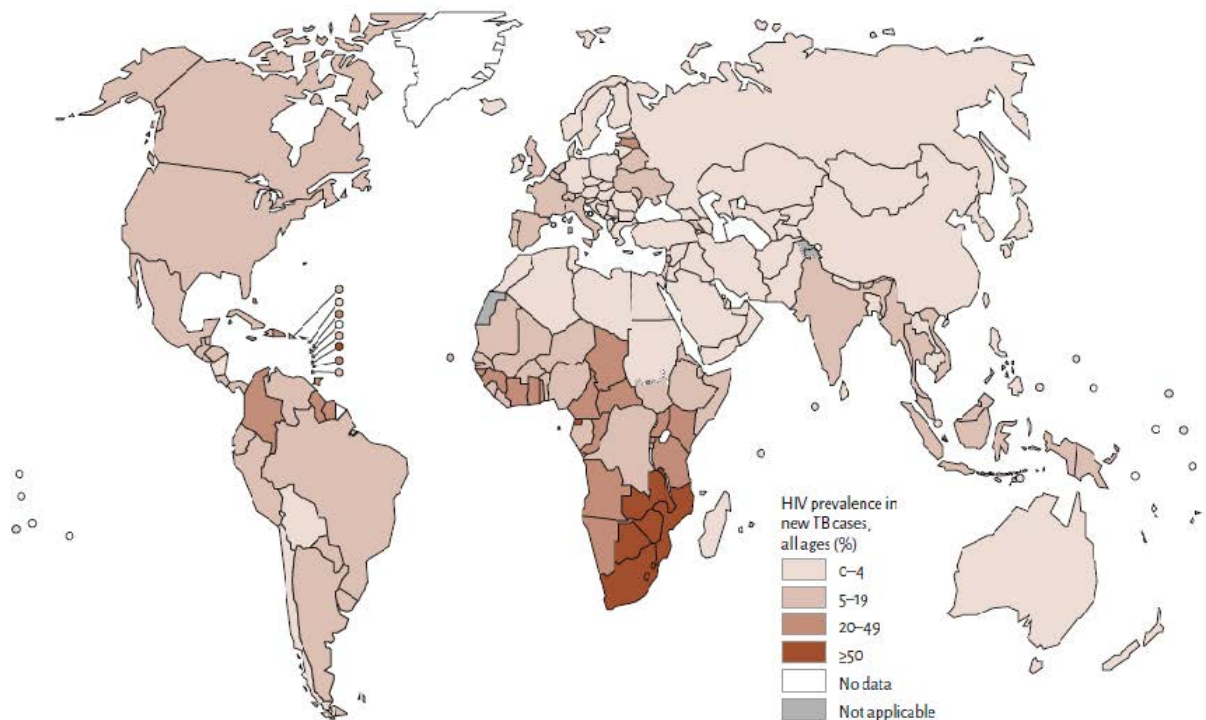


Figure 2.1. Estimated HIV prevalence in new and relapse cases of TB in 2014. Map shows percentage HIV-infected individual in cases of TB across all age groups in different regions of the world [1].

2.2 TB/HIV PATHOLOGY

In TB and HIV co-infection, *M.tuberculosis* and HIV act in synergy, accelerating the decline of immunological functions [24]. HIV causes a generalized depletion of CD4+ T-cells, a subset of T-cells known to be important in the control of TB infection. Additionally, it has been suggested that HIV-1 may preferentially infect and deplete *M.tb*-specific CD4+ T cells [25], therefore causing direct impairment of *M.tb* immune control. Treatment with highly active anti-retro viral therapy (HAART) restores these *M.tb*-specific CD4+ T cells leading to improved immune responses [26]. HIV has effects on other cells, including macrophages, and influences production of cytokines such as TNF- α , important in containing an initial *M.tb* infection.

Studies suggest that the increase in pathology associated with TB and HIV co-infection is caused by functional disruption of the local immune response within both established and newly forming granulomas [27]. This likely causes a decrease in the ability of granulomas to contain *M.tuberculosis* infection, leading to increased bacterial growth, more dissemination and eventually, severe pathology (Figure 1.6).

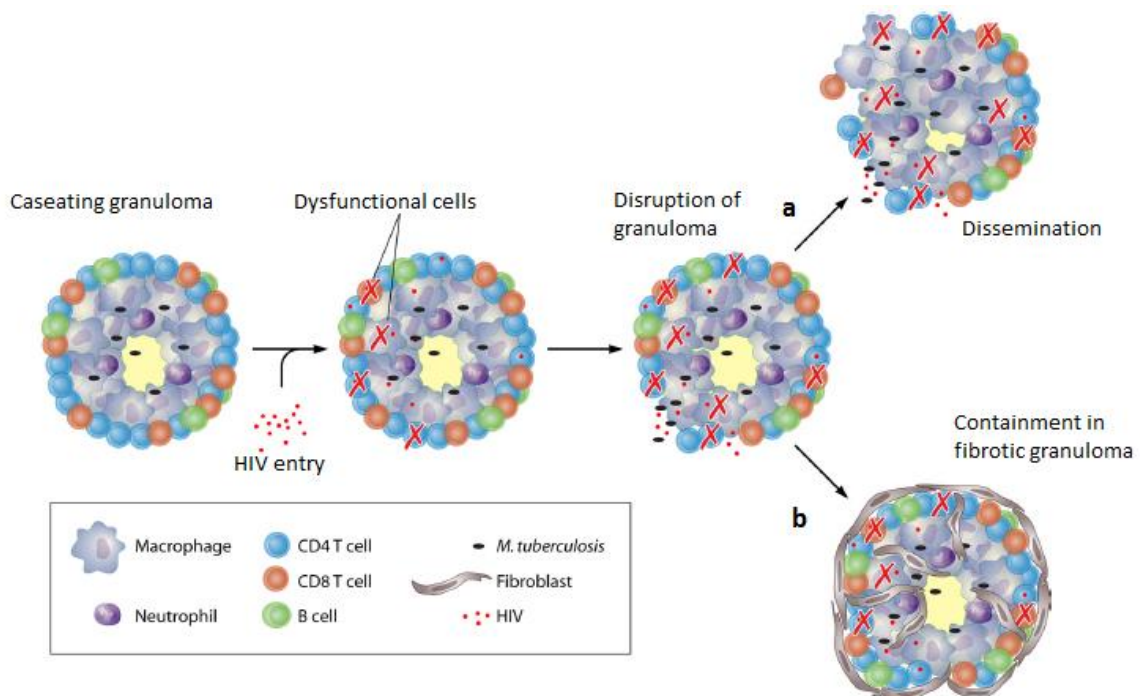


Figure 2.2. Proposed mechanism of HIV-induced reactivation of latent TB. HIV entry induces functional disruption and dissemination of granuloma leading to either a) continued *M.tb* dissemination and early TB reactivation or, b) temporary control in fibrotic granuloma, and prevents reactivation. Adapted from *Diedrich, & Flynn, 2011* [27].

2.3 HIV/TB TREATMENT OUTCOMES

The number of people dying from HIV-associated TB has been falling globally since 2002, as a result of antiretroviral therapy (ART) treatment in notified cases of TB associated with HIV infection [1]. WHO estimates that ART reduces the individual risk of TB disease among people living with HIV by 65% [1]. Initiation of ART results in sudden restoration of immune responses, accompanied by increase in CD4 T cell count and rapid decrease in viral load [28].

However, despite early initiation of ART, mortality in patients with TB/HIV co-infection, especially in developing countries, remains high in the first 3 months after initiation of highly active ART (HAART), in large part, as a result of a paradoxical reaction called immune reconstitution inflammatory syndrome (IRIS), or TB-IRIS in case of TB infection [28]. In these cases, the recovering immune system reacts to the high bacterial burden present, resulting in an uncontrolled and excessive immune response, termed a cytokine storm, leading to organ damage and death in the most extreme cases. Other complications that may result from concurrent treatment of both TB and HIV include drug-drug interactions and, poor patient adherence due to multiple treatments [29].

3.0 SEX-BASED DIFFERENCES IN TB AND HIV

3.1 INTRODUCTION

It has long been known that the incidence and severity of infectious diseases due to bacteria, parasites and viruses, are different between males and females, in both humans and a variety of other species [30]. In general, both the proportion of individuals infected and the severity of infection are higher in males than females [31]. For instance, gender disparities in favour of males are observed in most parasitic infections including schistosomiasis, leishmaniasis and malaria [reviewed in 32 & 33]. A study by Kadioglu et.al, showed that in mice infected with *Streptococcus pneumoniae*, male mice had increased susceptibility to infection and had significantly lower survival rates than their female counterparts [34].

A recent epidemiological meta-analysis study of infectious diseases in Brazil, attempted to address the hypothesis that sex-based differences may be due to, either physiological effects where genetic (chromosomal) differences and sex hormones explain the observed sex-bias in infectious diseases. On the other hand, the behavioral effects hypothesis proposes that exposure arising from differences in behavior renders one sex more exposed than the other to certain pathogens [35]. This study, carried across in different age groups, revealed a post-pubertal male bias in the incidence of cutaneous and visceral leishmaniasis, pulmonary tuberculosis (TB), lepromatous leprosy, and leptospirosis amongst others. However in some diseases such as typhoid fever, there was no sex-bias [35]. The most striking observation was that male bias in infectious disease susceptibility could also be observed in the first year of life, when behavior is unbiased, but sex hormone levels transiently rise [35].

A recent systems analysis of response to an influenza vaccine, showed that females generally had increased antibody responses and inflammatory cytokines and that a cluster of genes up-regulated by testosterone were correlated with poor virus-neutralizing response in male [36]. Increased levels of testosterone inversely correlated with antibody response [36], suggesting a role for sex differences in response to vaccination, likely modulated by sex hormones.

Animal model studies, which are free from confounding factors such as differences in exposure and access to treatment facilities, support the idea that biological mechanisms may drive the observed sex-bias in

infectious diseases. Also, the observation that incidence of autoimmune diseases is sex-biased towards females also provides insights on why these differences may be due to physiological mechanisms. Females are known to generally have more robust immune responses, and this sexual dimorphism in immune responsiveness is most accentuated post-puberty, suggesting that sex hormones play a critical role [30].

3.2 SEX-BASED DIFFERENCES IN TUBERCULOSIS

Tuberculosis incidence and severity also shows gender-based differences (Figure 3.1). The global TB epidemic is characterized by significant differences in prevalence between men and women. In 2014, the worldwide case notification rate of TB indicated a male: female ratio of 1.7, meaning that, generally, almost twice as many men were diagnosed with active TB compared to women, an observation seen in the past years as well [1, 37]. In high burden countries (HBCs), this ratio varied from 0.7 in Afghanistan to 3.0 in Viet Nam, which may be indicative of real epidemiological differences between regions [1]. The male: female ratio for bacteriologically-confirmed TB among adults is typically around 2–3 in Asian countries and 1–2 in Africa, and the ratio of prevalent to notified cases is systematically higher among men than women [1]. It is unclear why more men than women, are diagnosed with TB.

3.2.1 BEHAVIORAL EFFECTS

Whether these observed disparities are due to behavioral or biological effects, or both, is not fully known. There are some compelling arguments that socio –economic and –cultural factors may play a role in TB gender-bias [38]. For example, males are more likely to engage in risky activities that may increase the likelihood of contact with the pathogen and therefore are risk factors of TB transmission, such as cigarette smoking and alcohol consumption. In developing countries, working in mines is regarded as a risk factor for tuberculosis, a profession mostly carried by men [39]. However, household contact is an even stronger risk factor for TB and women tend to spend more time at home than men [39].

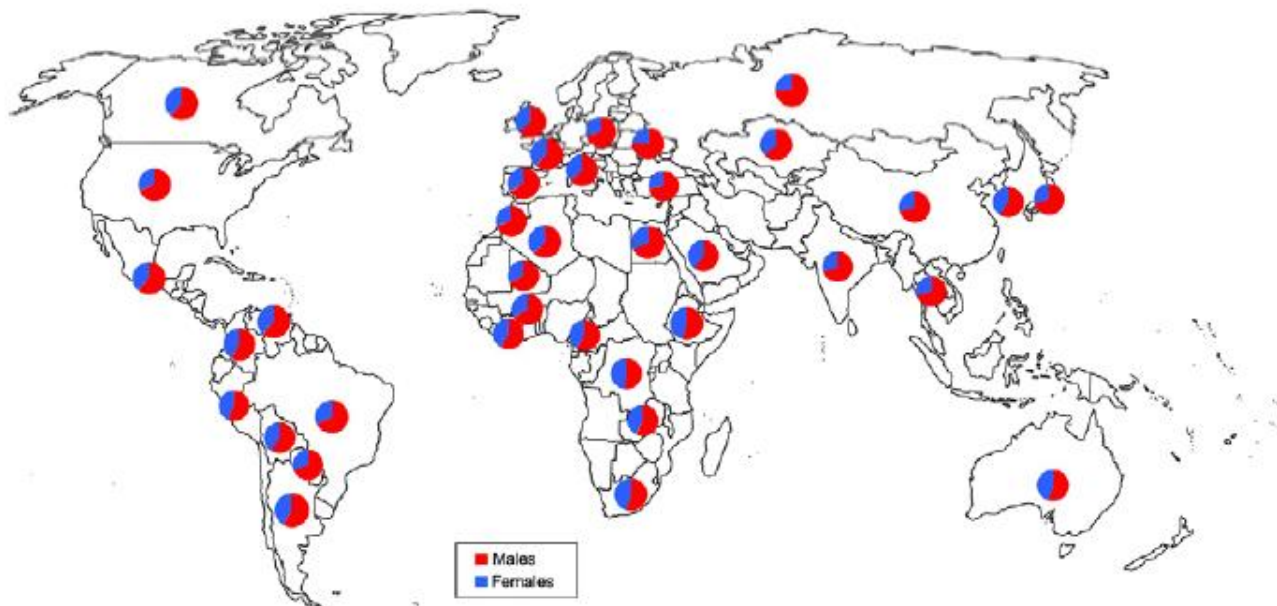


Figure 3.1. Sex distribution of new smear-positive TB cases, showing gender inequality in TB case notifications globally in 2007. *Adapted from Neyrolles & Quintana-Murci, 2009 [37].*

The strongest risk factor for tuberculosis is co-infection with HIV. Although approximately half of HIV-infected people globally are women, in sub-Saharan Africa this raises to 60%, and in South Africa >70% aged 20–30 years are women. Despite the high prevalence of HIV infection among South African women in this age group, however, incidence of tuberculosis remains higher in men [39]. Also, globally, mortality due to TB is generally higher in men in both HIV negative and positive cases, and the sex ratio varies in the different regions in the world (Figure 3.2). In 2014, amongst HIV-infected individuals, approximately 890 000 men died of TB compared to 490 000 females [1].

Some reports suggest that differences in help-seeking behavior and differential access to health care services may result in the possibility of under notification of women, particularly in developing countries. The case notification rate therefore may not reveal the many facets of inequality between male and female patients. However, a meta-analysis study by Borgdoff et al, showed that in prevalence surveys conducted

in 14 countries around the world, the number of TB cases were greater in men than in women in almost all cases [40]. Even though there were some limitations in the data from some of the studied countries, the sex bias was generally observed in suggesting that sex bias in TB prevalence may represent real epidemiological differences [40].

In a house-to-house survey done in Bangladesh, >260,000 individuals were recruited for detection of suspect TB cases, followed by confirmation with smear positivity. Despite nearly equal participation between males and females (51% males), an excess of cases in males was observed (ratio of 3:1), even when confounding factors such as income, awareness and stigma were taken into account [41]. These differences between sexes in active TB are real, however several potential confounding factors like smoking, drug use, and air pollution exposure were not adjusted in their study.

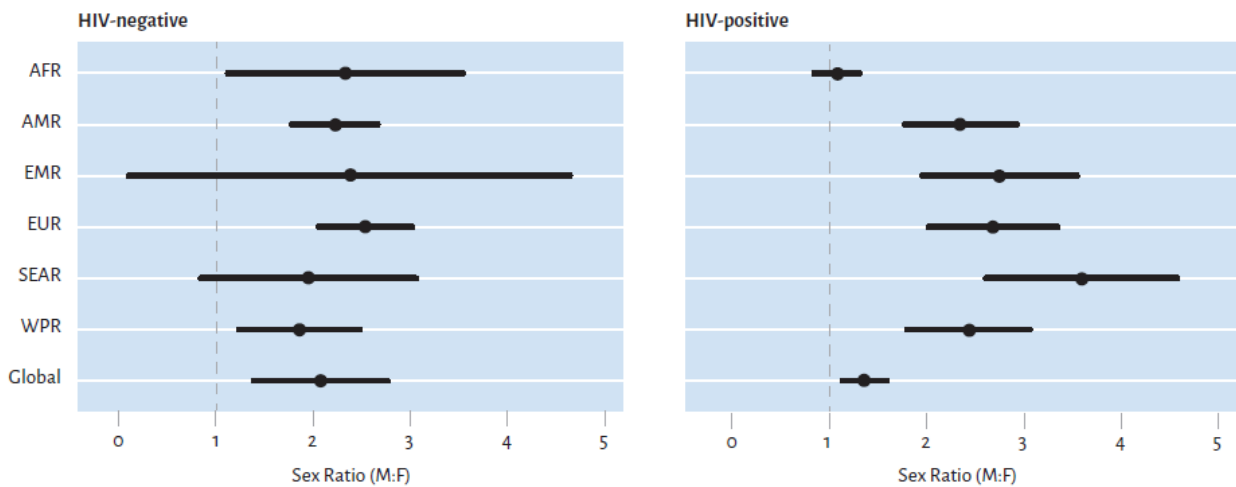


Figure 3.2. Global and regional male: female ratios of death due to TB among adults aged ≥ 15 years. Adapted from WHO Tuberculosis Report 2015 [1].

Taken together, although there is strong support in the literature for the role for behavioral or epidemiological factors in TB risk, it is highly likely that the sexual dimorphism observed in TB is explained at least in part by biological mechanisms.

3.2.2 PHYSIOLOGICAL MECHANISMS

Differences in physiological mechanisms, more specifically, sex-specific biological characteristics such as sex steroid hormones, genetic makeup of sex chromosomes and sex-specific metabolic features may account for the increased susceptibility to TB in men [37]. The study in Brazil, observed a post-pubertal male bias in pulmonary tuberculosis (ratio 1.9:1). Interestingly a stronger male bias was also seen in lepromatous leprosy (2.94:1) whereas mild tuberculous leprosy was female-biased [35]. Tuberculosis and leprosy have similar immuno-pathogenesis, with lepromatous disease being analogous to active tuberculosis and tuberculous leprosy representing cured or contained disease [39]. Crucially, both diseases are caused by the same pathogen, and thus male bias for the tuberculosis-like lepromatous disease is highly unlikely to result from differences in exposure [39]. It is worth noting that in the same study, male bias in these diseases was also observed in children ≤ 1 year of age. It has also been reported elsewhere, that primary TB infection in children under the age 2 and adolescents (≥ 10 years), frequently progressed to serious disease, whereas between the ages 2-10 years, infection rarely progressed to disease [42]. A common characteristic between infancy and adolescence, is a rise in sex hormone levels.

3.2.2.1 Sex hormones

Differences in sex steroid hormones have been suggested as one of the factors contributing to the male-biased susceptibility to TB. Under controlled laboratory conditions using animal models, a clear sex-bias in the susceptibility of males and females can be observed, supporting the argument that TB disease could be influenced by sex hormones.

Early studies in mice infected with *M.marinum*, a mycobacterial pathogen related to tuberculosis, showed higher susceptibility in males than females in terms of mortality, lesions and bacterial load in lungs and spleen [43]. Castration, in male mice improved their resistance, an effect substantially reversed by testosterone replacement. Testosterone treatment of female mice also increased their susceptibility to this infection, suggesting a role of the male sex hormone in reduced resistance to mycobacterial infections [43]. A more recent study showed similar results with *M.tuberculosis* infection, with female mice and castrated males having increased inflammation in the lung indicating better control of infection [44], whereas

ovariectomized female mice showed increased susceptibility to *M. avium*, an effect improved by estradiol therapy [45]. In addition, Depo Provera (depot medroxyprogesterone acetate or DMPA), a synthetic progesterone-based injectable contraceptive has been shown to impair *M. tuberculosis* infection control in mice [46], suggesting that hormonal contraceptives may play a role in TB risk.

In human, early observational studies also suggest sex hormones as a predisposing factor in TB incidence and mortality. A Swedish study showed an increase in death of oophorectomized females (7%), in comparison to TB mortality rates of 0.7% in the country [47]. Also, another study in a mental institution in America found that medically castrated males had better survival (only 8.1% died), compared with intact males (20.6% died). In addition, less intact females died from tuberculosis, compared with intact males (15.8%) [48].

Such studies have prompted investigation into the ability of sex-associated hormones to influence the immune system. It is now widely accepted that sex hormones, directly influence the immune system. Many types of immune cells, including T cells, B cells, dendritic cells (DCs), macrophages, neutrophils and natural killer (NK) cells, express cognate intracellular receptors which suggests that sex steroid hormones might have a role in the regulation of immunity [31]. Mechanisms of how sex-steroid hormones interact with the host immune responses are not yet fully known. However, given that the sex differences observed in animal model studies are similar to those reported by humans, the role of sex steroids in TB and on underlying immune responses need to be investigated.

Estradiol (17- β estradiol)

As a general rule, females exhibit more robust cell-mediated and humoral immune responses to antigenic challenges, such as infection and vaccination, compared with males [31]. Data from experimental mice studies suggest that, 17- β estradiol, the major female sex hormone, is the driving factor behind the enhanced immune responses in females. A T helper-1 (Th-1) cell mediated immune response, characterized by production of IFN- γ and TNF- α , in particular is crucial to control of TB infection [39]. Increased levels of these typical CD4+ T cell cytokines, together with IL-12 and IL-17 are observed in female and castrated male that show less susceptibility to TB infection, compared to non-castrated males, at least during the first month of infection [44]. Also, 17- β estradiol significantly increases activity of the IFN-gamma promoter in lymphoid cells that express the appropriate hormone receptor [49].

In macrophages, 17- β estradiol is thought to enhance activation. Treatment of ovariectomized female mice with either placebo or 17- β estradiol for 4 weeks, showed that macrophages from mice treated with 17- β estradiol had markedly increased expression of pro-inflammatory cytokines, IL-1 β , IL-6, and IL-12p40 and inducible NO synthase when stimulated ex vivo with LPS [50], probably mediated through the estrogen alpha receptor (ER α) expressed in these macrophages [51].

The role of neutrophils in the pathogenesis of TB infection is poorly understood, even though they have been shown to be the dominant infected cell type during pulmonary TB infection [52]. A study by Berry et al., revealed a neutrophil driven type-1 IFN signature in patients with TB infection [53], also suggesting an important role for neutrophils in TB. At high levels of circulating 17 beta-estradiol, during the follicular phase of the menstrual cycle, neutrophils produce high levels of nitric oxide synthase, an effect also observed in post-menopausal women after estrogen therapy [54]. The impact of this on the control of *M.tb* is yet to be established.

Testosterone

Animal model studies suggest an immunosuppressive role for testosterone in TB infection. In general, and in contrast to 17- β estradiol testosterone is thought to downregulate the T helper-1 (Th) - mediated immune response, and in the mouse model, TB infected male mice show increased expression of these cytokines upon castration [44]. Also, deprivation of androgens increases absolute numbers of T cells residing in peripheral lymphoid tissue in mice [55]. The role played by testosterone in regulating T cell function, and in relation to TB infection in humans, however remains to be investigated.

In tuberculosis, classically activated macrophages (CAMs) are induced by Th-1 signals and produce pro-inflammatory cytokines (TNF and IL-12), and chemokines. CAMs express inducible nitric oxide synthase (iNOS), responsible for production of nitric oxide that can kill *M.tuberculosis* [2]. However, again in contrast to 17- β estradiol, testosterone downregulates classical activation of macrophages by inhibiting TLR4 expression and oxygen radical formation [56].

Progesterone

Progesterone, a pregnancy-associated sex steroid hormone is generally known to be immunosuppressive. Downregulation of macrophage activity has been reported, where progesterone inhibits iNOS, activation

of nuclear factor-kappa B (NF- κ B) and TLR4 expression, while up-regulating expression of suppressor of cytokine signaling (SOCS1) in LPS-stimulated macrophages [57], all of which have been reported to play key roles in *M.tuberculosis* infection control *in vivo* and *ex vivo*. The relevance to mycobacterial control is perhaps also illustrated by the fact that *M.tb* can modulate the host eicosanoid and prostaglandin metabolism as a survival strategy [58]. The relative levels of prostaglandin E2 (PGE2) and lipoxin A4 (LXA4) govern whether macrophages undergo apoptosis, a phenomenon that correlates with pathogen containment; or necrosis, whereby *M. tuberculosis* is released from the infected cell, correlating with the spread of infection [58]. Progesterone may play a role in this balance as it increases PGE2 production monocytes [59]. The effect this will have on *M.tuberculosis* control or spread has yet to be studied.

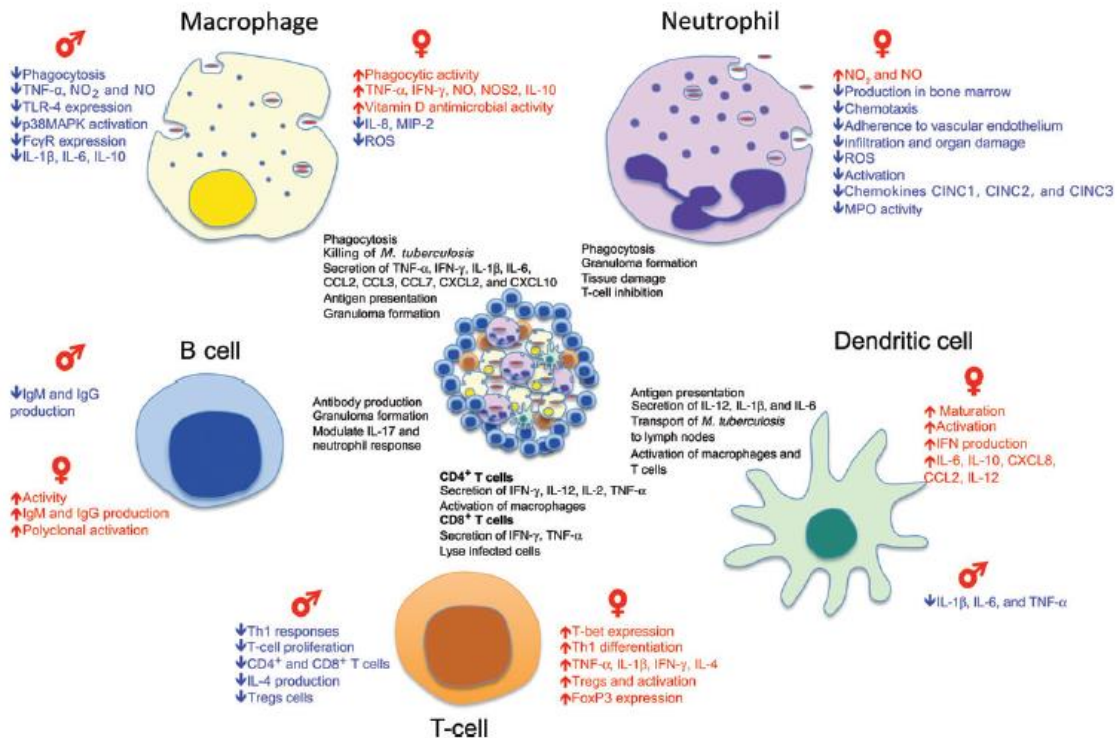


Figure 3.3. Proposed mechanisms by which sex hormones modulate immune responses to *M.tuberculosis* infection. Adapted from Nhamoyebonde & Leslie, 2014 [39].

3.2.2.2 Sex chromosomes

Differences in disease susceptibility observed in men and women can be attributed, at least in part, to the bias in the X chromosome. The X chromosome encodes approximately 1100 genes, the majority of which are immune-related whilst the Y chromosome encodes just a fewer than a 100 genes [31]. Mutations and/or polymorphisms of immune-related genes encoded by the X chromosome may be important determinants of sex-biased immune responses [31]. Women express XX, while men express the XY genotype. Therefore, deleterious recessive alleles in X-linked genes (e.g., TLR7, FOXP3, CD40L, and IRAK1) are more likely to cause immune phenotypes in males due to the heterogametic nature of their chromosomes [31].

A recent study on a Chinese population examined whether the X-linked single-nucleotide polymorphisms (SNPs) of the TLR8, CD40LG and IRAK1 genes were associated with presentation of pulmonary tuberculosis. The CD40LG variant alone, and in combination with the TLR8 and IRAK1 SNPs were associated with increased risk of pulmonary tuberculosis in males [60]. TLR8 polymorphisms have been linked with susceptibility to tuberculosis in other previous studies [37, 61]. IRAK1 proteins have critical roles in pathogen recognition and initiation of a pro-inflammatory immune response, resulting in type I IFN production and induction of the IFN-inducible genes [31]. Suggesting that polymorphisms of this gene may lead to an impaired IFN- γ driven Th-1 type response in tuberculosis infection.

Also, a possible role of the X chromosome in susceptibility to TB has been noted in studies of Mendelian susceptibility to mycobacterial diseases (MSMD), where two mutations in an X-linked gene have been identified in patients suffering from MSMD, some of whom also have presented TB [37]. These studies suggest that variation in the X chromosome may be involved in the genetic predisposition to TB, however, further research needs to be done with larger and well-controlled TB cohorts.

3.3 SEX HORMONES AND HIV RISK

Generally, risk of HIV acquisition is higher in females than males. More females globally, are HIV-infected and studies suggest a hormonal component to this observation. 17- β estradiol and progesterone levels in females fluctuate throughout the menstrual cycle. Hormonal control of the innate and adaptive immune

systems is supported by the fact that immune protection ,in general, is reduced during the secretory phase of the cycle to optimize conditions for tolerance of paternal antigens during reproduction, creating a 'window of vulnerability' during which potential pathogens can enter and infect the female reproductive tract [62]. Animal model studies on female pigtail macaques infected with SHIV, showed increased risk of infection during or following this high progesterone period [63].

In addition, concerns remain regarding HIV risk and progestin-based hormonal contraception DMPA, which prevent pregnancy by mimicking the luteal phase of the cycle. Rhesus macaque SIV model infected with DMPA showed significantly higher acute viremia and delayed virus-specific IFN- γ response [64].

Even though the data strongly suggest a role for sex hormones in HIV acquisition, limitations with these animal models are also apparent. Differences exist in the biology between human and the animals, therefore caution needs to be taken when extrapolating these findings to the context of human infection.

3.4 IMPORTANCE OF SEX CONSIDERATION IN CLINICAL STUDIES

The preceding discussion illustrates how sex-based differences in immune responses contribute to disease susceptibility and severity, which are regulated in part by interactions between sex steroid hormones and the immune system. Despite evidence that disease incidence is often different in men and women and response to treatment varies, sex-based differences in study design and analyses remain largely excluded in clinical trials [30]. Women, in particular are under-enrolled in these studies and this has led to 'one-drug' treatment regimens for both men and women. Inclusion of sex and gender as variables in clinical trials will eliminate discrepancies in both the understanding and the treatment of diseases in the individual sexes.

In addition, effects of synthetic hormonal contraceptives need to be addressed by conducting larger human clinical studies, to determine risk to TB and HIV. This will inform women on risk associated with these contraceptives and allow them the choice to use alternative methods available to them.

PROBLEM STATEMENT AND STUDY JUSTIFICATION

Global incidence rates for tuberculosis (TB) indicate a gender-bias to the disease, with almost twice as many men actively infected than women (male/female ratio of ~1.7). Sex hormones are known to regulate immune function and their role in tuberculosis has been suggested experimentally in animal models as described in previous chapters. However the role of sex hormones in human TB infection and whether there is any synergistic effect in HIV and TB co-infection is unknown. In addition, data suggests that manipulation of sex hormones with progesterone-based contraceptives increases susceptibility to both HIV and TB. Therefore our aims for this study were as follows:

Aim 1: To develop and optimize a method for quantitation of sex steroid hormones and contraceptive analogues in human plasma using a targeted LC-MS/MS approach.

Aim 2: To determine if use of progestin-based contraceptive DMPA is associated with risk of HIV acquisition.

Aim 3: To measure adaptive immune responses to TB and HIV infection by means intracellular cytokine staining using flow cytometry on peripheral blood mononuclear cells (PBMCs).

Aim 4: To determine phenotypic differences in memory T cell subsets, T regulatory cells and activation and/or exhaustion of CD4+ and CD8+ T cells.

4.0 QUANTIFICATION OF SEX STEROID HORMONES

4.1 INTRODUCTION

Quantification of sex steroid hormones has in the past, been commonly done using antibody-based immunoassays in clinical laboratories. These immunoassays often agree poorly, especially at normal and low concentrations due to problems resulting from low assay specificity, sensitivity, inadequate standardization, and poor optimization of the methods over the large range of concentrations seen clinically [65]. In addition, these assays often suffer from cross-reactivity between structurally similar compounds, as is the case for sex steroid hormones (Figure 4.1). Therefore, high-quality steroid hormone measurement assays with very good sensitivity, specificity, and reproducibility are therefore essential to the validity of epidemiologic studies [66].

The last decade has, therefore, seen an increase in the use of mass spectrometry (MS) for detection, identification and quantification of these molecules in complex biological matrices [67], as this technique offers superior analytical specificity and accuracy. However, there has been a slow adoption of this method as standard means of measuring sex steroid hormones as it is not commonly available in laboratories and hospitals due to associated high costs of MS instruments, lack of expertise, and time-consuming sample preparation procedures.

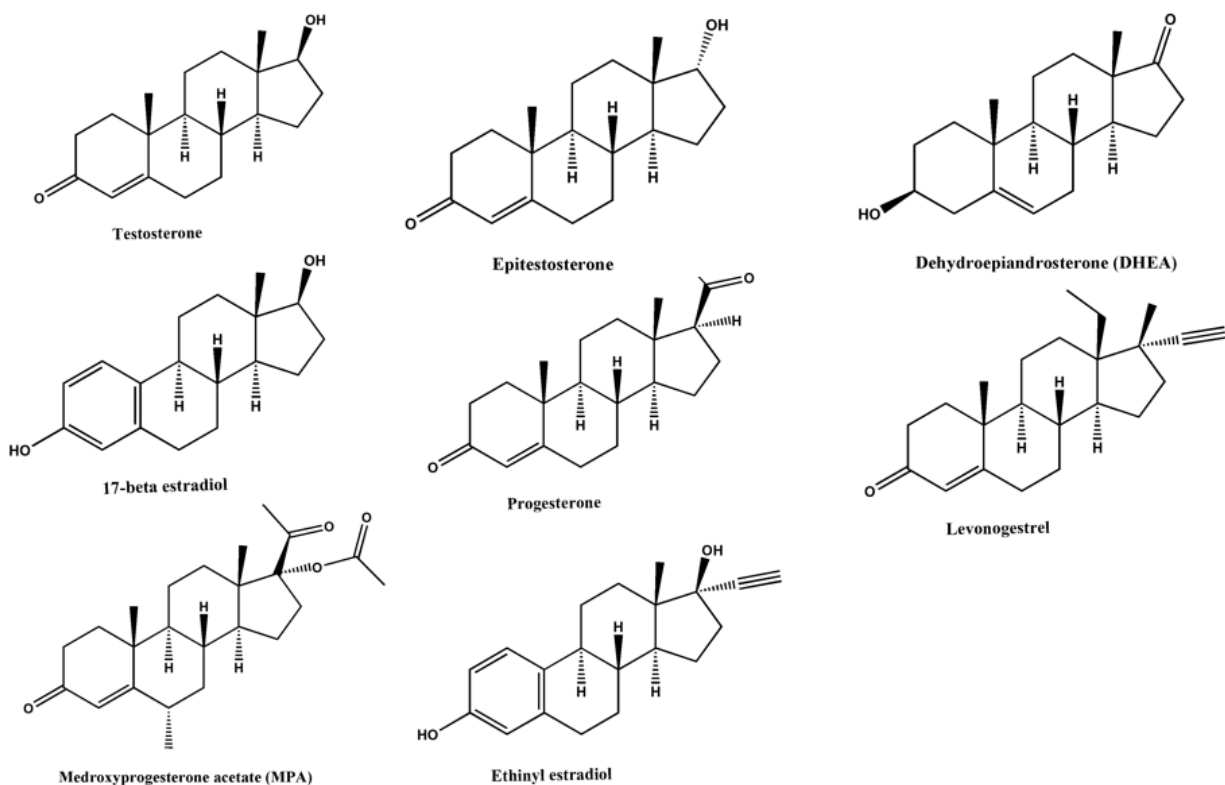


Figure 4.1 Some major endogenous sex steroid hormones, and contraceptive analogues. Similarities in chemical structures may result in cross-reactivity in antibody-based immunoassays such as ELISA.

4.2 MULTIPLE REACTION MONITORING (MRM)

Liquid chromatography-mass spectrometry (LC-MS) is a powerful analytic technique that combines the resolving power of LC with the high sensitivity and specificity of the mass spectrometer (Figure 4.2). The technique that is most frequently used in quantitative assays, selected reaction monitoring (SRM, plural form: multiple reaction monitoring, MRM) MS was first reported in 1979 during the introduction of the triple quadrupole (QqQ) mass spectrometer [67]. Modern liquid chromatography-tandem mass spectrometry (LC-MS/MS) with multiple reaction monitoring (MRM) mode provides accurate simultaneous quantitation of analytes on large sample sets in a single run, at the same time providing high quality information on target molecular profiles in complex biological samples. In this setting, high performance liquid chromatography (HPLC) with high resolving power (i.e. ability to resolve analyte peaks

to be eluted as discrete peaks) allows for separation of compounds in the chromatographic column. The mass spectrometer functions as a unique detector that provides structural information on individual analytes as they elute from the LC column [66]. This relies on the discriminating power of mass analyzers to select a specific analyte and ion current measurements for quantitation [68].

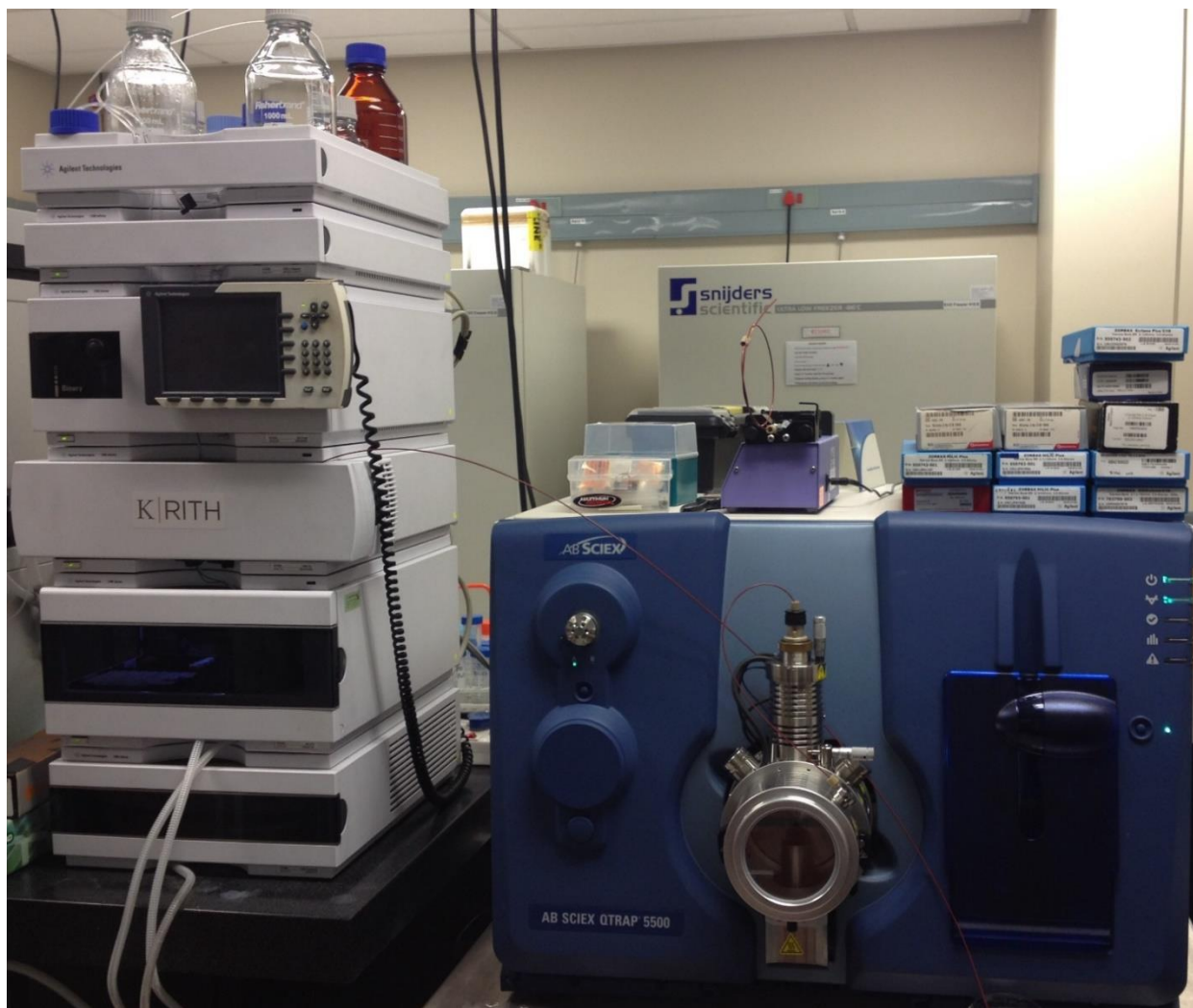


Figure 4.2 Liquid chromatography- mass spectrometry instrumentation used for MRM analysis.

MRM analysis employs a triple quadrupole MS. The mass spectrometer is equipped with an ionization source that impacts a charge, positive or negative depending on selected ionization mode, on each molecule as it elutes out of the column. Mostly such assays use electrospray ionization (ESI) followed by two stages of mass selection: a first stage (first quadrupole, Q1) selecting only the predefined mass-to-charge (m/z) value of the precursor ion of the intact analyte (molecular or parent ion), followed by fragmentation of the

parent by collision with argon or nitrogen gas atoms in a high vacuum collision cell (second quadrupole), resulting in low energy collisions and fragmentation of the selected precursor ion into many product ions. Finally, another mass selection stage (third quadrupole, Q3), selecting specific fragments of the parent (fingerprint daughter ions), collectively generates a SRM (or MRM) assay [67, 68]. The two mass filters produce a very specific and sensitive response for the selected analyte that can be used to detect and integrate a peak in a simple one-dimensional chromatographic separation of the sample [58] (Figure 4.3).

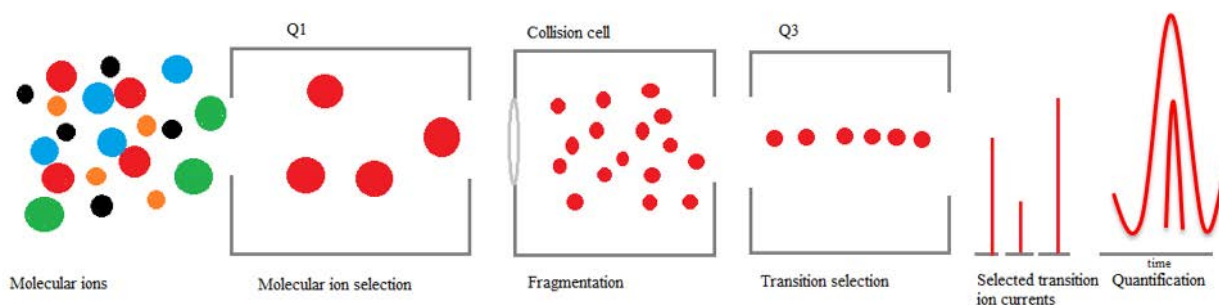


Figure 4.3 MRM scan mode in a triple quadrupole mass spectrometer. (Picture modified from mrmAtlas.org, accessed on 15 November 2015).

In principle, this mass spectrometry-based approach has the ability to provide absolute structural specificity for the analyte, as the resulting daughter transitions give a unique fingerprint for each analyte, and, in combination with appropriate stable isotope-labeled internal standards (ISs), it can provide absolute quantitation of analyte concentration [68].

4. 3 QUANTIFICATION METRICS

MRM assays are characterized and their performance assessed based on certain characteristics. As mentioned above, accurate and precise quantification is best achieved through addition of a stable isotopically labeled internal standard (IS). The ISs are usually spiked into samples far upstream of the sample preparation processes and because the ISs are structurally similar to analytes, they introduce only a minimal chromatographic shift in the reversed phase chromatography [67]. Signal intensity in the chromatogram, that is, area under curve of the IS peak, is compared to peak area of analyte to generate a peak area ratio as follows:

Peak area ratio (PAR) = peak of analyte / peak area of IS

Measured concentration = PAR X IS concentration

As shown in the equation above, measured concentration is determined by multiplying the PAR with concentration of IS. A calibration curve, generated from a dilution series of analyte standards prepared with IS of fixed concentration and amount is then used to plot these data. If IS concentration is unknown, data can be plotted as peak area ratio versus theoretical concentration of calibration standards. Calibration curve slope, represents analytical sensitivity of method for that specific analyte [67].

A limit of detection (LOD) for the assay is determined as the lowest concentration of analyte at which signal can be differentiated from noise (usually at ~ signal-to-noise ratio of 3). A limit of quantification (LOQ) is described as the lowest concentration of analyte at which quantitative measurement of analyte can be made with confidence (~S/N ratio of 10) [67]. Different methods for calculating LOD and LOQ have been described by Mani et al (2012) [67]. Analysis of samples in replicates allows for determination of precision, usually represented by coefficient of variance (%CV) or standard deviation and accuracy.

4.4 DEVELOPMENT OF LC-MS/MS METHOD FOR QUANTITATION OF SEX HORMONES AND CONTRACEPTIVE ANALOGUES IN PLASMA.

4.4.1 MATERIALS AND METHODS

4.4.1.1 Chemicals and reagents

Progesterone, progesterone-D₉, testosterone, epitestosterone, dehydroepiandrosterone and 17-β estradiol were purchased from Cerilliant. Norethisterone and ammonium formate were purchased from Sigma Aldrich. Commercially available Depo Provera (depot medroxyprogesterone acetate) was used (Pfizer Pharmaceuticals). Levonogestrel was extracted from commercially available Trigestrel tablets (Famy Care Ltd, India). Charcoal stripped plasma was purchased from Golden West Biologicals Inc. US. Lypocheck Immunoassay plus Control Levels 1, 2 and 3 were purchased from Bio-Rad. LC-MS grade methanol, hexane, ethyl acetate, acetonitrile, formic acid, acetone and water were purchased from Sigma Aldrich.

1.5ml auto sampler vials, caps with PTFE lined silicone septa, glass micro-inserts were purchased from Separations.

4.4.1.2 LC-MS/MS system

Analyte concentrations in plasma samples were measured by HPLC tandem mass spectrometry, using an Agilent Technologies 1200 Series HPLC with a Phenomenex C-18 (50 X 2.10mm) analytical column maintained at 4°C. The sample injection volume was 20 µl and mobile phases were water (A) and acetonitrile (B) with 0.1% formic acid. The column was developed by step-wise linear gradient elution as follows: 0.00min, 20%B; 0.10min, 20%B; 5.10min, 100%B; 15.00min, 100%B; 15.10min, 20%B and 23.00min 20%B. A constant flow rate of 200µl/min was maintained. Analytes were detected on an ABSciEx Q-TRAP 5500 mass spectrometer using electrospray ionization in a positive ion mode and multiple reaction monitoring to measure the precursor-to-product ion transition. All analyses were performed using Analyst Software (version 1.6) and quantitation values generated using the respective standard curves for each analyte. The total LC-MS/MS run time was 23 minutes per sample analyzed. Optimized settings are summarized in Tables 4.1 and 4.2.

Table 4.1 Electrospray ionization (ESI) settings

Curtain gas (CUR)	25
Collision gas (CAD)	medium
Ion spray voltage (V)	4500
Source temperature (°C)	350
Gas 1 (psi)	40
Gas 2 (psi)	40

4.4.1.3 Sample preparation

Frozen plasma samples, quality control samples and double charcoal stripped plasma (-80°C) were thawed on ice and 85µl removed for analysis. Calibration standards (0.01 to 10ng/ml) were prepared by adding

progesterone, testosterone, norethisterone, epitestosterone and DMPA in methanol to double charcoal stripped plasma. As an internal standard 10µl of progesterone-D₉ (103nM) was added to all samples, vortexed and equilibrated for an hour. Calibrators, quality control samples and plasma samples were then extracted by addition of 825ul ethyl acetate-hexane (80:20), vortexing for 2 minutes, and centrifuging at 300 xg for 5 minutes. The upper organic layer (600ul) was transferred to a new tube and washed with 50ul ammonium formate buffer (0.1M, pH 9.0). 500ul of organic phase was then removed and transferred to a new vial, evaporated to dryness under nitrogen gas and then re-constituted in 200ul H₂O: formic acid: MeOH (49: 0.1: 50), vortexed and transferred into micro-inserts and in auto sampler glass vials.

4.4.1.4 Preparation of internal standard (IS) solution

For quantitation accuracy and precision a deuterated internal standard (IS) with nearly similar chemical properties was used, namely progesterone-D₉. The progesterone-D₉ internal standard was purchased as a 100µg/ml solution in acetonitrile (Cerilliant) and stored at -20°C. Working stock of the IS (103nM) was prepared by dilution in methanol, instead of acetonitrile as this solvent caused precipitation of plasma sample when IS was added during extraction. IS stock and working solution were stored at -20°C.

4.4.1.5 Preparation of standard stock solutions

Stock solutions of all analytes were prepared at a concentration of 1 mg/ml in acetonitrile except for DHEA, which was prepared in methanol because of its solubility. For each analyte, a substock was prepared in acetonitrile at 10µg/ml. These substock solutions were used to prepare working solutions to spike double charcoal stripped plasma as required. All stock solutions were stored at -20°C.

Table 4.2 MS/MS settings

	Progesterone D₉	Progesterone	Testosterone	DMPA	Norethisterone	Levonogestrel	Epitestosterone	DHEA
Q1 mass [M+H]⁺	324.269	315.227	289.181	387.003	299.161	313.196	289.211	271.219
Q3 mass	100.000	97.000	97.000	327.100	91.000	107.000	97.000	253.100
De-clustering potential (V)	71	86	71	126	66	116	89	136
Collision energy (V)	29	33	29	19	55	33	29	15
Collision cell exit potential (V)	16	18	8	14	14	18	14	10
Scan type	MRM	MRM	MRM	MRM	MRM	MRM	MRM	MRM
Polarity	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Pause time (ms)	5.0070	5.0070	5.0070	5.0070	5.0070	5.0070	5.0070	5.0070

4.4.1.6 Preparation of calibration standards and quality control standards

A set of calibration standards were prepared volumetrically in double charcoal stripped plasma. A working stock solution of mixed analytes was prepared at a concentration of 105ng/ml mixed standard solution (MSS1) by adding 52.5µl of each 10µg/ml analyte substock in a 5ml volumetric flask and filled up to the mark with 90% methanol. MSS1 was then serially diluted with 90% methanol to attain the desired standard working solutions (STD1-8). 10ul of each STD was then spiked into 85µl double charcoal stripped plasma volume to give desired concentration range of 0.01 – 10ng/ml as presented in Table 4.3. From the calibration curve, the limit of detection (LOD) and lower limit of quantification (LOQ) were determined.

Quality control samples (QC1, low, QC2, medium and QC3, high) were purchased with added progesterone, 17β-estradiol and testosterone at fixed concentrations, however all other analytes were added into QC samples by spiking 10µl of prepared analyte solutions to give final concentrations of 0.7, 2.5 and 7 ng/ml for low, medium and high QCs respectively.

Table 4.3 Preparation of calibration standards for all analytes.

Standard	Volume MSS1 added (µl)	Volume of 90% MeOH diluent (µl)	Total volume (µl)	Volume added to double charcoal stripped plasma (µl)	Final standard concentration (ng/ml)
STD8	1000	0	1000	10	10
STD7	999	1	1000	10	5
STD6	995	5	1000	10	1
STD5	990	10	1000	10	0.5
STD4	950	50	1000	10	0.1
STD3	900	100	1000	10	0.05
STD2	500	500	1000	10	0.01
STD1 (blank)	0	1000	1000	10	blank

4.4.1.7 Calibration standard curve

Double charcoal stripped plasma and QCs stored at -80°C , were thawed, spiked with appropriate STD as indicated in Table 4.3, extracted using the protocol described in section 4.4.1.3 and analysed for each validation run. Using Analyst[®] software standard curve fitting was determined by applying the simplest model that adequately describes the concentration vs response relationship using appropriate weighting. The calibration curve is comprised of eight different STDs including the blank, with concentrations ranging from 0.01 ng/ml to 10 ng/ml. For quantitation the eight-point calibration standard curves were calculated based on peak area ratios of analyte to IS and fitted using $1/x$ (1/concentration) weighted linear regression, which was found to be the simplest regression model for quantification. Representative calibration curves for DMPA, progesterone and testosterone are presented in Figure 4.4a-c.

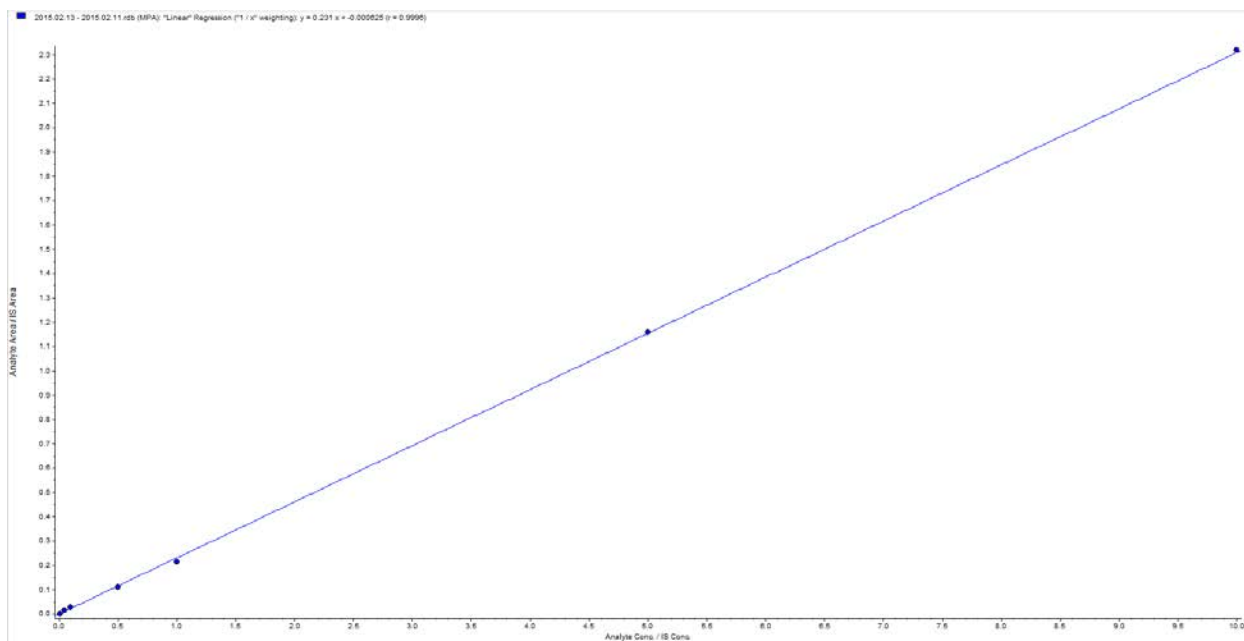


Figure 4.4a. Representative calibration curve for DMPA.

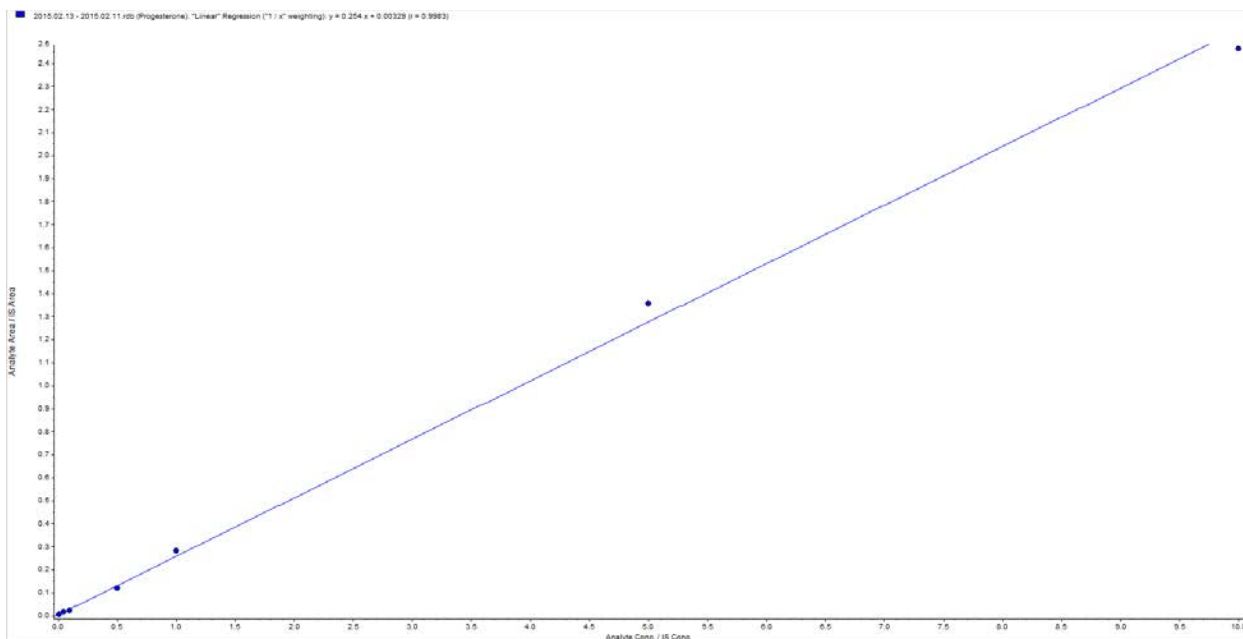


Figure 4.4b. Representative calibration curve for progesterone.

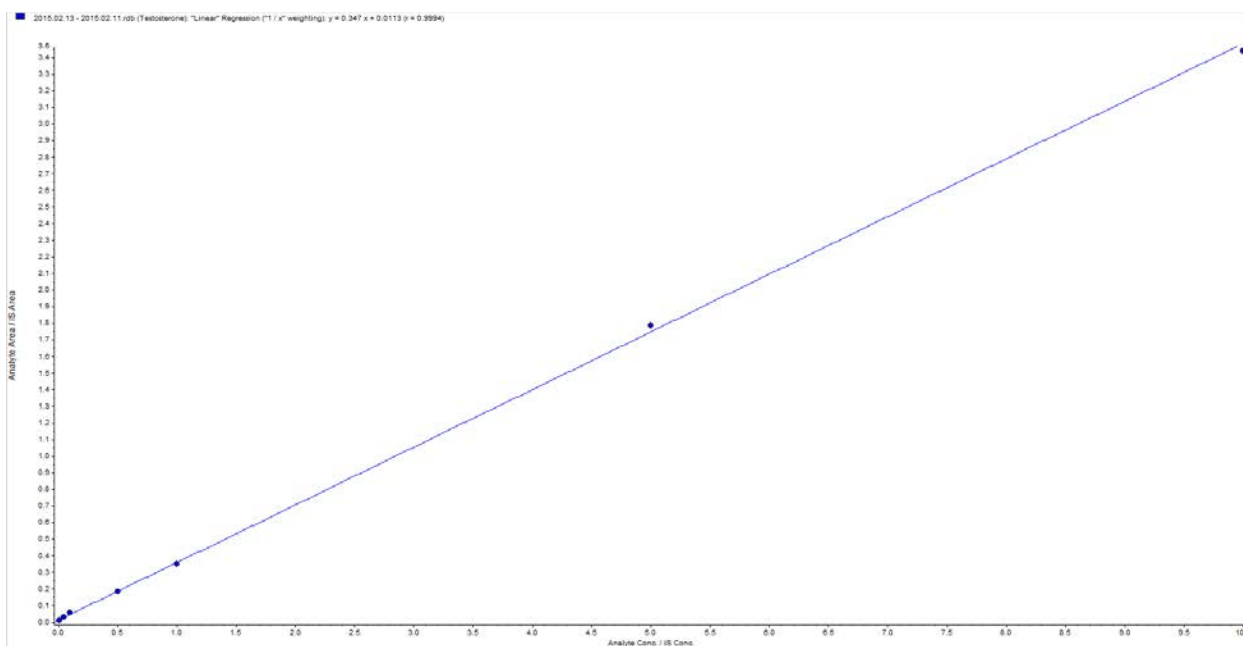


Figure 4.4c. Representative calibration curve for testosterone.

4.5 RESULTS AND DISCUSSION

4.5.1 Mass spectrometry

Since emergence of MRM analysis, a number of studies have been done on sex steroid hormone quantitation using MS. The first step was initial tuning of the mass spectrometer to determine ionization of analytes. Relevant literature was searched to determine expected parent ions and daughter transitions using ESI, and also previously established ionization mode for each analyte [69, 70]. Optimization of MS transitions was done by diluting analyte substock solutions in methanol, to lower concentrations, between 40 and 100ng/ml. Direct syringe infusion into the mass spectrometer, of a constant stream of an analyte at a given time was done and expected molecular ion was scanned in a pre-selected range of $[M+H]^+$ ions. Transitions (m/z), resulting from this specific parent ion were scanned and the most abundant 5 transitions were selected from the mass spectrum to maximize sensitivity. Furthermore, for each species, two ion pairs were chosen, with the most sensitive transition being used for quantitation (quantifier) and the other transition being used for confirmation (qualifier). Figure 4.5a and b, illustrate mass spectra of two of the analytes optimized, progesterone and the internal standard, progesterone- D_9 .

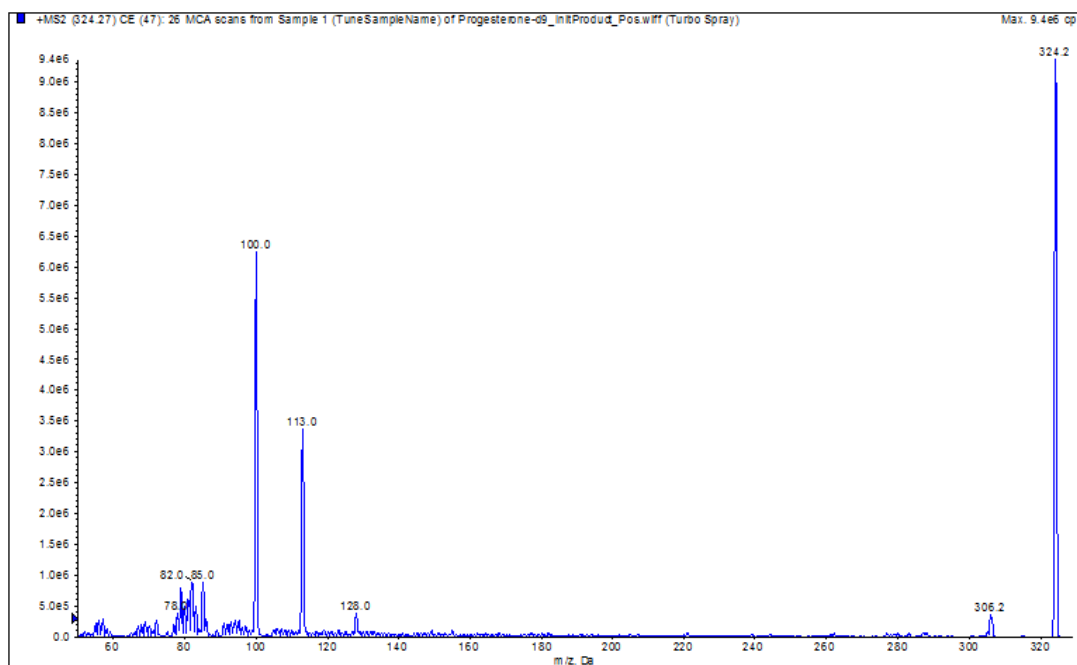


Figure 4.5 Mass spectrum for progesterone showing molecular ion peak $[M+H]^+$ m/z 315.2 \rightarrow 97.0 and 315.2 \rightarrow 109.0 m/z transitions.

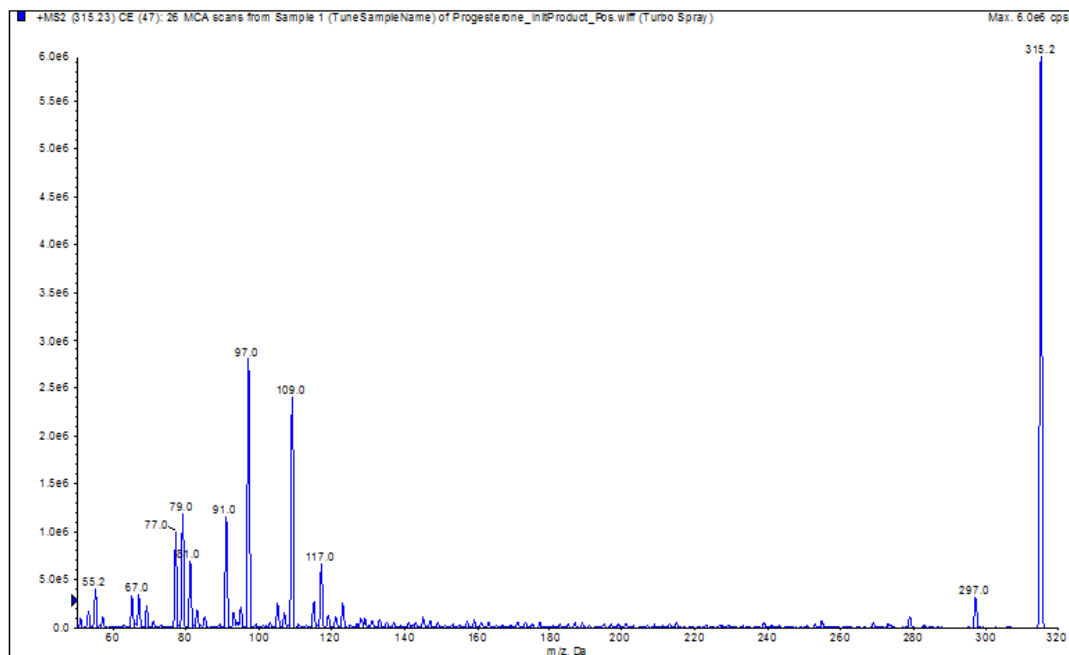


Figure 4.6 Mass spectrum for internal standard progesterone-D₉, showing molecular ion peak $[M+H]^+$ m/z 324.2 \rightarrow 100.0 and 324.2 \rightarrow 113.0 m/z transitions.

Mass spectra obtained during optimization in positive ESI mode, showed a protonated molecular ion $[M+H]^+$ for all steroids except for DHEA for which, a protonated molecular ion with loss of water, $[M-H_2O+H]^+$ was observed as previously reported in other studies. [71]. All optimized analytes and their respective transitions are shown in table.4.4 and mass spectrum for testosterone is included in Appendix section.

Table 4.4 Molecular ion, quantifier and qualifier transition, m/z for all optimized analytes in ESI positive mode

Analyte	Q1	Q3	
	Molecular ion	Transition 1	Transition 2
	(m/z)	(m/z)	(m/z)
Progesterone- D₉	324.2	100.0	113.0
Progesterone	315.2	97.0	109.0
Testosterone	289.2	97.0	109.0
Epitestosterone	289.2	97.0	109.0
DHEA	271.2	213.1	253.1
Levonogestrel (active compound in some oral contraceptives)	313.2	107.0	109.0
DMPA (Depo Provera)	387.2	122.9	327.1
Norethisterone (Nuristerate or Net-En)	299.2	65.1	91.0

4.5.2 Chromatography

Chromatographic separation was achieved using a step-wise linear gradient elution, with changes in mobile phase composition ratios with time. Mobile phase A, water was used in conjunction with mobile phase B, acetonitrile in the presence of 0.1% formic acid. Formic acid is added to mobile phase mixture, to aid in improving ionization of analytes by donating protons $[H]^+$. The column temperature was set at 40°C and a flow rate of 200 μ l/min was maintained. Optimal time of 23 minutes for each gradient run allowed for

elution of all analytes with good peak resolution and peak shape, as well time for clean-up of column for the next run.

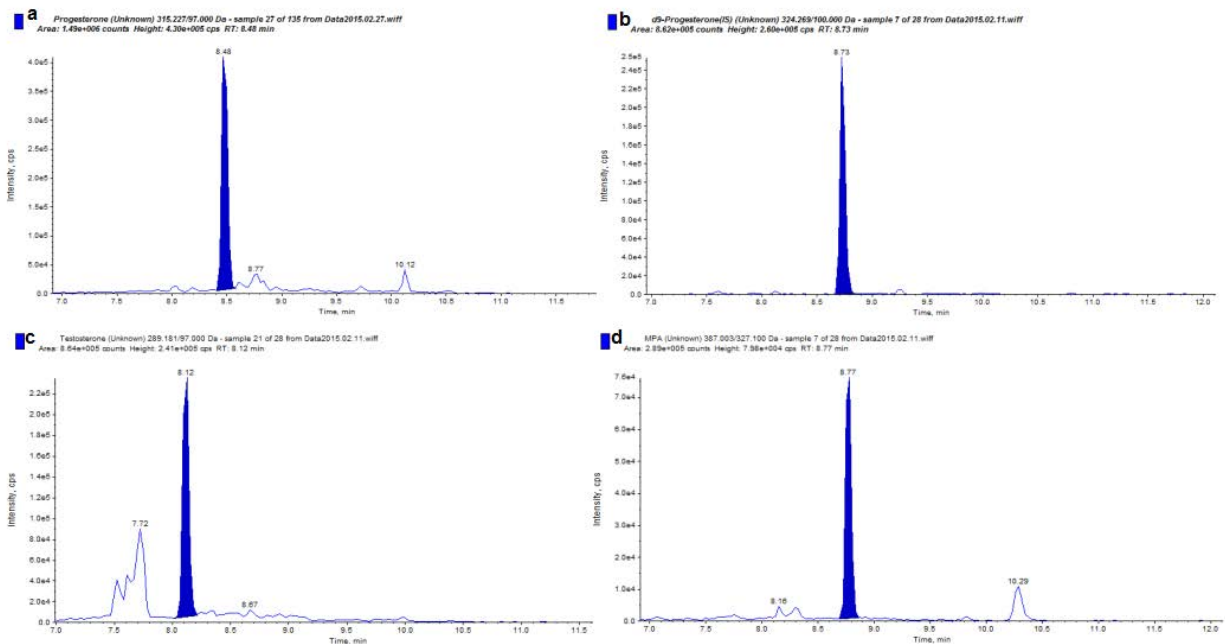


Figure 4.7 Examples of chromatograms showing analyte peaks and different retention times for A) progesterone, B) DMPA, C) testosterone and D) internal standard, progesterone-D₅

Quantitation of each analyte, as previously described, was done using analyte peak area ratio to peak area ratio of IS, progesterone-D₅ of known concentration. Peak area ratios were then computed against the calibration curve to determine the unknown.

4.5.3 LC-MS/MS METHOD VALIDATION

A good method validation consists of a set of calibration standards and quality control assessment run on different occasions to ascertain that the method is accurate, precise and reproducible. The QC samples are monitored over validation batches performed on three different occasions to determine intra-batch and inter-batch accuracy and precision.

Intra-batch and inter-batch accuracy and precision assessment

QC samples were extracted and prepared in parallel with the biological samples and injected at the beginning, in the middle and at the end of every analytical run to monitor the consistency of instrument performance throughout the analytical run – intra batch accuracy and precision. The QC samples were also monitored over successive batches performed on different days to ascertain inter-batch accuracy and precision. Pooled plasma sample were prepared from healthy controls (HCPP) and actual study samples (SPP) and were processed with each batch to determine batch processing variation.

From the results presented in Tables 4.5 and 4.6, it may be concluded that the method performed effectively between and within batch runs as all QC and pooled plasma samples have a calculated % CV of less than 15%.

Table 4.5 Summary of progesterone and testosterone inter-batch variation in quality control samples.

	Sample ID	Mean observed conc. (ng/ml)	Standard Deviation	CV (%)	n
progesterone	QC1	0.658	0.070	10.7	17
	QC2	5.559	0.46	8.3	17
	QC3	14.806	1.23	8.4	17
	HCPP	3.494	0.27	7.8	17
	SPP	1.498	0.15	10.4	17
testosterone	QC1	0.875	0.13	14.7	17
	QC2	3.879	0.47	12.2	17
	QC3	6.225	0.83	13.4	17
	HCPP	2.325	0.13	5.7	17
	SPP	0.992	0.12	12.0	17

Table 4.6 Summary of progesterone and testosterone intra-batch variation in quality control samples in a single batch.

	Sample ID	Mean observed conc. (ng/ml)	Standard Deviation	CV (%)	n
progesterone	QC1	0.678	0.080	11.8	6
	QC2	5.763	0.33	5.8	6
	QC3	16	0.74	4.6	6
	HCPP	3.667	0.24	6.5	6
	SPP	1.565	0.22	14.3	6
testosterone	QC1	0.884	0.068	7.7	6
	QC2	4.207	0.22	5.2	6
	QC3	6.650	0.61	9.2	6
	HCPP	2.475	0.081	3.3	6
	SPP	1.078	0.11	10.3	6

Stability Assessment

For method validation it is also necessary to ensure that the analytes are stable (do not degrade) throughout the sample preparation, extraction and analytical procedure including associated conditions such as temperature variations. Stability of steroid hormones in plasma or serum and stock solutions has been previously demonstrated. Previous studies have shown no significant analyte losses up to 3 freeze-thaw cycles and also post-extraction stability for up to 3 days at 10°C in the autosampler [71, 72]. All sample freeze-thaw cycles were kept to a maximum of 2, to minimize degradation of analytes and batches were run over a maximum period of 3 days. Manufacturer's storage instructions for standard stocks were followed (storage at -20°C). Plasma sample handling was kept to a minimum and on-bench processing was done on ice.

Specificity and sensitivity

A method should be selective for a specific analyte and not be affected by interfering or co-eluting components in the biological matrix. Any interference would be most apparent at low analyte concentration

levels. The selection of a specific precursor ion followed by the formation and detection of a specific product ion renders quantitative mass spectrometry highly specific.

A representative chromatogram of a plasma sample is presented in Figure 4.8, indicating both progesterone and progesterone-D₉ peaks with no interfering peaks. The method is specific for the quantitation of each analyte from human plasma samples as indicated by the different *m/z* transitions resulting from the molecular ions and different elution times of both analytes, even though progesterone and the deuterated form are identical in structure, with only a difference of heavier isotope of hydrogen in the deuterated form.

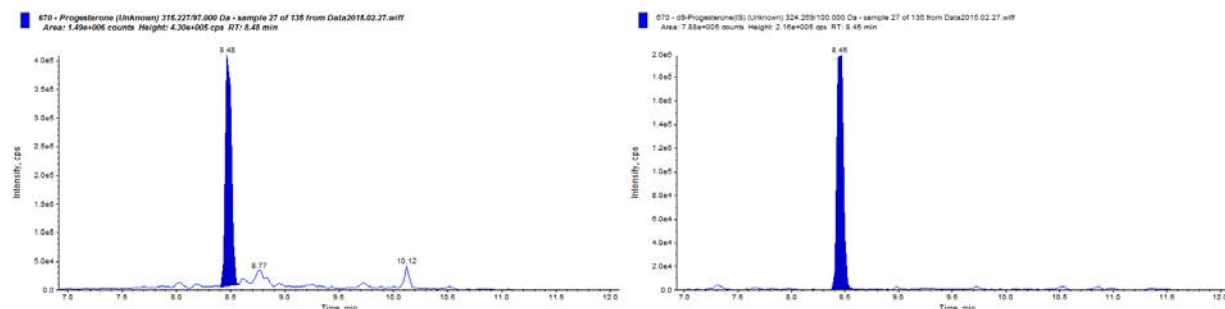


Figure 4.8 An example of a chromatogram of progesterone and internal standard, progesterone-D₉.

A linear calibration curve can be used to determine sensitivity of method for a specific analyte. This allows for determination of LOD and LOQ parameters using the regression equation of the calibration curve. The standard error $S_{y|x}$ of the measured concentration (*y*-estimate in the regression equation) and slope of the equation are to determine LOD and LOQ as follows:

$$\text{LOD} = 3 S_{y|x}/\text{slope}$$

$$\text{LOQ} = 10 S_{y|x}/\text{slope}$$

Table 4.7 Determination of LOD and LOQ from linear calibration curve.

Compound	r	slope	y-intercept	$S_{y x}$	Estimated LOD (ng/ml)	Estimated LOQ (ng/ml)
DMPA	0.9993	0.213	0.00055	0.0010	0.015	0.046
Progesterone	0.9977	0.254	0.00608	0.0022	0.029	0.087
Testosterone	0.9987	0.351	0.00695	0.0042	0.039	0.11

$S_{y|x}$ is the standard error of *y*-estimate (*y*-intercept) of the regression line.

Matrix effects

Also known as ion suppression, is a phenomena that occurs because ionization of analyte of interest is susceptible to disturbance from other components of the biological samples that co-elute with the analyte. This can either cause suppression or enhancement of analyte peak. This effect was not tested during method development, however to reduce this effect two steps were taken,

- i) During sample preparation, a wash step with ammonium formate (pH 9.0, 0.1M) was performed. This removes molecules that may cause interference in steroid ionization such as phospholipids
- ii) Compensation with a stable isotopically labelled internal standard (IS) ensures a consistent effect as both IS and analytes undergo same amount of matrix effect.

Sample recovery

A sample preparation method is required that can balance cleaning up of samples to remove as many interferences as possible while achieving maximal recovery of analytes. To achieve this, the isotopically labeled internal standard, progesterone-D₉ was spiked pre- and post- extraction of quality control samples. Ideally spiking a blank matrix such as charcoal stripped plasma is preferred, however due to limited resources at the time, QC samples were used. Percentage recovery is calculated using the equation:

$$\text{Peak area of analyte before extraction} / \text{peak area of analyte after extraction} \times 100 = \% \text{ recovery}$$

In Table 4.8, recovery of progesterone-D₉ was 56-63%, which is a moderate recovery indicating a certain level of analyte losses. This may be due to the additional wash step for removal of interfering molecules in the plasma samples. Also, for extraction process, plastic microtubes were used, and this may not be ideal for steroids hormones since they are lipid-like in nature and could form non-polar interactions with microtube walls, causing losses of analyte. This could, in the future be improved by use of glass microtubes. For this project, the obtained recoveries were considered acceptable.

Table 4.8 Determination percentage recovery of progesterone-D₉.

	Mean peak area spiked before extraction	Mean peak area spiked after extraction	% recovery
QC1	1.89 x10 ⁶	3.29 x10 ⁶	56
QC2	2.17 x10 ⁶	3.43 x10 ⁶	63
QC3	2.26 x10 ⁶	3.92 x10 ⁶	58

4.5.4 Challenges with 17-β estradiol optimization

Previous studies have reported measurement of 17-β estradiol (E2) in serum or plasma samples as a major challenge due to low concentrations normally found in these samples (pg/ml range). This represents a challenge in particular, in serum or plasma from children, men and postmenopausal women who have very low circulating levels of E2 and therefore would require highest level of sensitivity for analysis. In premenopausal women, E2 levels also fluctuate during the menstrual cycle, however, even if E2 concentrations are higher in this group, they are still below 55pg/ml except at ovulation and in pregnancy, which requires considerable assay sensitivity [69].

Previous studies have described MRM analysis of E2 using ESI ionization mode. MS optimization of ion pair of molecular ion to daughter ion was done in both negative and positive mode of ESI. The molecular ion was scanned successfully in both modes, [M-H]⁻ -->271 and [M+H]⁺ --> 273 respectively, however as seen in both Figure 4.9a and b, ionization efficiency for E2, which lacks highly ionizable functional groups is relatively low using ESI mode as indicated by absence of prominent daughter transitions. Successful E2 ionization in ESI using both negative and positive mode, has been described before, however a number of studies describe poor ionizability therefore sensitivity at pictogram levels is difficult to achieve. Therefore to improve ionization, studies have used other more harsh ionization modes such as atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization (APPI) [73]. However, due to technical issues with our mass spectrometer the APCI mode could not be utilized and this method was not explored further.

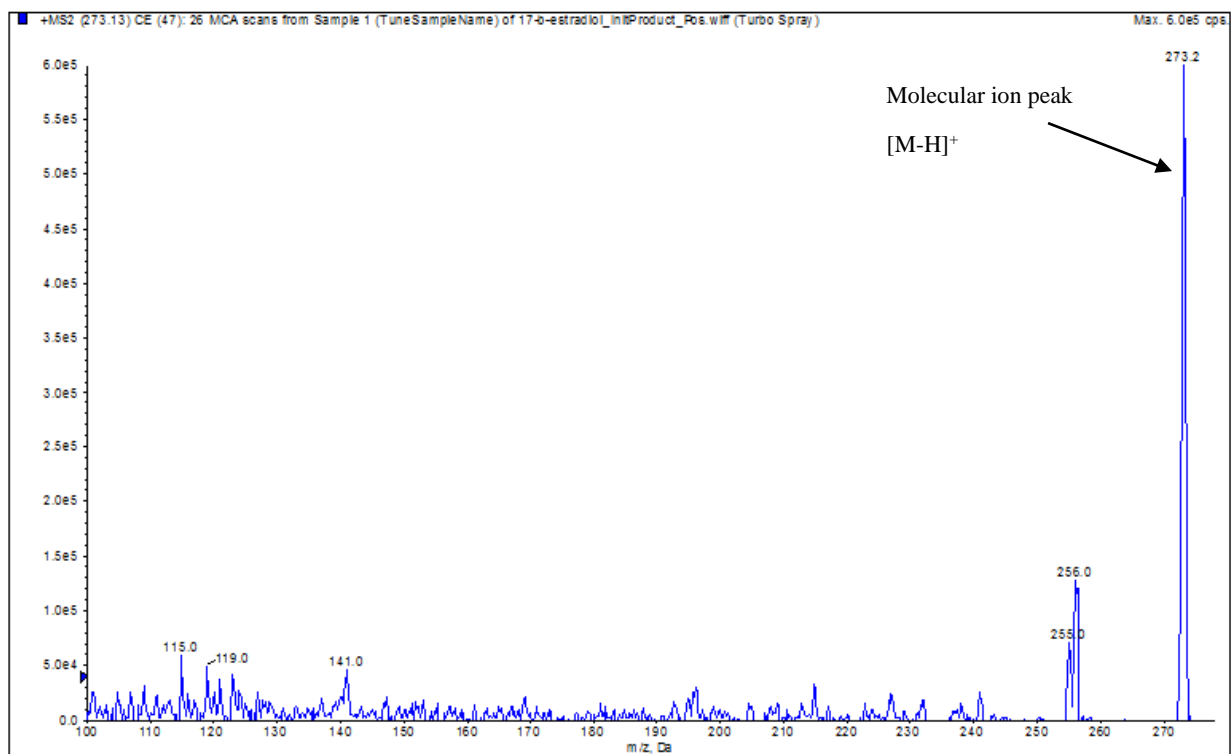
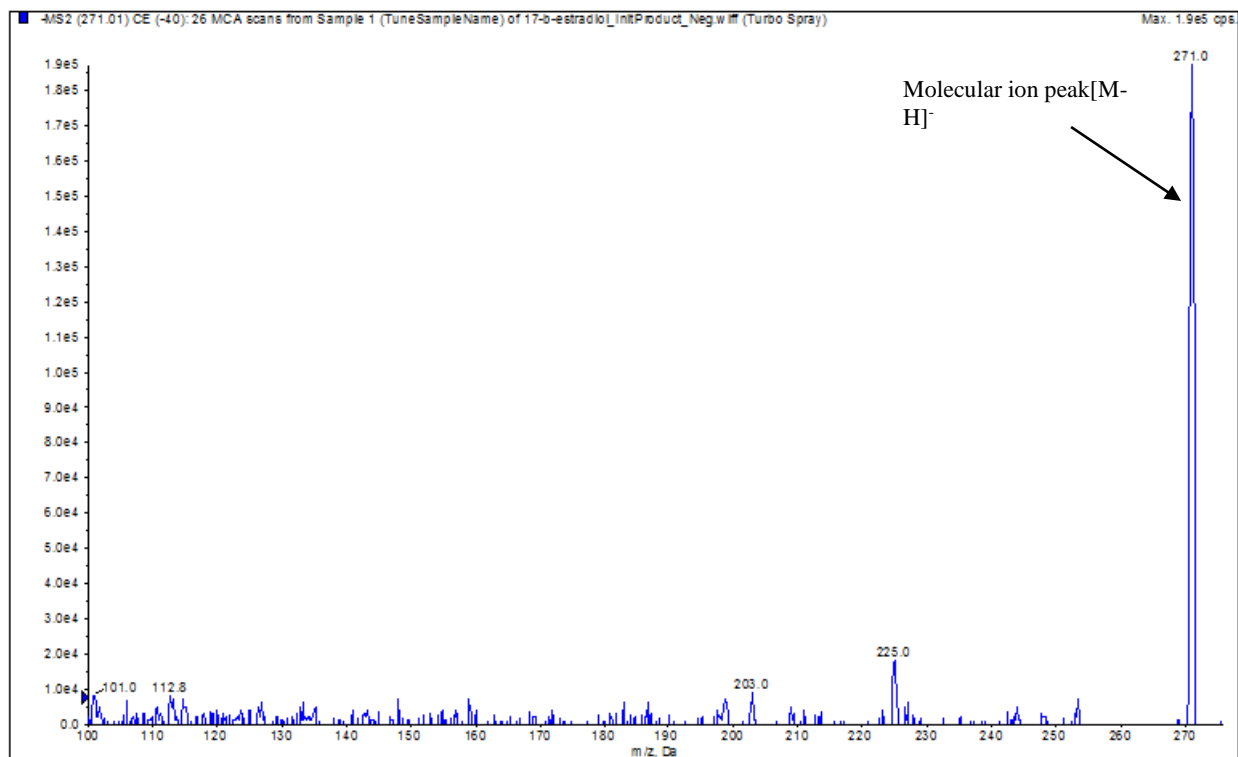


Figure 4.9a and b, mass spectra of E2 in negative and positive ESI mode respectively.

Recent years have seen new methods that utilize the introduction of a highly ionizable functional group into steroid molecules to significantly enhance the ionization efficiencies of various steroid hormones, a process called derivatization. The derivatization of estrogens with a dansyl functional group has been reported [74, 75], using ESI, APCI or APPI positive ionization mode. Use of 4-(dimethylamino) benzoyl chloride (DMABC), as a more versatile and more sensitive derivatizing agent for ESI source has also been described. Hence, we optimized a reaction between DMABC dissolved in dry acetone and excess E2 standard solution (100ng/ml) by heating at 60°C for 6min (heating for longer, 25 minutes, did not improve peak intensity). In **Figure.4.10**, the MRM transition 420.2 -->166.0 was observed as expected.

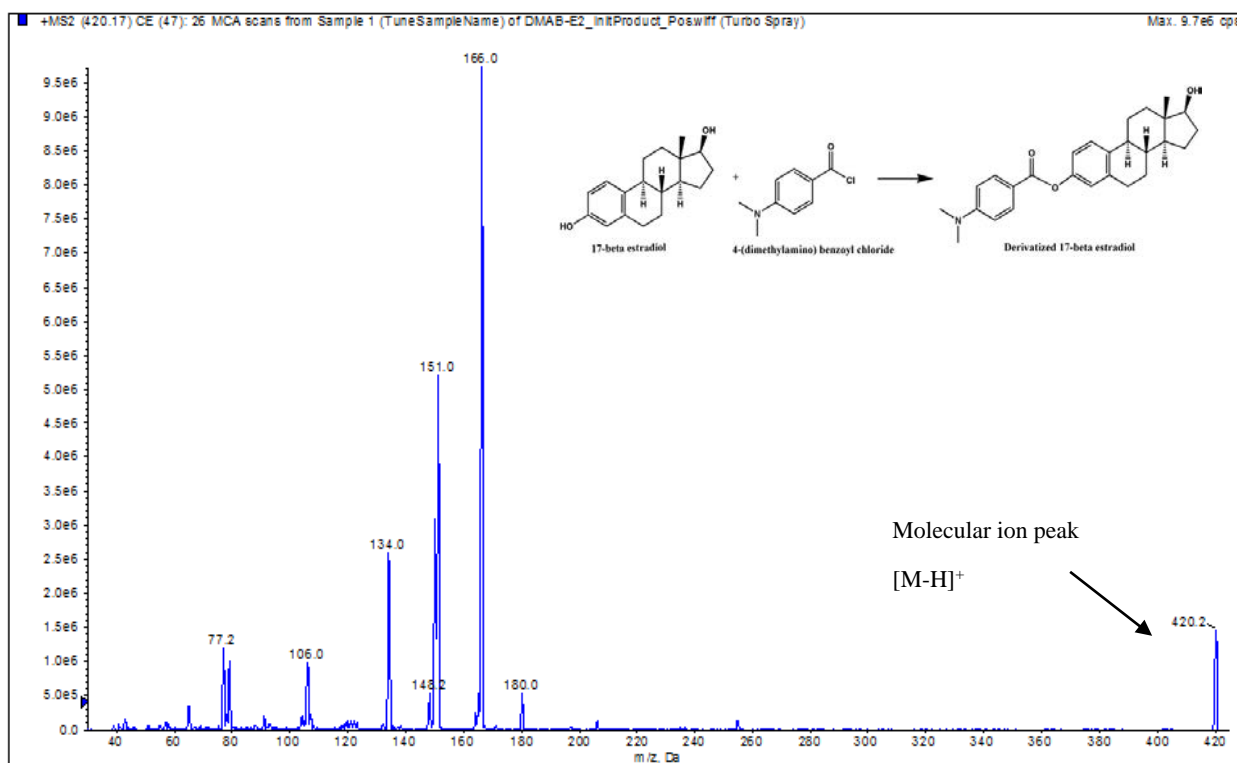


Figure 4.10 Mass spectrum of DMABC-derivatized 17-β estradiol.

Using the same method of E2 derivatization, E2 standard solution of 100ng/ml, and female plasma samples were prepared. However, we did not observe the expected peak in actual female plasma samples, only in high concentration derivatized E2 standard. From this we conclude that even though we successfully optimized derivatized E2 transitions, and column separation, as seen in high concentration E2, our method however is not sensitive enough to detect E2 at physiological level. We propose that the sample extraction

method may be limiting. Other studies have overcome this by using solid-phase extraction (SPE) to concentrate the sample before injection into the LC system. Therefore, our future work will investigate use of SPE to improve sensitivity.

4.6 SUMMARY

We have developed a LC-MS/MS method that allows specific and simultaneous detection of multiple sex hormones and contraceptive analogues in human plasma, and quantification with good sensitivity, with the exception of 17- β estradiol. Investigation of SPE in sample processing and use other ionization techniques such as APCI and APPI, for measurement of 17- β estradiol, need to be investigated to improve method sensitivity for this analyte. Observed limits of detection and quantification were in the lower pg/ml range and comparable with what has been shown in other studies. This method is simple, requires a small sample volume and sample preparation is minimal. It also provides sufficient sensitivity, which allows for meaningful interpretation of results in clinical application, as shown in the next chapter.

5.0 INJECTABLE HORMONAL CONTRACEPTIVES AND ASSOCIATION WITH HIV RISK

5.1 LITERATURE REVIEW

A number of studies have associated the use of hormonal injectable contraceptives, and particularly progestin-based DMPA, with increased risk in HIV acquisition [76, 77, 78]. A systematic review by Polis et al, (2014) points out that, some studies however report no statistical significant increased risk with both DMPA and Net-En [79]. Therefore a lot of controversy still remains about the validity of these observations given the high potential for confounding factors such as potential risky sexual behavior among hormonal contraceptive users.

Studies in non-human primate models using simian immunodeficiency virus (SIV) and simian-human immunodeficiency virus (SHIV) represent a good model to study this effect as these are free from confounding factors. For example, treatment of macaque with DMPA results in increased susceptibility to both SIV and HIV infection compared to untreated controls [64, 80]. This increased susceptibility in SIV/SHIV infection in DMPA treated macaques was attributed to changes in the vaginal epithelium [80]. Limitations, however, exist in these studies, which include small numbers of animals and differences in species, dosage, and study design. Human studies specifically designed to determine impact of DMPA or other hormonal contraceptives on HIV risk need to adjust for the variability of human behavior such as sexual exposure, condom use amongst other factors, which presents a significant challenge [81].

AIMS

- a) To validate LC-MS/MS method for quantitation of sex hormones and hormonal contraceptives, in real biological samples.***

- b) To determine if hormonal contraceptive use is associated with higher risk of HIV acquisition.***

To study this we collaborated on two separate cohorts, in which DMPA levels in plasma samples were measured. In the CAPRISA cohort, cervicovaginal samples were analysed using shotgun proteomics and in the FRESH cohort, immunological responses were measured, to determine risk factors associated with HIV acquisition.

5.2 MATERIALS AND METHODS

5.2.1 Ethics statement

Ethical approval for the FRESH cohort study was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Durban, South Africa) and the Massachusetts General Hospital Institutional Review Board (Protocol 2012P001812/MGH; Boston, MA).

Ethical clearance for the CAPRISA 004 study was obtained from the BREC of the University of KwaZulu-Natal, (Durban, South Africa), ref. E111/06 and University of Manitoba Institutional Review Board (Canada).

5.2.2 Study design

For the CAPRISA pilot study, study participants were a subset of 38 cases and controls selected from the CAPRISA CAP004 study. This study enrolled high risk HIV negative women between 18-40 years, in a double-blinded, randomized, placebo-controlled trial to assess effectiveness of tenofovir gel against HIV infection. If any of the women became HIV-infected, they were then enrolled in the CAP002 arm. Women were enrolled at CAPRISA Vulindlela clinic and eThekweni Research clinic for. All participants provided written informed consent.

The FRESH cohort study was part of an observational, closed cohort, prospective study conducted in Umlazi, South Africa. Participants were HIV-negative women ages 18-23, recruited for the Females Rising through Education, Support, and Health (FRESH) study. Twice per week, participants attended classes focused on personal empowerment, job skills training, and HIV prevention. HIV-1 testing is done twice-weekly and demographic and behavioral data is collected in addition to blood and cervical samples. Informed consent was obtained from all participants.

5.2.3 Measurement of sex hormones and contraceptives using LC-MS/MS.

All instrumentation set-up, reagents and plasma sample preparation protocols for quantitation of sex steroid hormones has been described in Chapter 4.

5.2.4 Proteomic analysis of vaginal lavage samples from the CAPRISA study

Proteomic analysis of matched cervicovaginal lavage samples was done at the University of Manitoba by collaborators. (See contributions).

5.2.5 Statistical analyses

All sex hormones measurement analysis was performed using GraphPad Prism software, version 6.0. In the CAPRISA 004 study, statistical differences in DMPA, progesterone and testosterone levels were determined using one-way ANOVA tests, and Bonferroni multiple comparisons test. Correlation between progesterone and DMPA was determined using a Pearson's r correlation (p-value, one tailed). Shotgun proteomic analysis of CVL samples, was performed by our collaborators, and is not discussed here.

5.3 RESULTS

To validate our LC-MS/MS method in real biological specimens, we collaborated on two separate blinded studies, looking at risk of hormonal contraceptives in HIV. In the first instance, we analyzed female plasma samples from the CAPRISA CAP004 study ($n = 38$). Samples were prepared and analysed using the extraction protocol and optimized instrument settings, described in Chapter 4. Once un-blinded, samples were stratified according to contraceptive use. We had 21 samples for Depo-Provera contraceptive users, 8 samples of oral contraceptive (OC) users, 5 samples of Nuristerate (Net-EN) users, and 1 sample from a patient who underwent tubal ligation (not included in analysis). DMPA, is the active compound in the long-term injectable contraceptive Depo Provera. As shown in Figure 5.1, Depo Provera users had significantly higher levels of DMPA in their plasma compared to OC and Net-EN users ($p = 0.0167$ and 0.0262 respectively). For OC and Net-EN users, DMPA levels were near or below both the LOD and LOQ, indicating absence of DMPA except for 1 patient sample in the Net-EN group who had significantly high DMPA levels.

Next we examined levels of natural progesterone in these subjects, as Depo Provera is known to directly inhibit the production of endogenous progesterone [82, 83]. As expected subjects with detectable DMPA levels in general have lower levels of endogenous progesterone than those not taking Depo Provera (Figure 5.1). This difference is not significant, although that is perhaps not unexpected as subjects not using Depo Provera are still subject to natural cyclic changes in progesterone. In addition, one might expect quantity of DMPA present to impact on the extent to which natural progesterone is suppressed. To test this we correlated progesterone and DMPA in the subjects taking Depo Provera and observed a significant inverse correlation ($r^2 = 0.1537$, $r = -0.3920$, $p = 0.0437$), despite the small sample size (Figure 5.2). This indicates

that as DMPA levels wane, their modulatory effect is also likely to decline, which in turn may influence their impact on the immune response. No significant differences were observed in testosterone levels.

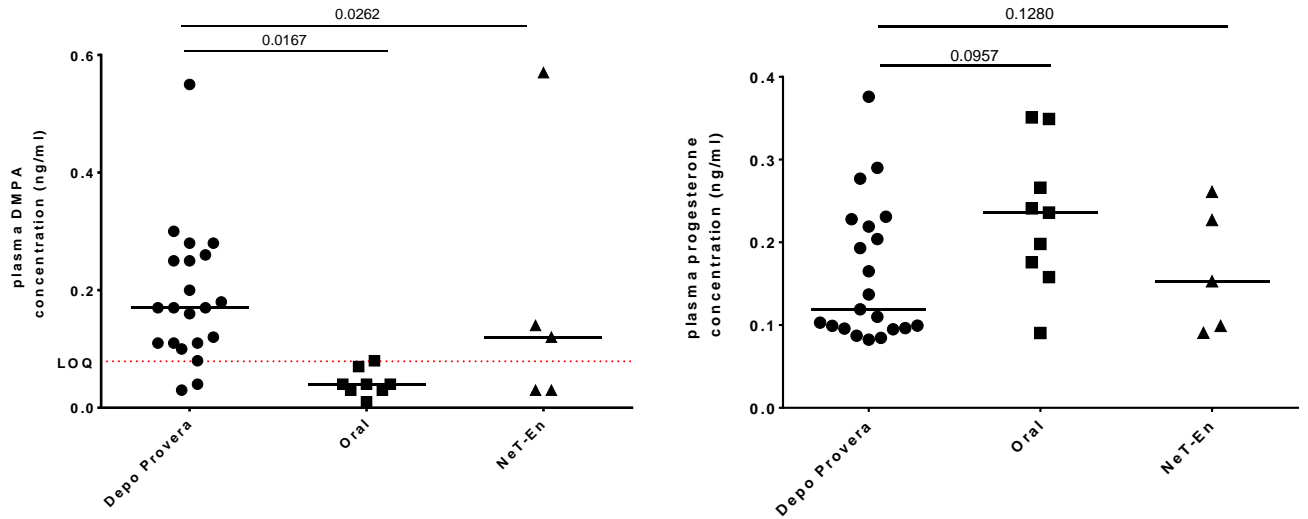


Figure 5.1. Plasma DMPA and progesterone levels measured using LC-MS/MS in individuals on different contraceptive methods.

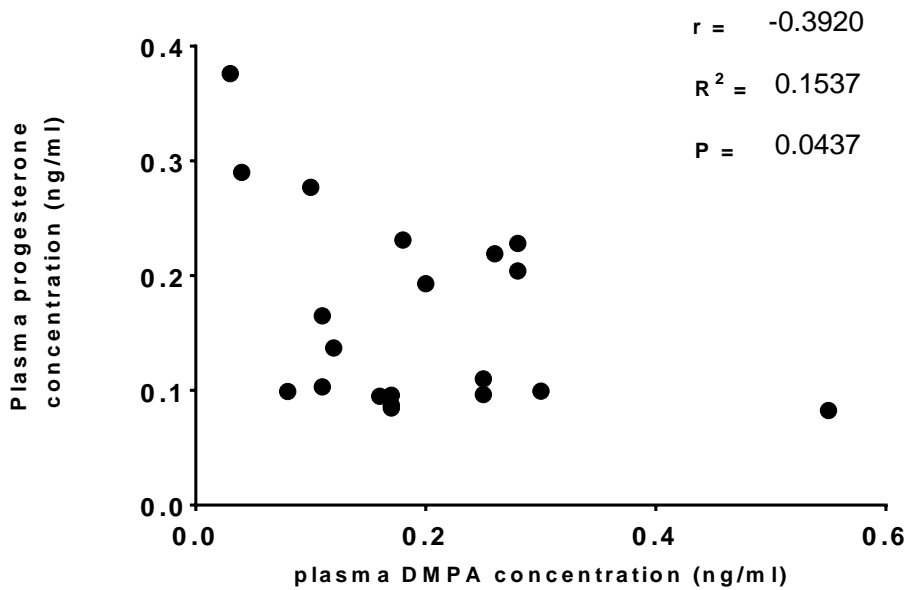


Figure 5.2 Plot showing DMPA-progesterone correlation.

Sex hormones and hormonal contraceptives are known to modulate changes in the female reproductive tract environment. There are several biologically plausible mechanisms by which hormonal contraceptives could increase HIV risk including disrupting epithelial barriers, causing changes in inflammatory responses that could in turn enhance HIV replication locally, or by altering the vaginal microbiota which itself effects local immunity and genital inflammation [84].

To investigate if hormonal contraceptive DMPA directly affects the female reproductive tract we next examined the proteome of the cervix of Depo- Provera users, measured by our collaborators in cervicovaginal lavage (CVL) samples obtained from the same subjects and at the same time point. CVL proteome was analyzed using shotgun proteomics, and the proteins identified were correlated with DMPA levels using the linear univariate Pearson correlations. Table 5.1 shows the proteins significantly associated with Depo Provera use, most of which were positively correlated with DMPA levels and a few that were negatively correlated.

Table 5.1 Correlation of CVL proteins with DMPA levels in the CAPP004 pilot study.

Positive Correlations	r	Adjusted P value	Rank
ANXA1_HUMAN	0,6657	0,0019	1
RL40_HUMAN	0,6552	0,0023	2
LMO7_HUMAN	0,6488	0,0027	3
GSTM3_HUMAN	0,663	0,0037	4
TRI29_HUMAN	0,6071	0,0058	5
FILA_HUMAN	0,5902	0,0078	6
PSCA_HUMAN	0,588	0,0081	7
ANXA2_HUMAN	0,54	0,017	8
ZN185_HUMAN	0,5344	0,0184	9
K1C15_HUMAN	0,5338	0,0186	10
POF1B_HUMAN	0,5168	0,0235	11
MUC4_HUMAN	0,5096	0,0258	12
EF2_HUMAN	0,4964	0,0306	13
AHNK_HUMAN	0,4908	0,0329	14
PERM_HUMAN	0,4908	0,0329	15
TGM1_HUMAN	0,4892	0,0335	16
PEPL_HUMAN	0,4858	0,035	17
IL1RA_HUMAN	0,4797	0,0377	18
K2C78_HUMAN	0,4736	0,0405	19
ECM1_HUMAN	0,4729	0,0409	20
MDHM_HUMAN	0,4681	0,0433	21
GBP6_HUMAN	0,4807	0,0434	22
SUMO2_HUMAN	0,4672	0,0437	23
S10AG_HUMAN	0,4572	0,049	24
K2C5_HUMAN	0,4572	0,049	25
TPM4_HUMAN	0,4567	0,0493	26
KRT85_HUMAN	0,4559	0,0498	27
Negative Correlations	r	P value	Rank
MUC5A_HUMAN	-0,5278	0,0202	1
SEMG2_HUMAN	-0,4793	0,0379	2
CALL5_HUMAN	-0,4759	0,0394	3

All samples (DMPA, Net-En and OC users), were then grouped into high, medium and low DMPA levels using arbitrary cut-offs based on distribution of data. A LASSO (least absolute shrinkage and selection operator) and PLSDA (partial least-squares discriminant analysis) were used to identify a set of proteins that best differentiated the groups. In Figure 5.3 LV1 (latent variable 1) is a regression score for the linear combination of proteins that best differentiate the groups, and LV2, is the second best. Each dot is an individual, coloured by 3 groups: red-high, green-medium and blue-low DMPA levels. PLSDA tries to pull these three groups apart to see maximum differences. The loadings plot shows the proteins that are associated with the groups and a full description of the proteins is given in Table 5.1. Although preliminary, these data strongly suggest that Depo Provera use significantly impacts on the female genital tract proteome that may in turn modulate HIV transmission. The specific function of the top protein hits are under investigation.

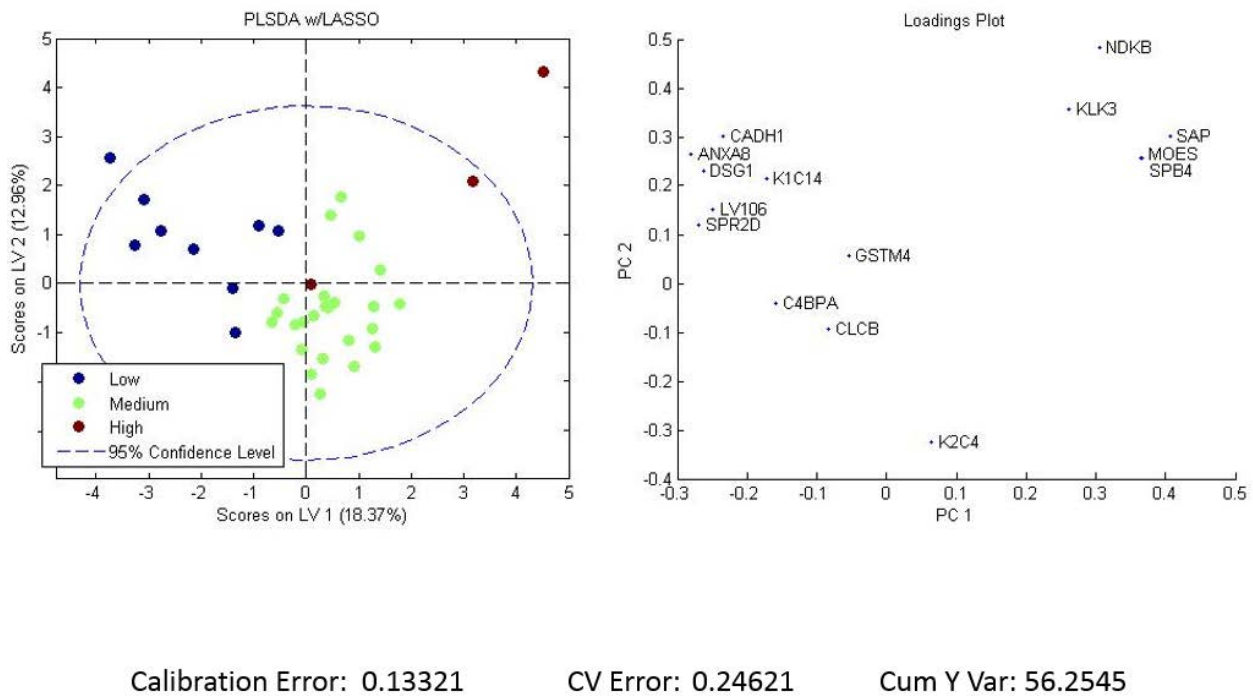


Figure 5.3 Protein signature associated with high, medium and low DMPA levels. PLSDA w/ LASSO (Least Absolute Shrinkage and Selection Operator) is used to identify a protein signature that best classified high (red), medium (green) and low (blue) groups, with calibration error = 0.13321 and cross validation = 0.24621. Protein contributions to the identified signature can be visualized in the loadings plot.

Table 5.2 List of CVL proteins found to be differentially expressed in high, medium and low DMPA levels.

Protein	Full Name	¹General function
CADH1	Cadherin-1	Epithelial adhesion protein
ANXA8	Annexin A8	Anti-coagulation
DSG1	Desmoglein-1	Intercellular junctions, cell-cell adhesion
K1C14	Keratin, type I cytoskeletal 14	Structural, keratin filament binding
LV106	Ig lambda chain V-I region WAH	Antigen binding
SPR2D	Small proline-rich protein 2D	Keratinization
GSTM4	Glutathione S-transferase Mu 4	Enzyme binding, glutathione transferase activity
C4BPA	C4b-binding protein alpha chain	Compliment activation, classical pathway
CLCB	Clathrin light chain B	Intracellular protein transport, endocytosis
K2C4	Keratin, type II cytoskeletal 4	Structural, cytoskeleton organization
SPB4	Serpin B4	Protease inhibitor
MOES	Moesin	Cell-adhesion binding molecule
SAP	Proactivator polypeptide	Enzyme activator activity
KLK3	Prostate-specific antigen	Hydrolase activity, liquefaction of seminal coagulum
NDKB	Nucleoside diphosphate kinase B	Nucleotide metabolism

¹ General functions are based on each protein's gene ontology obtained from the UniProt website.

This preliminary data forms part of an ongoing study, investigating changes in CVL proteomic signatures in Depo Provera users followed over time. Longitudinal analysis of DMPA concentrations in biological samples will allow us to profile the DMPA decay curve over time and determine if the high or low levels are associated with infection time points. For example in Figure 5.4, we show DMPA decay profiles of 5

of the 14 patients that have already been analyzed. This is an ongoing study, with approximately 700 more samples yet to be analyzed. Although also somewhat preliminary in nature it is apparent from these data that decay rates of DMPA are variable between individuals. Presumably, levels remain above that required for activity as a contraceptive agent. However, the level at which DMPA influence the immune response may be quite different. This, therefore again, highlights the potential importance of being able to directly quantify DMPA levels rather than rely on reporting times for the last injection.

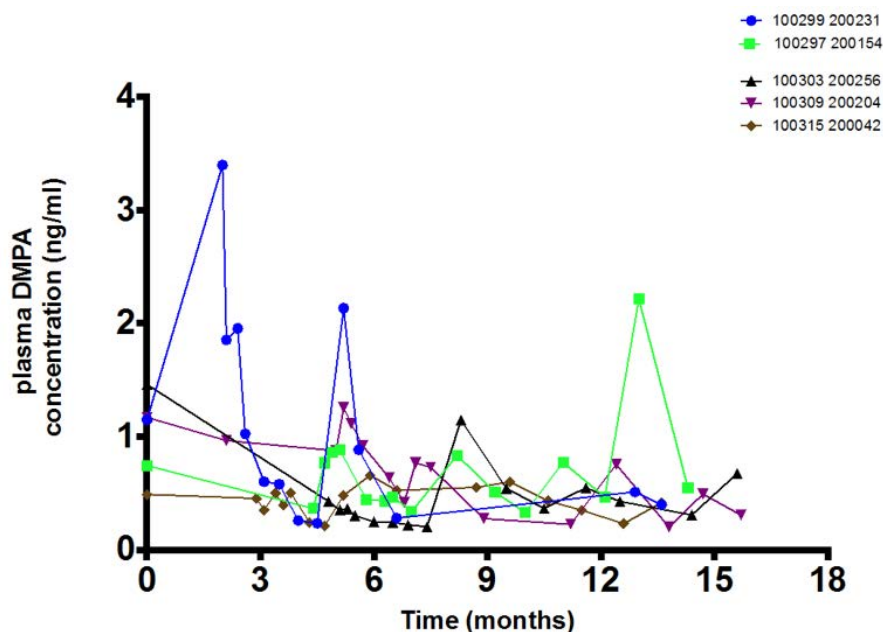


Figure 5.4 Longitudinal profiling of DMPA decay curve in 5 patients using Depo Provera (DMPA).

A second cohort was also used for validation of our LC-MS/MS method, the FRESH cohort. To investigate effects of DMPA levels on risk of HIV acquisition, a blinded analysis of female plasma samples (n = 62) was done to determine DMPA levels. When un-blinded, patients were stratified according to their contraceptive use (Table 5.2). 38 of the total of 44 self-reported Depo-Provera users had significant levels of DMPA while 4 were negative (below limit of quantification) by mass spectrometry. Of the non- Depo-Provera users, 1 of 9 in the no family planning group and 1 of 9 in the Net-En group were positive for DMPA.

Table 5.3 Confirmation of self-reported hormonal contraceptive use by quantification of DMPA levels using LC-MS/MS.

n = 62	Number of patients	DMPA MS results	
Depo Provera users	44	38 (positive)	86%
		4 (negative) *	
No Family Planning	9	8 (negative)	88%
		1 (positive)**	
Net-En users	9	8 (negative)	88%
		1 (positive)***	

- * - 3 Only used Depo for less than 6months, 1 no longer using Depo.
- ** - briefly used Depo approximately 5months prior.
- *** - positive for Depo, but only reports using Net-En for 10 months.

Individuals in this study, were enrolled as HIV negative. Despite intensive counseling and education, 24 women became HIV infected by the time of analysis. An analysis done by our collaborators showed that injectable progestin-based contraceptive users (DMPA and Net-En) combined had a higher risk of HIV acquisition compared to non-family planning users ($p=0.0142$) (Figure 5.4). There was no association between increase in DMPA levels and increase in risk of HIV acquisition.

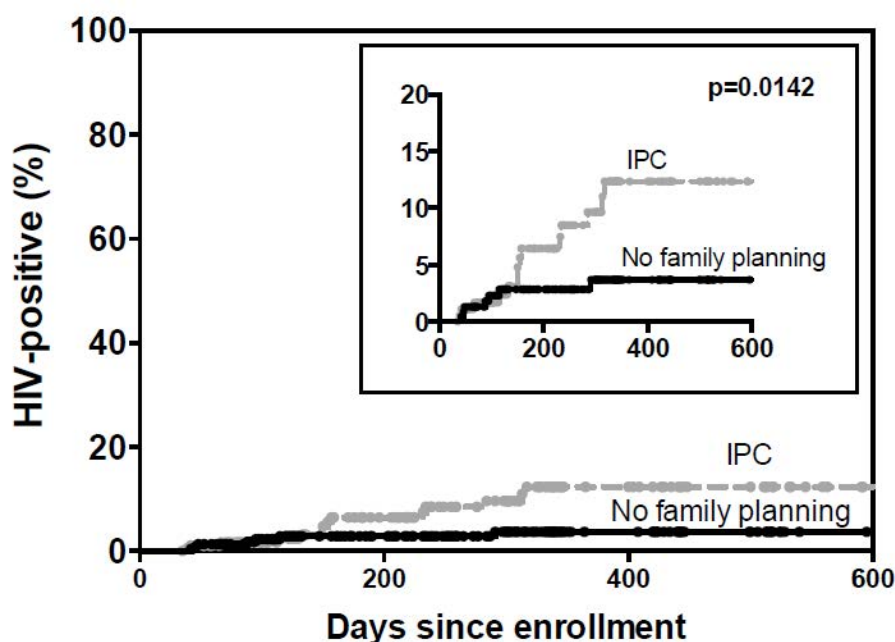


Figure 5.5 HIV incidence comparison between no family planning group and injectable progestin-based contraception (IPC) group. P-value in Kaplan-Meier curve determined by a log-rank test (*Manuscript accepted for publication*- in Appendix).

5.4 DISCUSSION

Measurement of sex hormones and hormonal contraceptives using mass spectrometry allows for identification and quantitation of these biological molecules with high specificity and sensitivity. With regards to contraception use, this method has the potential in clinical studies, to eliminate the need to rely on self-reported information, on what contraceptive they could be using. For example, in Figure 5.1, a patient classified in the Net-En users group had significantly high levels of DMPA in their plasma. In Table 4.4, we show that these two molecules have structurally different molecular ion peaks and m/z transitions (norethisterone: 299.161--->91.000; 299.161--->65.100 and DMPA: 387.003--->327.100; 387.003--->122.900). Such highly levels of DMPA could mean that this patient misreported information on their health questionnaire or alternatively, since injections are administered at hospitals and clinics, patients may be misinformed on which contraceptive they are using. Another explanation could be that the patient was using Net-EN upon enrolment but had subsequently changed to Depo-Provera injection when the sample was obtained. Even in a tightly followed cohort as the FRESH cohort, there were some inconsistencies in reporting, which could be explained as indicated in Table 5.3. Therefore this suggests a huge potential for

mass spectrometry methods, not only for quantitation of molecules but for identification of exogenous molecules in biological samples to eliminate errors associated with self-reported information.

In Figure 5.1, a decrease in progesterone levels in both Depo-Provera and Net-EN users is observed compared with OC users, though not significant, suggesting that Net-En may also modulate progesterone levels in a similar mechanism to DMPA. However since Net-En is only a two-month acting injectable compared to the three-month for DMPA, it is not as strong a suppressor of progesterone as DMPA. DMPA inhibits ovulation by suppressing levels of the luteinizing hormone (LH) and follicular-stimulating hormone (FSH), subsequently serum progesterone levels remain low (< 0.4 ng/mL) for several months following an injection of DMPA [82, 83]. As such, we observed a negative correlation between progesterone and DMPA levels, as expected. There were no significant differences in testosterone levels between the groups, suggesting that testosterone levels may remain unaffected by contraception use.

The female genital tract possesses several mechanisms of protection including epithelial barrier of the vagina and cervix, immune cells, and various molecules secreted by epithelial cells, immune cells, and microbiota [81]. For initiation of infection in the FRT, HIV must infect the highly susceptible cervical and vaginal immune cells in the submucosa, which are protected by either the squamous or single layer columnar epithelium of the cervical or vaginal compartments, respectively. [85]. FRT secretory proteins have been shown to have the ability to provide protection against HIV and other sexually transmitted infections *in vitro*, but changes in their expression may facilitate HIV infection by activating target cells or increasing viral replication. Changes in the expression of these soluble mediators have been observed throughout the menstrual cycle and during pregnancy, providing support for the hypothesis that reproductive hormones may increase HIV risk through modulation of soluble mucosal defense. [81]. Comprehensive qualitative proteomic studies on cervicovaginal samples show that this matrix contains proteins/peptides with intrinsic anti-HIV activity such as defensins, lactoferrin, lysozyme, cathelicidin and SLPI [86, 87]. A decrease expression of an anti-inflammatory serine protease inhibitor, SPLI, known to have anti-HIV activity was observed after treatment with DMPA [88]. However, the effect of exogenous hormones, particularly DMPA on changes in cervical and vaginal proteome has been less studied.

Our aim was therefore to study changes in the proteome of CVL samples in women with varying levels of DMPA. A number of proteins were positively correlated with DMPA levels, as shown in Table 5.1. Some of these proteins form part of the cytoskeleton and are necessary for structural integrity of the epithelia, such as, K1C15, POF1B, FILA and KRT85 amongst others. PCSA and ZN185 are known for regulation of cell proliferation and/or differentiation. SEMG1, a semen protein was negatively correlated with DMPA

levels. Some proteins have been previously shown to play a role in HIV infection, or at least in the susceptibility of the female reproductive tract to viral infections, such as ANXA2, MUC4, and MUC5A. Annexin 2 (ANXA2), was shown to bind Gag protein and associated with increase in infectivity of virion particles in monocyte-derived macrophages [89]. However, Rai et al (2010), showed that Annexin 2 is rather involved in a cell type-dependent role in regulating production of infectious HIV-1 by macrophages than HIV-1 virion particle production [90]. MUC4 and MUC5A are mucus proteins secreted by epithelial cells for mucosal protection. These mucins are found in the cervicovaginal mucus, which has been shown to have activity that hinders HIV-1 movement. [91]. Therefore downregulation of gel-forming MUC5A by DMPA may aid in increasing risk of HIV acquisition.

In the PLSDA analysis, a cluster of 5 proteins in the PC1 vs PC2 plot, were found to be associated with high levels of DMPA: MOES, NDKB, KLK3, SAP and SPB4. A study by Barrero-Villar suggests that Moesin (MOES), a cell adhesion molecule may facilitate HIV-1-induced CD4-CXCR4 interaction and therefore efficient HIV-1 viral fusion and infection [92]. Therefore upregulation of MOES at high DMPA levels may play a role in increased risk of HIV infection. Serpin B4 (SPB4) is known to have anti-HIV activity, and was shown in the study by Burgener et al, (2008) to be overexpressed in HIV-resistant women [93]. On contrary, we observe an association of SPB4 with high DMPA levels and DMPA has been suggested to increase risk of HIV.

In the FRESH cohort study, a higher risk of HIV acquisition is observed among the IPC users compared to individuals not using any form of contraception (Figure 5.5). Although both DMPA and Net-En were associated with greater risk of HIV acquisition, Net-En conferred a particularly high risk compared to DMPA. The findings of association of IPCs with increased HIV risk, are in agreement with some of the published studies [79], however, contrary to other studies who have reported no risk for Net-En or even for DMPA [94, 95, 96]. Limitations of prior studies are confounding factors, mainly due to differences in behavioral risk between non- hormonal contraceptive users, and long-term injectable DMPA and/or Net-En users. In this cohort, one of the strengths is that potential confounders such as condom use, sexual behavior, and differences in age were assessed and controlled for. A biological explanation for this observed increased risk amongst IPC users, was the observation of an increase in CCR5+CD4+ T cells and also an increase in expression level of CCR5 in CD4+ T cells in the cervix compared to no family planning group (*manuscript in Appendix section*). CCR5 co-receptor is used for entry by HIV into CD4+ T cells, therefore increased expression of these HIV target cells in the cervix provides a possible explanation for increased HIV acquisition in women using injectable contraceptive.

5.5 CONCLUSION

We have used mass spectrometry methods for detection and quantitation of exogenous and endogenous sex hormone levels, which is a very reliable tool as it eliminates uncertainties associated with self-reported information by patients. We observe an association of injectable contraceptives with protein signatures that have been previously shown to play a role in HIV infection, and also an increase in HIV target cells in the cervical compartment of IPC users. These findings suggests a role for IPC in HIV acquisition.

Therefore women using progestin-only injectable contraceptives such as DMPA or NET-EN should be informed on the current observation of possible increased risk of HIV acquisition associated with these hormonal contraceptive methods. This will help women make more informed choices about birth control. In addition, new strategies are needed for alternative effective and cheaper methods of contraceptives to then find a balance between contraceptive use and unwanted pregnancies especially in the high risk countries.

6.0 EFFECT OF SEX HORMONES ON ADAPTIVE IMMUNE RESPONSES

6.1 LITERATURE REVIEW

The effect of sex steroid hormones on the immune responses to human TB infection remains less studied. Even though evidence of influence of sex hormones on immune functions have been shown in simple animal experiments involving castration and hormone replacement [44]. Literature relevant to this topic is discussed in Chapter 3, generally, progesterone and testosterone have been shown to play an immunosuppressive role in TB infection. In addition, progestin-based DMPA has been shown to affect control of mycobacterial infection and BCG-induced cytokine expression in mice studies [97], and is linked to increased risk of HIV acquisition, as described in the previous chapter. However effect of these hormones in human TB infection and, whether there is any synergistic effect in HIV and TB co-infection is unknown.

AIMS

- a) *To quantitate levels of progesterone, testosterone and DMPA in a cohort of HIV-infected females and males using LC-MS/MS.*
- b) *To determine their effects on adaptive immune responses to TB and HIV by:*
 - i) *Evaluating changes in phenotype expression of T regulatory cell subsets, naïve and memory subsets as well as changes in activation and exhaustion of CD4 and CD8 T cells.*
 - ii) *Evaluating changes in cytokine expression in response to TB, HIV and other (CMV and SEB) antigens.*

6.2 MATERIALS AND METHODS

6.2.1 Ethics statement

Ethical approval for the Cryptococcal cohort study was obtained from the University of KwaZulu-Natal Medical Research Ethics Committee in Durban and Partners Healthcare's Institutional Review Board in Boston.

6.2.2 Study design

The study design used for the Cryptococcal cohort study was cross sectional. Participants were initially recruited as part of a larger cohort for screening of cryptococcal meningitis at iThembalethu clinic in Durban. 271 participants, composed of 172 females and 99 males were randomly selected. All participants were HIV infected adults aged ≥ 18 years. Patients were recruited at time of initial HIV diagnosis, before initiation of antiretroviral therapy. Clinical and demographic data such HIV status, gender, contraceptive use, CD4 cell count were captured. All the study participants gave written informed consent.

Table 6.1 Cohort characteristics

	Total samples (n = 271) All HIV + No ART	Age range (years)	Age median (years)	TB culture positive (n)	Reported DMPA contraception use	Reported Net-En use
male	99	18 – 65	31	11	-	-
female	172	19 - 58	31	11	3	3

6.2.3 Screening tests

HIV-1 testing was done at iThembalethu clinic in Umlazi Clinic before enrolment into the study. Spot and induced sputum samples were collected and active TB infection was confirmed by both positive Ziehl-Neelsen (acid fast) stain and growth of one or more colonies cultured in Middlebrook 7H11 agar.

6.2.4 Specimen collection

Blood was drawn into EDTA collection tubes. Plasma samples were collected after centrifugation of blood and PBMCs were isolated using the standard Ficoll gradient protocol. All samples (1ml plasma and 5million cells/ml) were stored at -80°C and subsequently transferred into liquid nitrogen for long term storage.

6.2.5 Measurement of sex hormones and contraceptive analogues in plasma using LC-MS/MS

Measurement of sex hormone and contraceptive levels was achieved using the method and materials described in Chapter 4.

6.2.6 Preparation of peripheral blood mononuclear cells (PBMC) for intracellular cytokine staining (ICS) and immunophenotyping

Frozen PBMC sample vials were thawed in a water bath at 37°C until a small ice pellet was left. The vials were then disinfected with 70% ethanol and immediately transferred into the biosafety cabinet. Vial contents were emptied into a 15ml Falcon tube with R10 media (8ml) containing DNase, to prevent clumping of cells. Media was topped-up to 10ml and cells were washed by centrifugation (Beckmann Coulter Allegra X) for 5minutes. Supernatant was discarded and cells were re-suspended in 10ml of R10 media and counted using the T-C20 automated cell counter (Bio-Rad). For cell counting 10µl of cell suspension was mixed with 10µl of Trypan blue dye (Bio-Rad) and transferred into counting slides. Cells were then centrifuged and the pellet suspended in 4ml R10, without DNase. The cell suspension was transferred to 12-well culture plates and incubated at 37°C, 5% CO₂ for 4 hours.

After a 4 hour incubation period, cells were transferred into a Falcon tube, centrifuged and re-suspended in R10 media such that, there were at least 500 000 cells per 200µl volume. Cells were then partitioned for immediate phenotype staining and for ICS.

Immediate immunophenotype staining

200ul volume containing at least 500 000 cells was transferred into a FACS tube and 1ml phosphine buffered saline (PBS) added. Cells were centrifuged and supernatant was discarded, blotting off extra supernatant on a clean paper towel. 25µl of surface stain master mix (prepared as indicated in table 6.2), was added to into each sample tube and incubated in the dark, at room temperature for 20 minutes. Cells

were washed with 1ml PBS twice, and 100µl of eBioscience fixation/permeabilization solution (prepared by mixing 1 part concentrate to 3 parts diluent) was added and incubated in the fridge, in the dark (4°C) for 20 minutes. Cells were removed from the fridge and washed with 1ml, 10X eBioscience buffer. Extra supernatant was blotted off and 25µl of intracellular stain master mix was added into each sample (preparation shown in table 6.2) and samples were incubated in the dark at room temperature for 20minutes. Cells were then washed once with the eBioscience buffer, re-suspended in 500µl of 2% paraformaldehyde (PFA), and stored at 4°C until ready for acquisition on Flow Cytometer.

Intracellular cytokine staining

Another portion of the cells were used in ICS. Approximately 180µl of cell suspension of each sample was transferred into a 96-well plate for 5 different stimulation conditions including non-stimulated control (table 6.2). Different stimulatory antigens (purified protein derivative (PPD), staphylococcus enterotoxin B (SEB), cytomegalovirus peptide pool (pp65) as well as HIV peptide pool mixture (Gag, Pol, Env, Nef, Reg and Acc peptides) for general HIV response were added to their respective wells. 15µl CD107a antibody added was also added at this time and plate was incubated at 37°C, 5% CO₂ for 18 hours. Two hours into stimulation, 0.4µl Golgi stop and 0.4µl Golgi plug were added to each sample to block cytokine release from cells. After 18 hour stimulation, the plate was centrifuged and washed with PBS twice.

Extra supernatant was blotted off and 25µl of surface stain master mix (prepared as indicated in table 1), was added to into each sample well and incubated in the dark, at room temperature for 20 minutes. Cells were washed with 200µl PBS twice, and 100µl of BD Cytofix/Cytoperm solution was added and incubated in the fridge, in the dark (4°C) for 20 minutes. Cells were removed from the fridge and washed with 200µl, 10X BD Perm/Wash buffer. Extra supernatant was blotted off and 25µl of intracellular stain master mix was added into each well (preparation shown in table 1) and samples were incubated in the dark at room temperature for 20minutes. Cells were then washed once with the 10X BD Perm/Wash buffer and cells re-suspended in 500µl of 2% paraformaldehyde (PFA), transferred into FACS tubes and stored at 4°C until ready for acquisition on Flow Cytometer.

6.2.7 Titration of antibodies to determine optimal concentration

Antibodies were titrated to determine optimal working concentration for maximal staining of the CD4+ and CD8+ T cell populations with minimum background staining under the conditions of the experiment. Serial titrations of each antibody were used to stain PBMCs using protocol in section 6.2.6. The samples were

analyzed by flow cytometry to determine the concentration at which the sample is near, but not above, the saturation point (Table 6.2).

Table 6.2. Antibody panel used for ICS and phenotype staining of PBMCs.

INSTRUMENT	BD LSR FORTESSA		
	Fluorochrome	Optimal antibody dilution	Vendor, Cat. No., Clone
ICS panel			
<i>Surface Antibody</i>			
CD4	Alexa Fluor 700	1:100	BD Pharmingen, 557922, RPA-T4
CD8	HV500	1:100	BD Horizon, 560774, RPA-T8
CD14	APC-Cy7	1:50	BD Pharmingen, 557831, MΦP9
CD16	BV650	1:50	Biolegend, 302042, 3G8
CD56	BV711	1:50	Biolegend, 318336, HCD56
CD19	APC-H7	1:50	BD Pharmingen, 560252, SJ25C1
Live/Dead fixable near-IR		1:200	Life Technologies, L10119
<i>Intracellular Antibody</i>			
CD3	PE-CF594	1:100	BD Horizon, 562280, UCHT1
TNF α	PE-Cy7	1:25	Biolegend, 502930, Mab11
IFN γ	PerCP-Cy5.5	1:25	Biolegend, 502526, 4S.B3
IL-2	PE	1:25	Biolegend, 500307, MQ1-17H12
IL-13	V450	1:25	BD Horizon, 561158, JES10-5A2
1L-17	BV605	1:40	Biolegend, 512326, BL168
Mip-1 β	FITC	1:25	R&D Systems, IC271F, IgG ₂ B
CD107a	CD107a	15 μ l	BD Pharmingen, 555802
Phenotype panel			
<i>Surface Antibody</i>			
CD38	PE-Cy7	1:25	Biolegend, 303516, HIT2
PD-1	BV421	1:25	BD Horizon, 562516, EH12.1
CD8	HV500	1:100	BD Horizon, 560774, RPA-T8
CD161	BV605	1:50	Biolegend, 339916, HP-3G10
CD4	BV711	1:50	Biolegend, 317440, OKT4
CD25	BV785	1:50	Biolegend, 302637, BC96
HLA-DR	FITC	1:25	BD Pharmingen, 555560
CD62L	PE	1:50	Biolegend, 304806, DREG-56
CCR7	PerCP-Cy5.5	1:50	Biolegend, 353220, G043H7
CD127	PE-CF594	1:25	BD Horizon, 562397, HIL-7R-M21
CD45RA	Alexa Fluor 700	1:25	Biolegend, 304120, HI100
CD14	APC-Cy7	1:50	BD Pharmingen, 557831, MΦP9
CD19	APC-H7	1:50	BD Pharmingen, 560252, SJ25C1

L/D fixable near-IR		1:200	Life Technologies, L10119
<i>Intracellular Antibody</i>			
CD3	eFluor 650	1:100	eBioscience, 95-0037-42, OKT3
FoxP3	APC	1:10	eBioscience, 17-4776-41, PCH101

6.2.8 Flow cytometry analysis

Calibration of flow cytometer

In flow cytometry, fluorochromes have different emission spectra, however when using a multi-color panel (such as 15-color used in this study), it requires good compensation as different fluorochromes tend to spillover. Compensation allows for correction of spillover by quantifying and subtracting the amount by which each fluorochrome overlaps into another. For this study, a compensation beads kit containing BD negative control beads which have no antibody-binding capacity, BD IgK mouse compensation beads which bind to antibodies was used. To set-up the 15-color panel compensation, 16 tubes were prepared by adding a drop of negative and positive control beads into each tube. Fifteen tubes were each added with a specific antibody with the following fluorochromes: FITC/Alexa Fluor 488, PE, PerCP-Cy5.5, PE-Cy5, PE-Cy7, APC-Cy7, APC, BV421, BV711, BV785, BV650, BV605, PE- Texas Red, Alexa Fluor 700, HV500, and one of the tubes, a negative control was left unstained. The tubes were then incubated for 30 minutes, washed and PBS added. Acquisition was done on the LSR BDFortessa using FACSDiva Software version 7.0. Positive and negative population gates for each fluorochrome were set, while adjusting the PMT values to reduce emission spectral overlap. Compensation was calculated and overlap of below 50% between any two fluorochromes was acceptable. The compensation settings were used in all further analysis. BD rainbow fluorescent particles were used for each acquisition thereafter, to monitor instrumental day-to-day variation. All settings for the flow cytometer were based on instrument laser settings provided in Table 6.3.

Data acquisition

The BD FACSDiva software version 7.0 was used for creation of the acquisition template with the scatter plots forward scatter (FSC) vs. side scatter (SSC) used to define lymphocyte populations. The subsequent gating strategies that were used to for analysis of lymphocyte subsets are shown in the results section of this chapter.

Table 6.3. Flow cytometer instrument settings.

Instrument	BD LSR FORTESSA	
Fluorochrome	Laser (wavelength)	Emission Filter
PerCP-Cy5.5	Blue (488nm)	695/40
FITC/Alexa Fluor 488		530/30
APC-Cy7 /APC-H7	Red (640nm)	780/60
Alexa Fluor 700		730/45
APC		670/30
BV785/ QDot800	Violet (405nm)	780/60
BV711/ QDot705		710/50
BV650/ QDot655		670/30
BV605/ QDot605		610/20
HV500		525/50
BV421/ PacBlue		450/50
PE-Cy7	Yellow-Green (561nm)	780/60
PE-Cy5		670/30
PE- Texas Red/ PE-CF594		610/20
PE		585/15

6.2.9 Materials

Table 6.4 List of materials and reagents used in the methods described above.

Reagents and Materials	Product No., Supplier
RPMI 1640, without L-Glutamine	Lonza, BE12-167F
Pen-Strep	Lonza, DE17-602F
L-Glutamine	Lonza, BE17-605F
Tuberculin PPD	Statens Serum Institut, 2391
Phosphate buffered saline (PBS) tablets	Sigma-Aldrich, P4417-100TAB
BD Perm/Wash buffer 10x	BD Biosciences, 51-2091KZ
BD Cytotfix/Cytoperm	BD Biosciences, 51-2090KZ
Fixation/Permeabilization diluent	eBioscience, 00-5223-56
Permeabilization buffer 10x	eBioscience, 00-8333-56
Compensation beads kit (Anti-mouse Ig-κ/ Negative control)	BD Biosciences, 552843
Rainbow fluorescent particles (mid-range FL1 fluorescence)	BD Biosciences, 556298
FACS clean	BD Biosciences, 340345

FACS rinse	BD Biosciences, 340346
Staphylococcal enterotoxin B (SEB)	Sigma Aldrich, S4881-1MG
pp65 cytomegalovirus peptide (CMV)	Donation from HIV Pathogenesis Programme
HIV peptides (Gag, Pol, Env, Nef, Acc, Reg)	Donation from HIV Pathogenesis Programme
50ml Falcon polypropylene tubes	Corning, 352070
15ml Falcon polypropylene tubes	Corning, 352096
5ml Falcon polystyrene tubes	Corning, 352054
Golgi stop	BD Biosciences, 554724
Golgi plug	BD Biosciences, 555029

6.3 STATISTICAL ANALYSES

Data for quantitation of sex hormones was analysed using Analyst software version 1.6 and Graph Pad Prism version 6. Flow cytometry data was analysed using FlowJo version 9.8 and Graph Pad Prism version 6. Mann-Whitney tests were used to compare median values between two groups. Pearson's correlation was used to determine correlation between DMPA and progesterone. Kruskal-Wallis tests were used for comparisons with more than two independent variables and the Bonferroni and Dunn's tests, used for multiple comparisons. P values ≤ 0.05 were considered significant.

6.4 RESULTS

6.3.1 Mass spectrometry

All 271 patient plasma samples were processed and analysed using mass spectrometry. The method provided information on progesterone, testosterone and DMPA concentration levels on both males and females (Figure 6.1).

Progesterone concentrations amongst females clustered into two groups (Figure 6.1A), with some females in the low group having concentrations below the limit of quantification (LOQ). Generally, females had significantly higher progesterone levels than males as expected ($p = 0.0014$). Most males had progesterone levels near or below the LOQ. However, three reported male participants had significant levels of progesterone, comparable to the group of women with highest progesterone levels.

The method also positively identified patients using the injectable contraceptive Depo-Provera, and informed the levels of DMPA in their plasma (Figure 6.1B). All male patients had no DMPA in their

plasma, indicated by levels below the LOD and LOQ. Females plasma DMPA concentrations, on the other hand, revealed two groups of females with significant DMPA (above LOQ) and non-significant or no DMPA (below LOQ). Correlation plot of DMPA and progesterone levels in females (Figure 6.1D), revealed three clusters or groups with i) high DMPA, low progesterone, ii) no DMPA, low progesterone and iii) no DMPA, high progesterone. This was a significant negative correlation ($p = 0.0016$, Pearson $r = -0.2446$).

Plasma testosterone concentrations (Figure 6.1C), were significantly higher in males than females, as expected ($p < 0.0001$). However there were some outliers, either males with very low or females with very high testosterone levels.

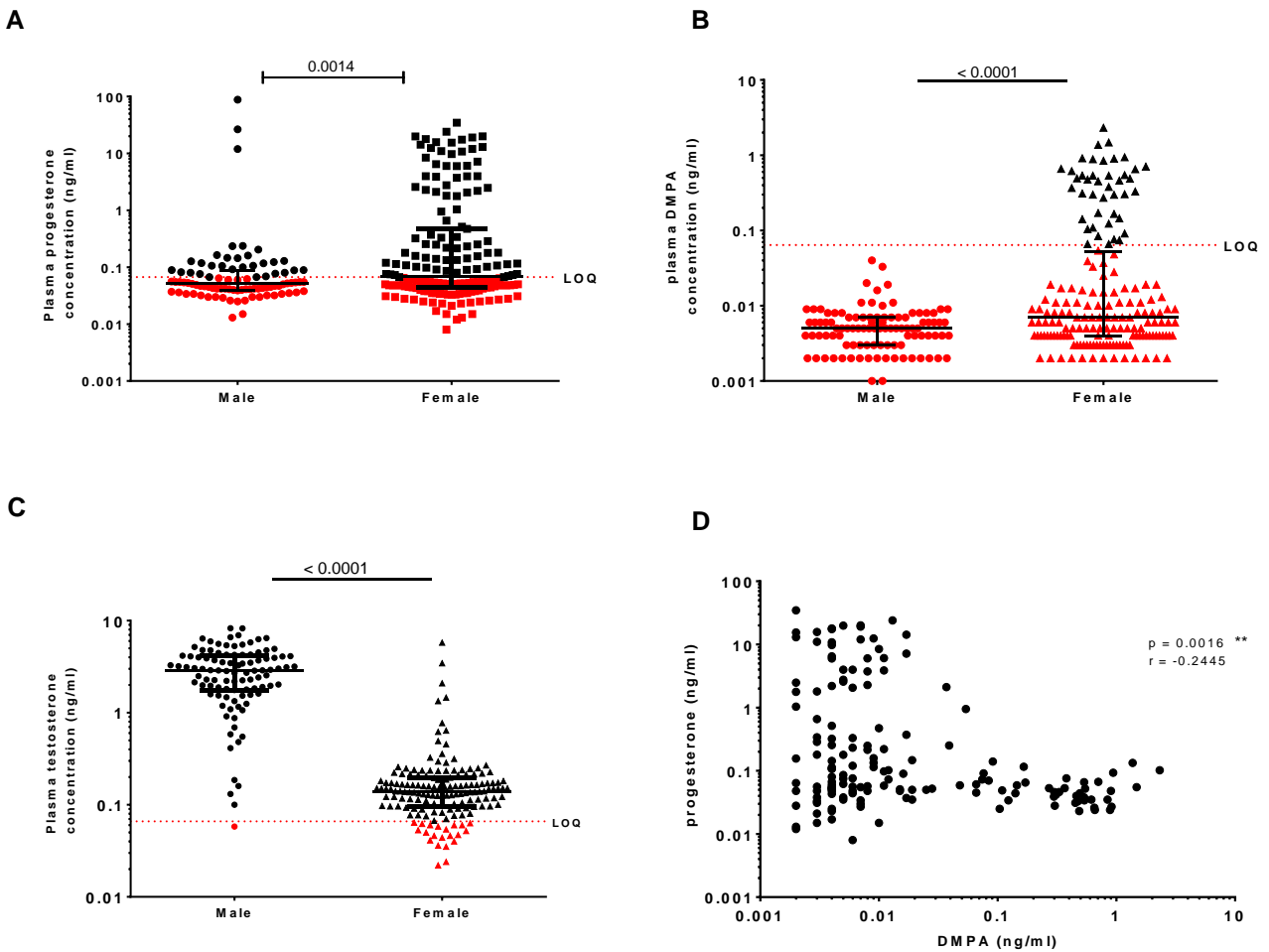


Figure 6.1 Quantification of sex hormones in male and female plasma samples. We illustrate differences in A) progesterone, B) DMPA and C) testosterone levels between males and females. D) Correlation of progesterone and DMPA levels in females (Pearson correlation). Mann-Whitney test was used to compare the groups. Horizontal lines on graphs represents the median. P-values < 0.05 were considered significant.

6.3.2 Patient selection for flow cytometry

All 271 patient plasma samples were used for quantitation of progesterone, testosterone and DMPA. For flow cytometry analysis however, specific groups that would allow us to determine if there were any differences in immune responses as a result of differences in natural hormones levels, differences in contraceptive status, or disease status, were selected from the mass spectrometry data. For females, four groups were selected, one group consisted of 11 patients with active TB disease (culture positive) and from the mass spectrometry data, because of the huge spread in data and in order to see maximum effect if there were any differences at all, we selected two groups with highest progesterone and lowest progesterone levels and were not using injectable contraceptive DMPA. The fourth group were individuals who had DMPA levels above the LOQ, and these patients were selected randomly. For males, we had three groups; 11 individuals with active TB disease and from the mass spectrometry data, two groups were selected with highest and lowest testosterone levels.

6.3.3 Gating strategy used in flow cytometry analysis

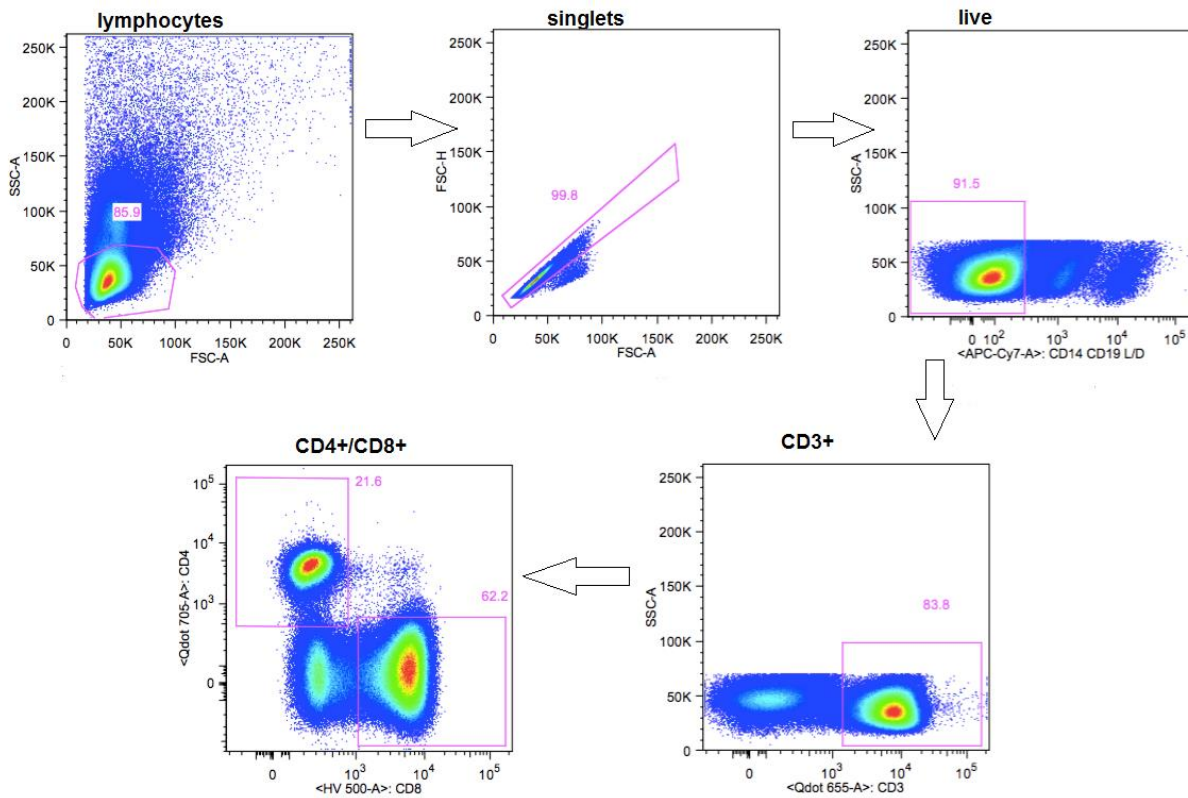


Figure 6.2 Gating strategy. Lymphocyte populations were defined using the SSC-A (side scatter-A) against FSC-A (forward scatter-A). Doublets were excluded by gating on forward scatter-height (FSC-H) against FSC-A. A dump channel was used to gate on live cells, while excluding CD14, CD19 expressing lymphocytes and dead cells. CD3+ lymphocytes were selected by gating on SSCA against CD3. CD3+ T cells were further gated into CD4+ and CD8+ T cell subsets.

6.3.4 Expression of activation and exhaustion markers, CD38, HLA-DR and PD-1

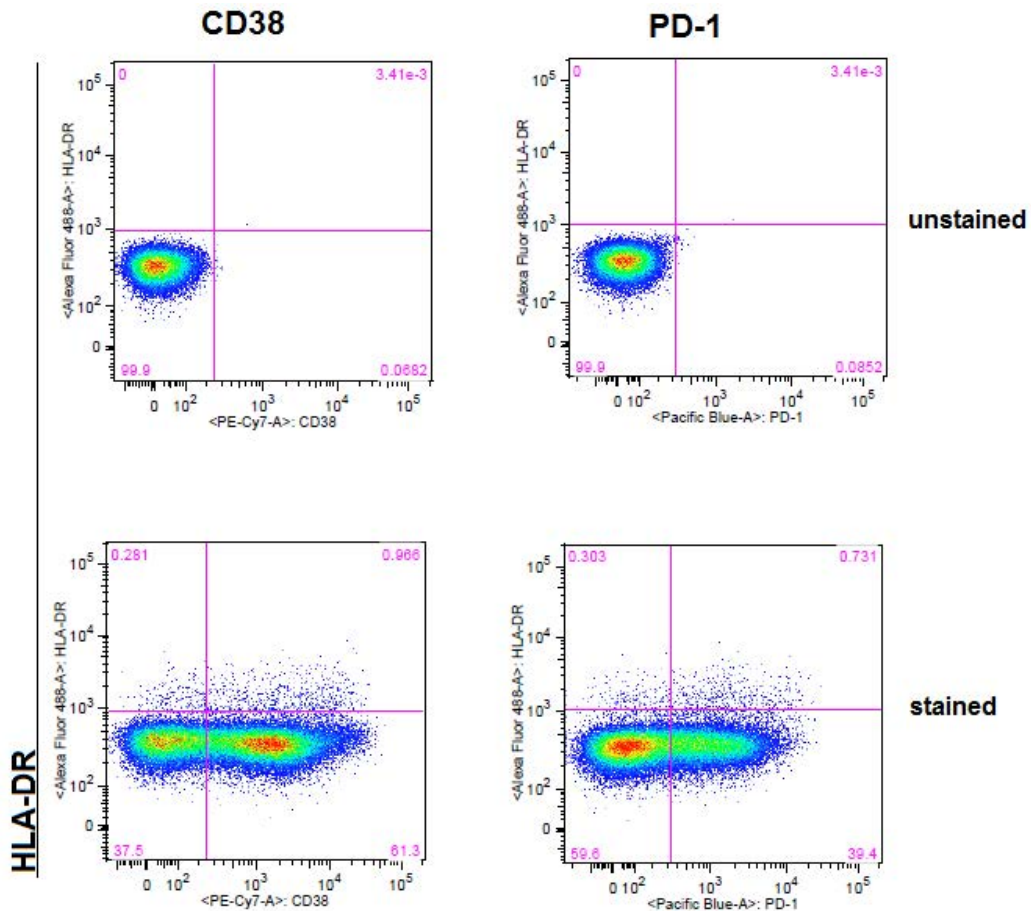
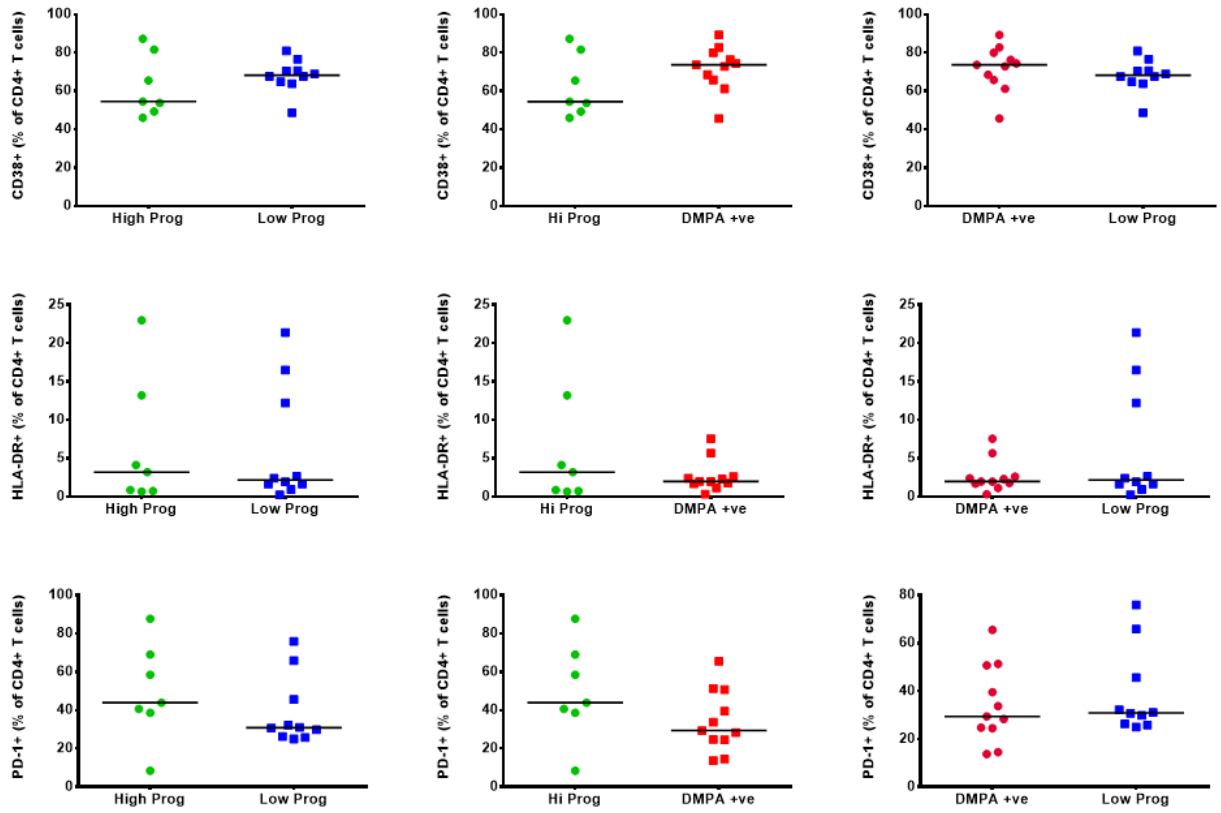


Figure 6.3 Representative illustration of expression of CD38, HLA-DR and PD-1 in CD4+ and CD8+ T cells. Gates were set using an unstained control.

CD4



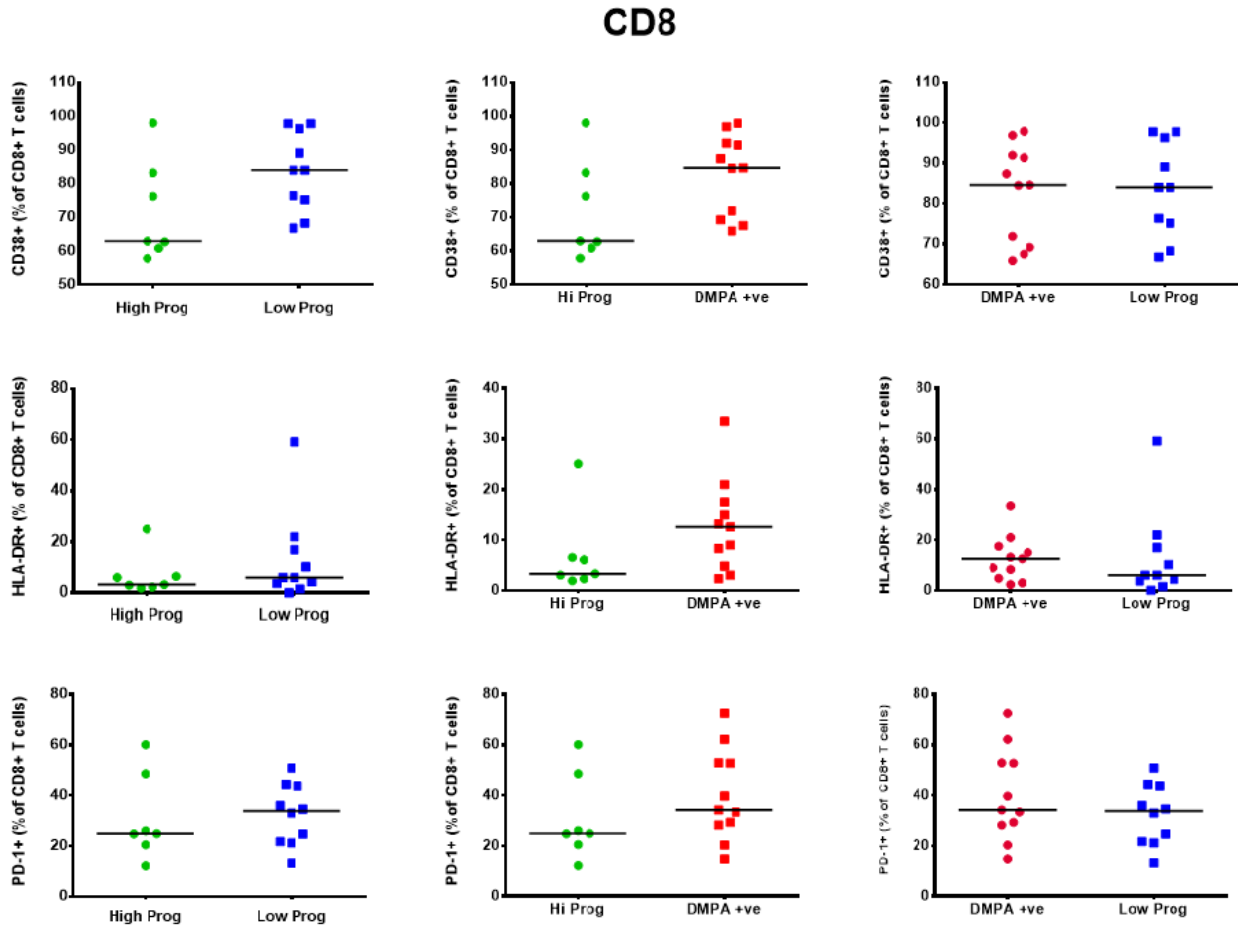


Figure 6.4 Expression of CD38, HLA-DR and PD-1 in both CD4 and CD8 T cells in female subgroups with high progesterone, low progesterone and DMPA positive. Mann-Whitney test was used to compare the groups. Horizontal lines on graphs represents the median. P-values < 0.05 were considered significant.

To examine if differences in progesterone levels and DMPA use had an effect on activation and/or exhaustion of CD4 and CD8 T cells, unpaired non-parametric T tests (Mann-Whitney tests) were used to compare the different groups in Figure 6.4. There were no significant difference in expression of activation markers, CD38 and HLA-DR or exhaustion marker, PD-1. However, a general trend of lower CD38 expression in CD4 and CD8 and lower PD-1 in CD8 T cells amongst female with high progesterone levels compared with the other two groups was observed. DMPA positive and low progesterone groups had comparable CD38 and PD-1 expression for both CD4 and CD8 T cells and PD-1 was, generally, more expressed in CD4 T cells from high progesterone group. Similar to the female subgroups, there were no

significant differences in expression of CD38, HLA-DR and PD-1 between high and low testosterone male subgroups (Figure 6.5).

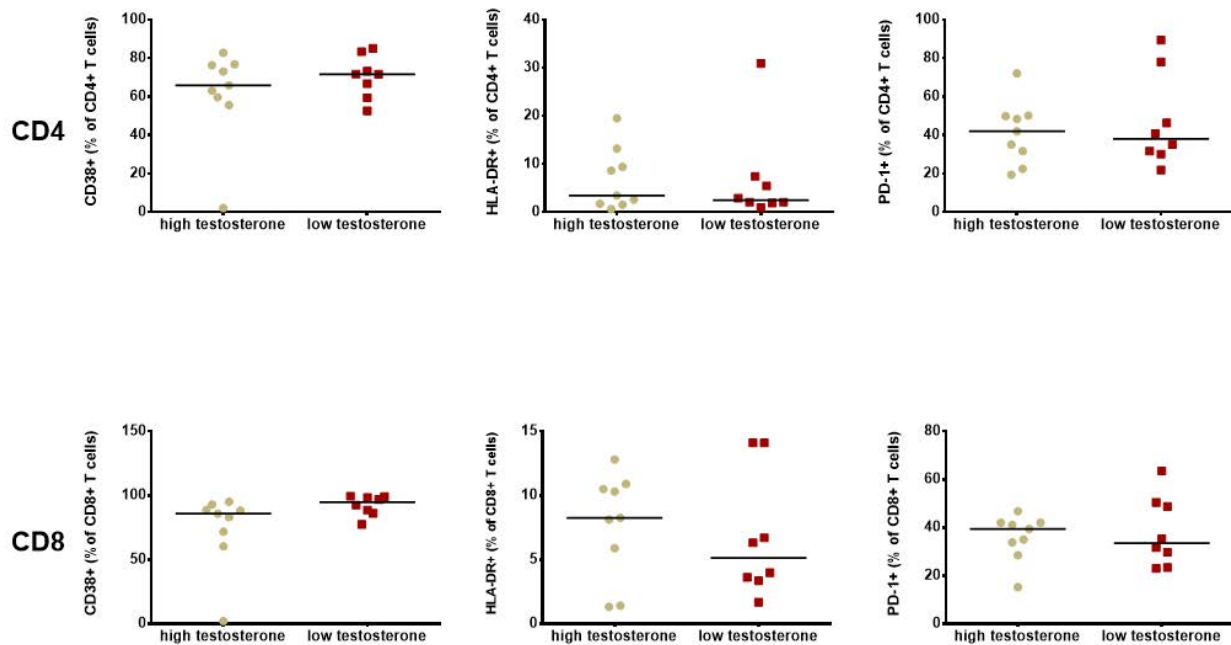


Figure 6.5 Expression of CD38, HLA-DR and PD-1 in both CD4 and CD8 T cells in high and low testosterone male groups. Mann-Whitney test was used to compare the groups. Horizontal lines on graphs represents the median. P-values < 0.05 were considered significant.

6.3.5 Differences in T regulatory subsets

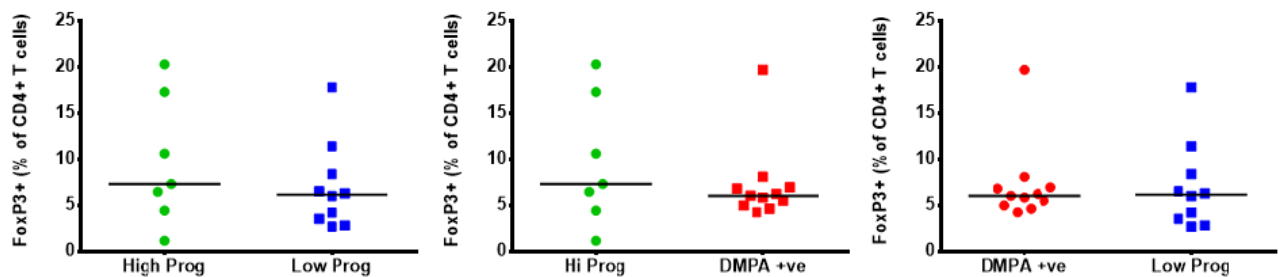


Figure 6.6 FoxP3 expression of CD4 T cells in high progesterone, low progesterone and DMPA positive groups. Mann-Whitney test was used to compare the groups. Horizontal lines on graphs represents the median. P-values < 0.05 were considered significant.

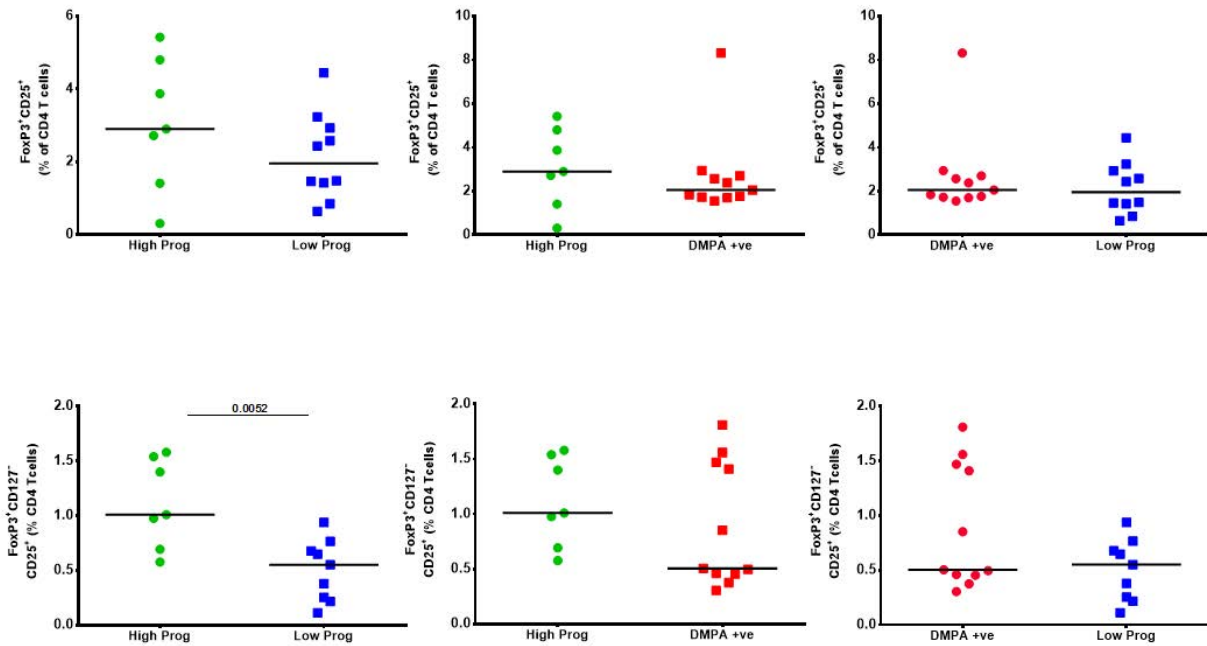


Figure 6.7 Expression of FoxP3+CD25+ and FoxP3+CD25+CD127-/lo CD4 T regulatory cells in high progesterone, low progesterone and DMPA positive groups. Mann-Whitney test was used to compare the groups. Horizontal lines on graphs represents the median. P-values < 0.05 were considered significant.

The effect of differences in progesterone, testosterone and DMPA use on T regulatory cells were analysed. There were no significant differences in expression of FoxP3 in CD4 T cells (Figure 6.6), or in percentage of CD4+Foxp3+CD25+ T cells (Figure 6.7), in females with high and low progesterone levels as well as DMPA positive groups. Significantly higher expression of CD4+FoxP3+CD127-/loCD25+ cells were observed in females with high progesterone levels compared to those with low levels ($p = 0.0052$) In general, the high progesterone group had higher expression of T regulatory subsets compared with low progesterone and DMPA positive groups, whereas the latter groups had comparable expression of these cell subsets.

There was no significant difference in T regulatory subsets between high testosterone males and low testosterone males (Figure 6.8).

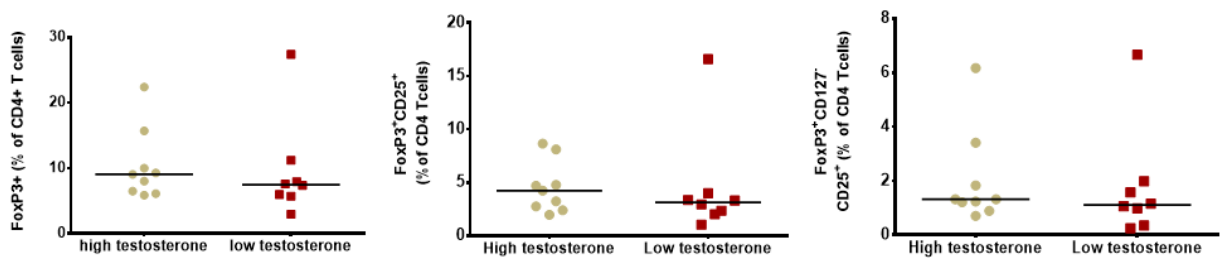


Figure 6.8 Comparison of Foxp3+CD4+ T regulatory subset expression between high and low testosterone males. . Mann-Whitney test was used to compare the groups. Horizontal lines on graphs represents the median. P-values < 0.05 were considered significant.

6.3.4 Differences in expression of naïve and memory T cell subsets

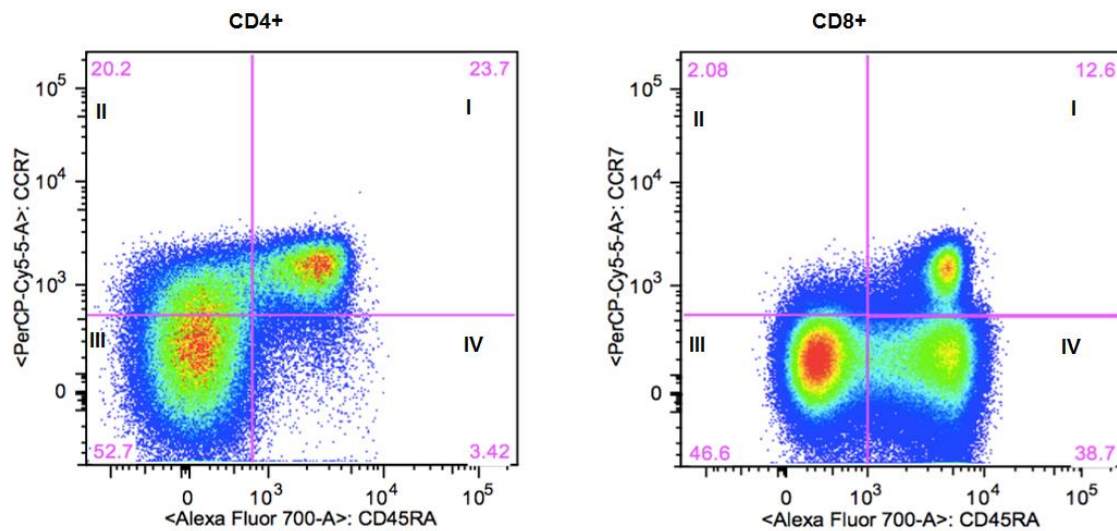


Figure 6.9 Gating naïve and memory T cell subsets, using CCR7 and CD45RA expression. **I.** Naïve T cells. **II.** Central memory T cells. **III.** Effector memory T cells. **IV.** Terminally differentiated effector memory T cells.

Memory and naïve T cell subsets were defined by expression of CCR7 and CD45RA (Figure 6.9). No significant differences in naïve and memory CD4 and CD8 T cells, were observed between high and low progesterone as well as DMPA positive and low progesterone female groups. DMPA positive females had significantly higher expression of CD45RA+CCR7+ (naïve) and CD45RA+CCR7- (terminally differentiated effector memory) CD4 T cells compared with high progesterone group, with p values of 0.0436 and 0.0343 respectively (Fig 6.10). CD45RA+CCR7- subset was also significantly expressed in

CD8 T cells of DMPA positive group ($p = 0.0153$), while high progesterone significantly expressed the central memory subset ($p = 0.053$), as shown in Figure 6.11.

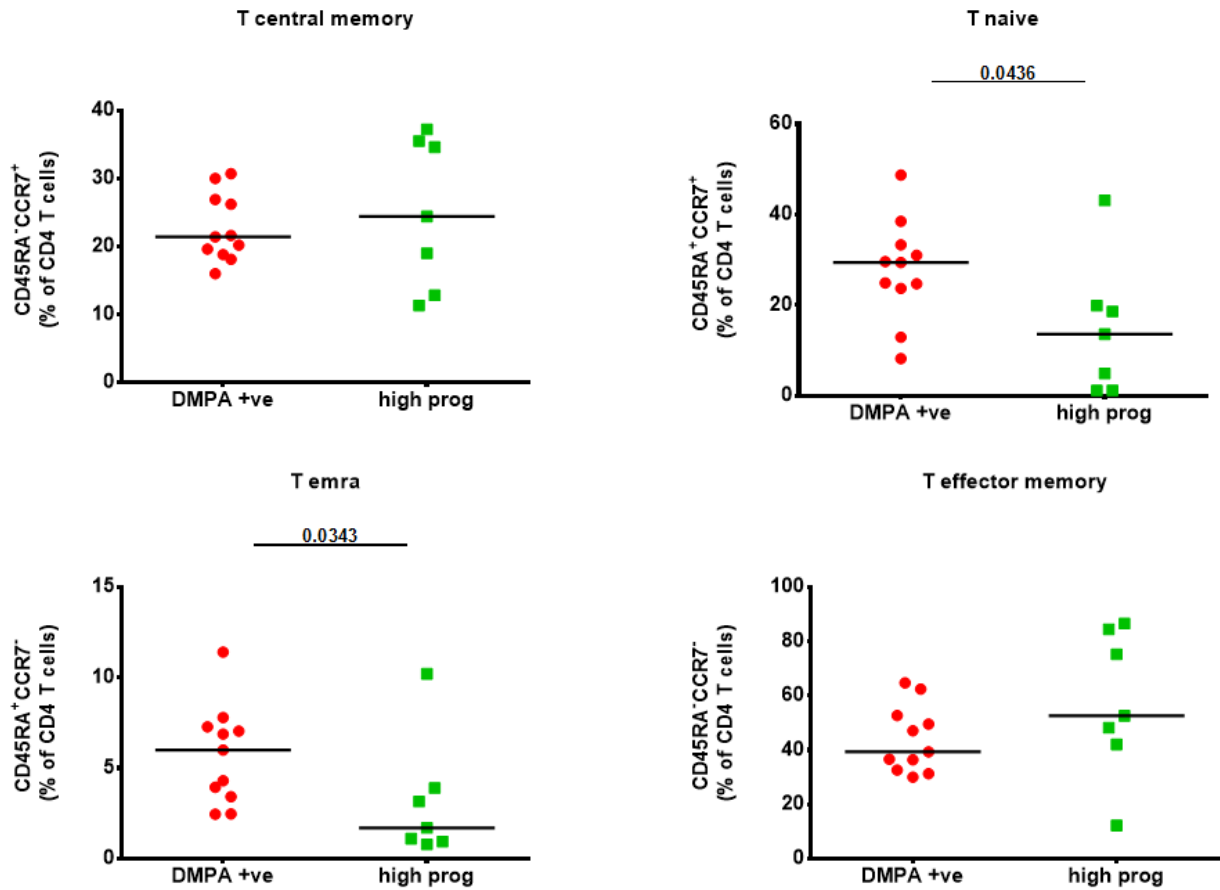


Figure 6.10 Comparison of CD45RA and CCR7 expression as a percentage of CD4 T cells in DMPA positive and high progesterone females. Temra represents terminally differentiated effector memory T cells. Mann-Whitney test was used to compare the groups. Horizontal lines on graphs represents the median. P-values < 0.05 were considered significant.

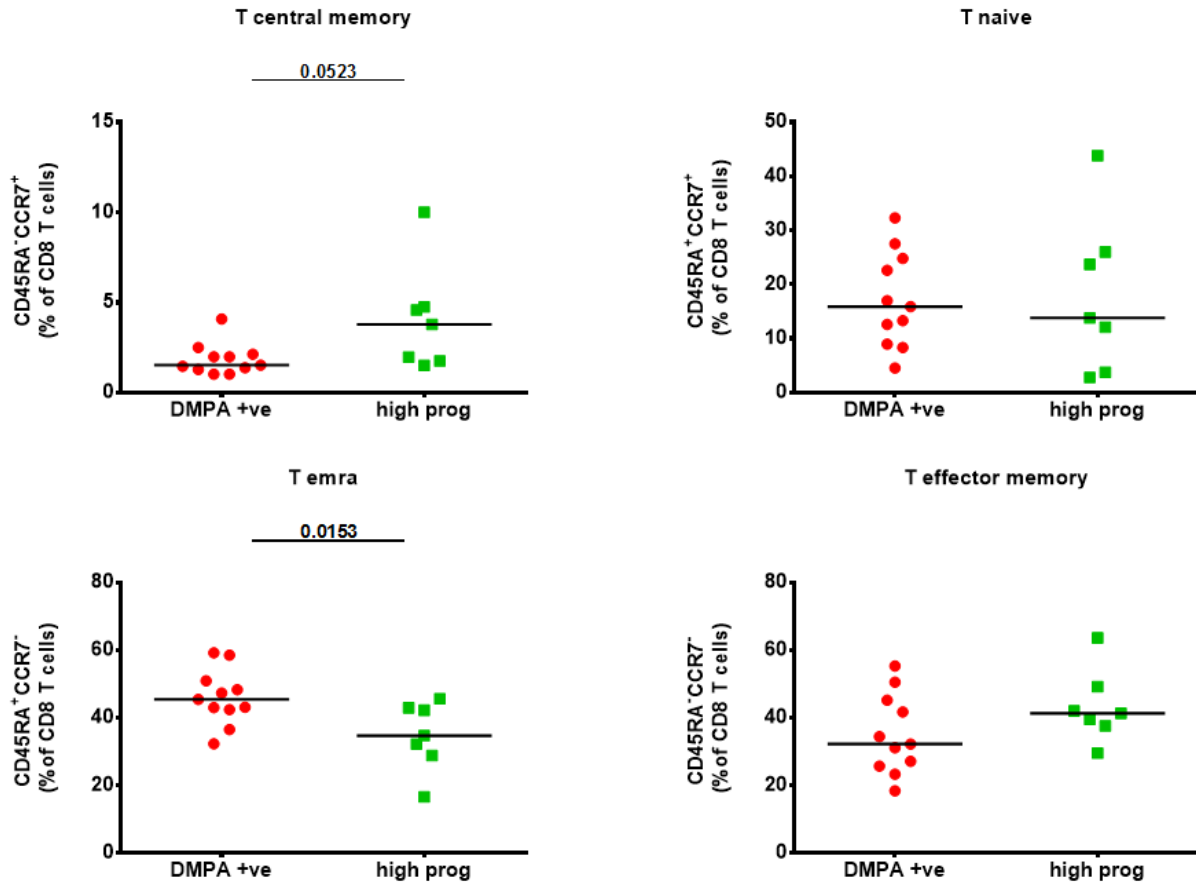


Figure 6.11 Comparison of CD45RA and CCR7 expression as a percentage of CD8 T cells in DMPA contraceptive users and high progesterone females. Temra represents terminally differentiated effector memory T cells. Mann-Whitney test was used to compare the groups. Horizontal lines on graphs represents the median. P-values < 0.05 were considered significant.

Expression of CD45RA and CCR7 was also analysed in high and low testosterone males. In the CD4 T cells, no significant differences were seen naïve and memory subsets. In CD8 T cells however, males with high levels of testosterone had a higher expression of CD45RA+CCR7+ and CD45RA-CCR7+, with $p = 0.0590$ for both subsets. Males with low testosterone had significantly higher expression of CD5RA+CCR7- CD8 T cells (Temra) ($p = 0.0299$), shown in Figure 6.12.

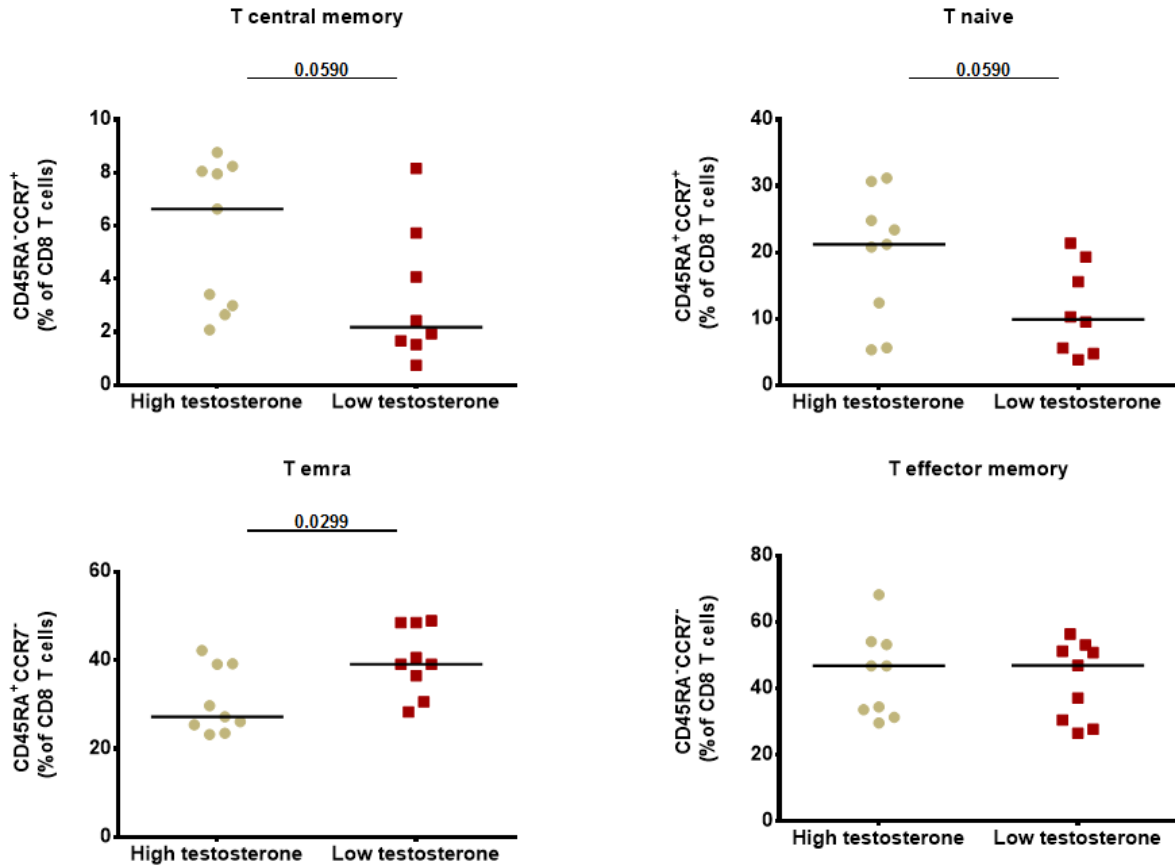


Figure 6.12 Comparison of CD45RA and CCR7 expression as a percentage of CD8 T cells in high and low testosterone males. Temra represents terminally differentiated effector memory T cells. Mann-Whitney test was used to compare the groups. Horizontal lines on graphs represents the median. P-values < 0.05 were considered significant.

6.3.5 Effect of sex hormones on CD4 and CD8 T cell cytokine expression

We next investigated the effect of sex hormones on antigen- specific immune responses of CD4+ and CD8+ T cells. To do this, PBMCs were stimulated with SEB (positive control), PPD, CMV, and HIV peptide pools over 18 hours. An unstimulated sample (negative control) was set-up for each sample for fluorescence background subtraction (Figure 6.13). We first compared differences in expression of immunological factors, TNF α , IFN γ , IL-2, IL-17, Mip-1 β and CD107a, between high progesterone, low progesterone and DMPA positive groups.

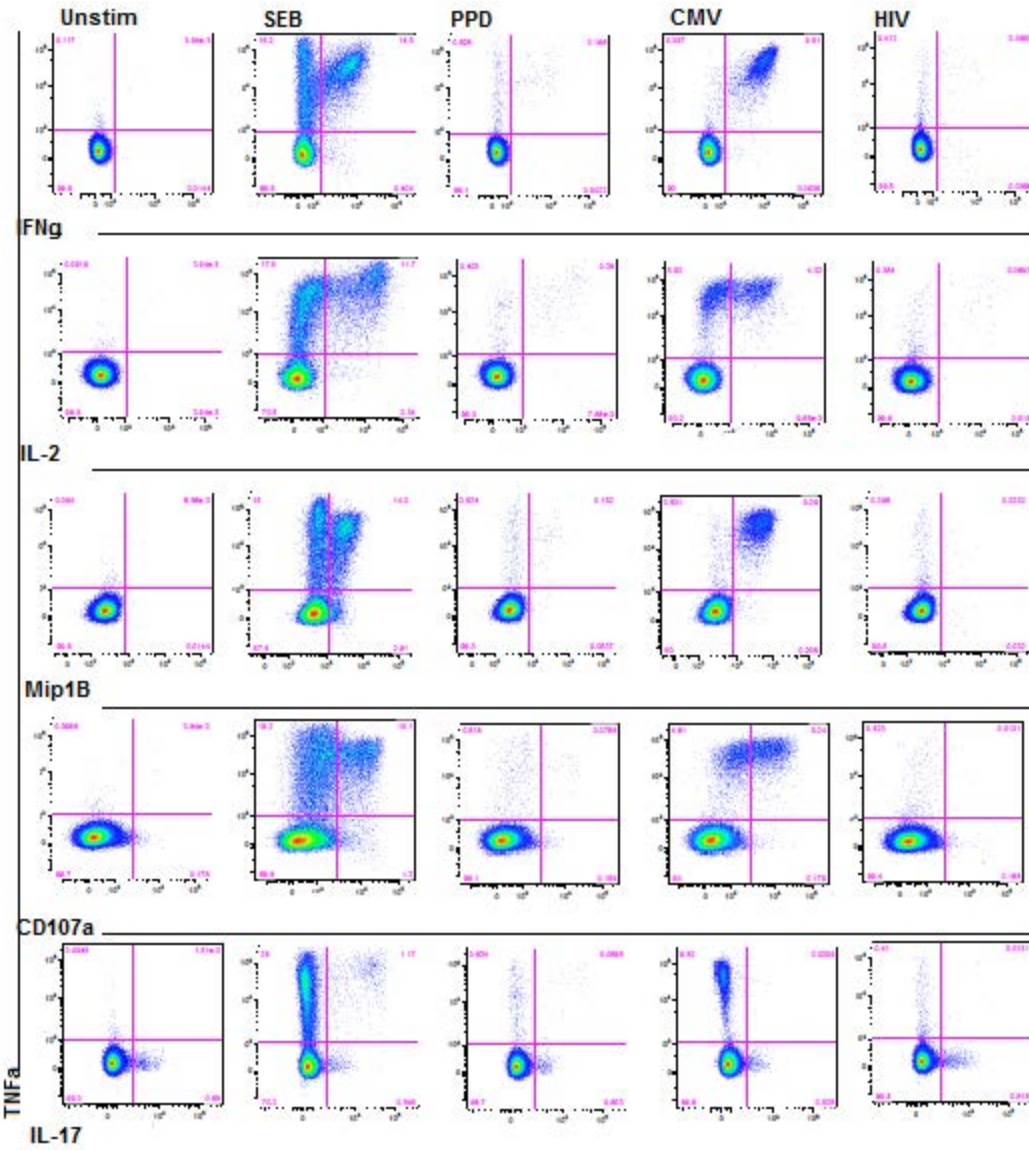


Figure 6.13 PBMCs were stimulated with SEB, PPD, CMV and HIV peptides and flow cytometry was used to determine antigen-specific CD4⁺ and CD8⁺ T cells expressing TNF α , IFN γ , IL-2, IL-17, Mip-1 β and CD107a. Antigen-specific T cell responses were defined by subtracting background signal in the unstimulated value from each antigen stimulated value.

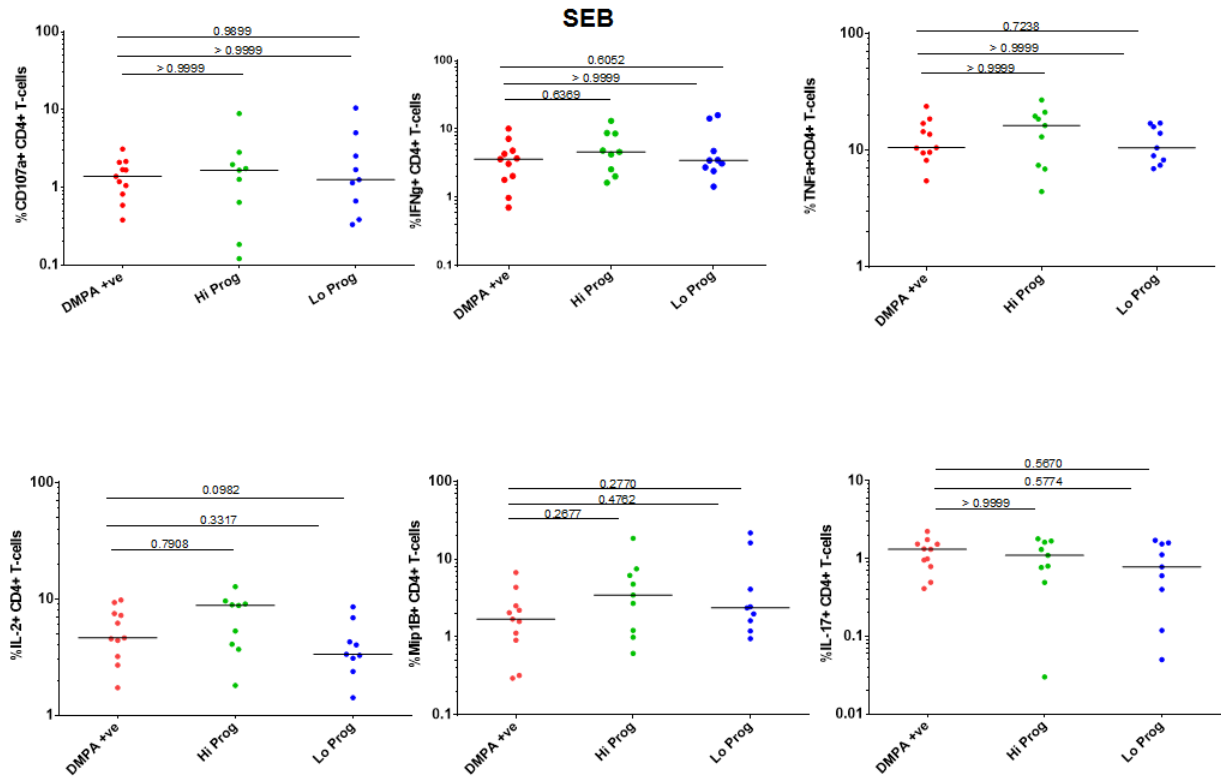


Figure 6.14 Expression of TNF α , IFN γ , IL-2, IL-17, Mip-1 β and CD107a as frequency of parent population CD4+ T cells in SEB stimulated PBMCs. Multiple comparisons using the Kruskal-Wallis test was used to compare the groups. Horizontal lines on graphs represents the median. P-values < 0.05 were considered significant.

Analysis using the Kruskal-Wallis tests and Dunn's multiple comparisons, showed no significant differences observed in CD4 expression of TNF α , IFN γ , IL-2, IL-17, Mip-1 β or CD107a upon stimulation with SEB (Figure 6.14), PPD, CMV and HIV (Appendix). Using Mann-Whitney tests for comparison between two groups at a time, also showed no significant differences in any of the cytokines, except for increased IL-2 expression in high progesterone compared to low progesterone (Figure 6.15), in CD4 T cells stimulated with SEB, ($p = 0.0399$). However, generally, CD4 expression of TNF α , IFN γ , IL-2, and Mip-1 β responses was increased in high progesterone group compared to both low progesterone and DMPA positive groups. Comparison between high testosterone males and low testosterone showed no differences in cytokine expression.

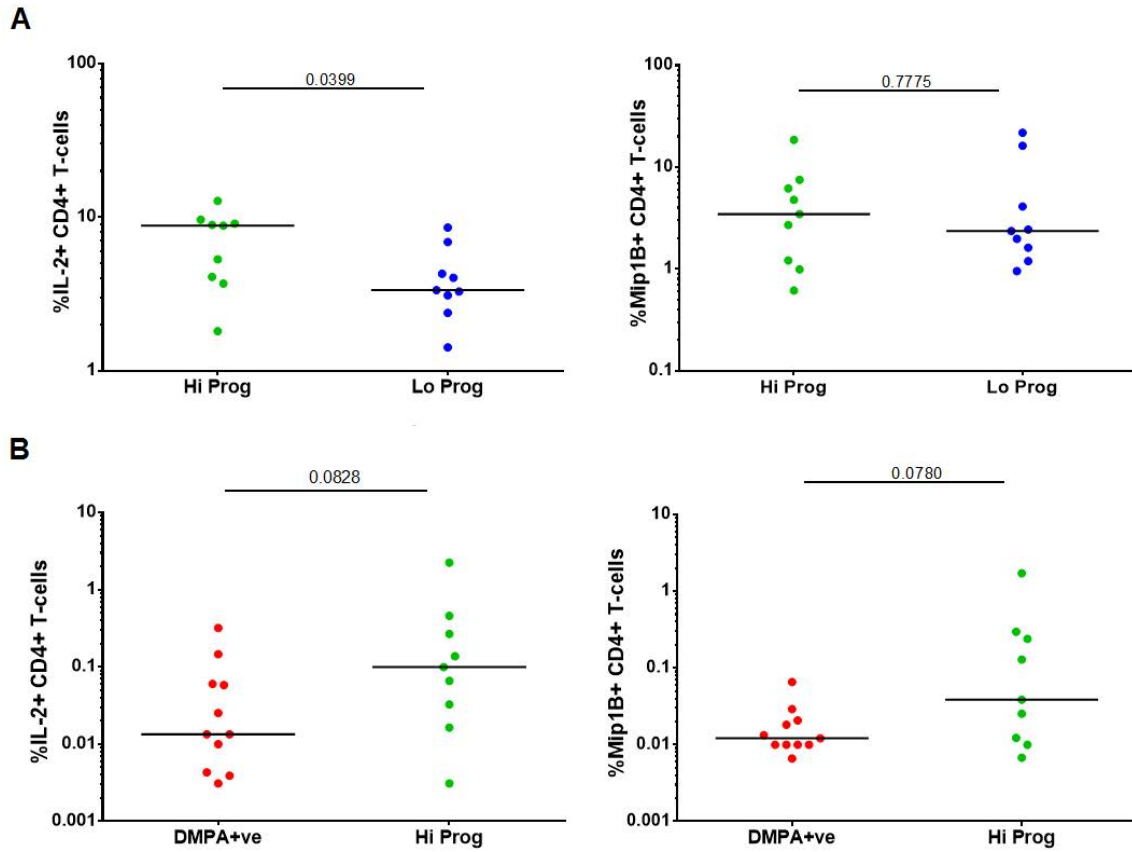


Figure 6.15 High progesterone group shows increased CD4 T cell cytokine expression compared to DMPA positive and low progesterone groups. An example showing expression of IL-2 and Mip-1 β as frequency of parent population CD4+ T cells stimulated with SEB (A) and PPD (B). Mann-Whitney test was used to compare the groups. Horizontal lines on graphs represents the median. P-values < 0.05 were considered significant.

The same general trend was observed in CD8 T cell cytokine expression. High progesterone group showed increased expression of IFN γ ($p = 0.0310$), TNF α , and IL-2 when compared to DMPA positive group (Figure 6.16). Also, in response to HIV stimulation, this group expressed significantly higher levels of TNF α , $p = 0.0483$.

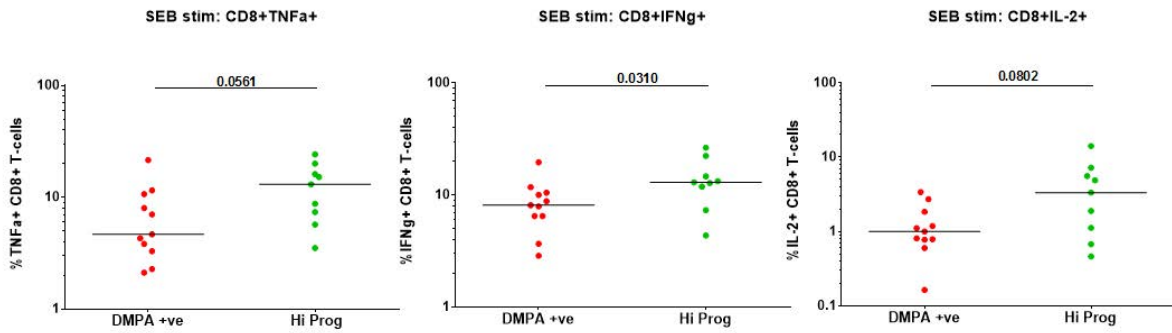


Figure 6.16 High progesterone group shows increased CD8 T cell cytokine expression compared to DMPA positive and low progesterone groups. An example showing expression of TNF α , IFN γ and IL-2 as frequency of parent population CD8+ T cells stimulated with SEB (A). Mann-Whitney test was used to compare the groups. Horizontal lines on graphs represents the median. P-values < 0.05 were considered significant.

To determine differences in immune responses between males and female, with and without TB disease, comparisons were done using Kruskal Wallis tests and multiple comparisons and showed no significant differences between the groups, for example Figure 6.17. Using Mann-Whitney tests, we compared males and females, with or without TB disease, however observed differences were non-significant.

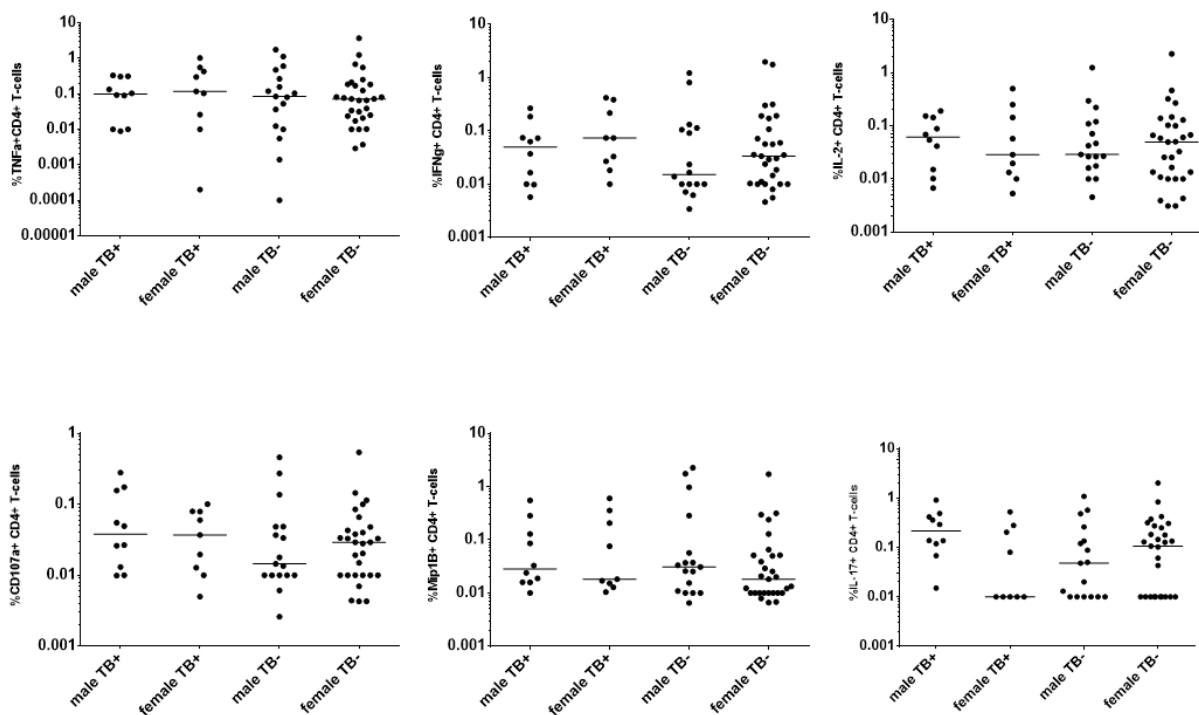


Figure 6.17 Expression of TNF α , IFN γ , IL-2, IL-17, Mip-1 β and CD107a in PPD stimulated cells, as frequency of parent population CD4 $^{+}$ T cells. Multiple comparisons using the Kruskal-Wallis test was used to compare between male and females, with or without TB. Horizontal lines on graphs represents the median. P-values < 0.05 were considered significant.

6.4 DISCUSSION

In this chapter, we demonstrate the application of an LC-MS/MS method for quantitation of endogenous sex hormones and hormonal contraceptive DMPA. As previously described in chapter 5, LC-MS/MS analysis of sex hormones has gained attention in recent years owing to the high sensitivity and specificity of the technique. Using this method, we were able to quantify levels of progesterone, testosterone and DMPA on plasma samples from HIV-infected males and females.

Progesterone levels in females vary with the menstrual cycle, but generally, two phases occur, that is, the pre-ovulatory phase when progesterone levels are low, known as the follicular phase and post-ovulation when progesterone levels rapidly rise in preparation for pregnancy, the luteal phase. Analysis of progesterone levels in females clustered into two groups with high and low progesterone, suggesting luteal and follicular phases respectively. Male progesterone levels, are generally known to be in the low picomolar

range, and we observed low progesterone levels in males, near or below the limit of quantification however three outliers had significantly higher progesterone which may suggest misreporting of gender in the study or possibly males with XXY genotype.

Only three participants of the 196 females selected for this study reported to be using the injectable contraceptive DMPA. Our analysis however, found 42 patient samples to have DMPA levels higher than the limit of quantification. A possible explanation for this observation maybe misreporting by patients or alternatively, since DMPA is a slow-release long acting injectable contraception, patients will have different retention periods of the contraceptive and it remains in the circulatory system for longer after its administration is stopped. This may mean that even though patients are no longer using DMPA, significant levels of the contraceptive can still be detected and quantified in their plasma. As described in chapter 5, DMPA acts as a suppressor of progesterone and the same negative correlation between progesterone and DMPA levels was observed in this study. Also testosterone levels in both males and female were quantified, and generally men had significantly higher testosterone levels than women as expected. However, some males had very low testosterone levels while some females had high testosterone, which maybe indicate androgen deficiency in males, misreporting of gender in the study or XYY and XXY genotypes.

Many previous studies studying effects of sex hormones on immune responses, using animal models, particularly involving castration or ovariectomization of males and females, followed by hormone supplementation have suggested a role for these hormones in susceptibility to TB. We therefore set to investigate the effects varying levels of endogenous sex hormones on CD4 and CD8 T cells phenotypes as well as T cell cytokine production. Patients with highest and lowest progesterone and testosterone levels in females and males, respectively, were selected to analyze this effect. Injectable contraception, DMPA positive individuals were used to compare the effect of synthetic progestin on the immune response.

Effects of sex hormones and hormonal contraception on cellular activation, remains less studied in HIV [88] and TB as well. Expression of activation markers CD38, HLA-DR and exhaustion marker PD-1 was analysed. We observed no significant differences in either activation or exhaustion of both CD4 and CD8 T cells. However females with naturally low progesterone or induced low progesterone (DMPA positive group) generally showed higher CD38 expression in both CD4 and CD8 T cells. In the context of HIV infection, the FRESH study shows that hormonal contraception seems to be associated with activation of CCR5+CD4+ T cells, in the cervix and not blood. Which may suggest a possible mechanism for increased risk of HIV, as this CCR5+CD4+ phenotype is known to be target cells for HIV.

Analysis of T regulatory cells, defined using FoxP3, CD25 and CD127, also revealed no significant differences. We also investigated differences in naïve and memory T cell subsets. These T cell subsets have

been well characterized before, using a number of markers including CCR7 and CD45RA [99, 100], as shown in Figure 6.7. In this study we observed no significant differences in CD4⁺ naïve and memory populations between males with high and low testosterone, as well as CD4⁺ and CD8⁺ populations between females with high progesterone and low progesterone or DMPA positive and low progesterone. The small number of sample used for these comparisons, may not be able to show any significant effect, if any is present. However, high progesterone females showed significantly higher expression of central memory and terminally differentiated memory CD4 and CD8 subsets compared to DMPA positive group, an observation also seen in CD8 subsets of high testosterone males compared to low testosterone. With lack of previous evidence studies investigating effect sex hormones on these cells, with regard to TB and HIV, it is difficult to conclude what these observations mean. Further investigations, with larger sample size may be needed to confirm these observations.

We also investigated antigenic stimulation of CD4 and CD8 T cell responses, to determine differences in cytokine expression. We observed no significant differences in cytokine production, however found a trend towards higher production of TNF α , IFN γ , IL-2, Mip1 β and CD107a by females with high progesterone levels compared to DMPA positive group and low progesterone group, in CD4 and CD8 T cells. Low numbers of patients in each group may possibly mean this analysis was under powered to observe any significant effect, however if this trend can be confirmed, it would be in line with the literature as DMPA has been shown to alter cytokine production in human and animal models [46, 97].

CHAPTER 7: GENERAL CONCLUSION

The significant influence of sexual inequality observed between males and females in susceptibility to, and severity of infectious diseases need to be considered in development of therapeutic interventions against diseases. Tuberculosis, is no exception to this observed sex-based bias. Understanding factors that predispose males to increased susceptibility is key, and sex hormones have been suggested as one of the biological factors that play a role in this observed bias in TB incidence. In addition, changes in hormones during the menstrual cycle, and use of DMPA have been linked to increased risk of HIV. Therefore this study aimed at quantitation of sex hormones and injectable contraceptive, DMPA with the particular aim of evaluating relationship with immune responses to TB and HIV.

A highly specific method using LC-MS/MS method was developed and allowed for identification and quantification of sex hormones in study patients. A key observation was misreporting, in particular by female participants with regard to contraceptive use. This large under-reporting of contraceptive use indicates the value in direct measurement as opposed to self-reporting. The ability to clearly distinguish different phases of menstrual cycle, informed by progesterone levels detected, allows us to study hormonal changes and how they may impact immune responses to TB and HIV.

A clear relationship between injectable contraceptive use and risk of HIV acquisition is shown in the FRESH cohort. The presence of activated CCR5+C4+ HIV target cells in the cervix of injectable contraceptive users, may suggest a mechanism for increased susceptibility to HIV. Study of mucosal changes in the cervical region reveals proteins which are differentially expressed in DMPA and non-DMPA users. This preliminary data shows downregulation of certain proteins which are important in protection of the FRT against infections and in particular HIV. This data may suggest that DMPA changes the environment in the FRT to increase susceptibility to HIV.

In this study, generally, there were no significant effects of hormonal differences in immune responses. A limitation to this study is the sample size, therefore further work will have to involve more study patients to investigate if the effect of sex hormones levels on immune responses to TB and HIV observed in animal studies is seen in humans. Finally, the increased risk associated with DMPA is an important finding that should inform development of alternative contraceptive strategies for women, especially in developing countries where DMPA is used the most. Also women, should at least be warned on the effects of this contraceptive, if they have no alternate methods of contraception.

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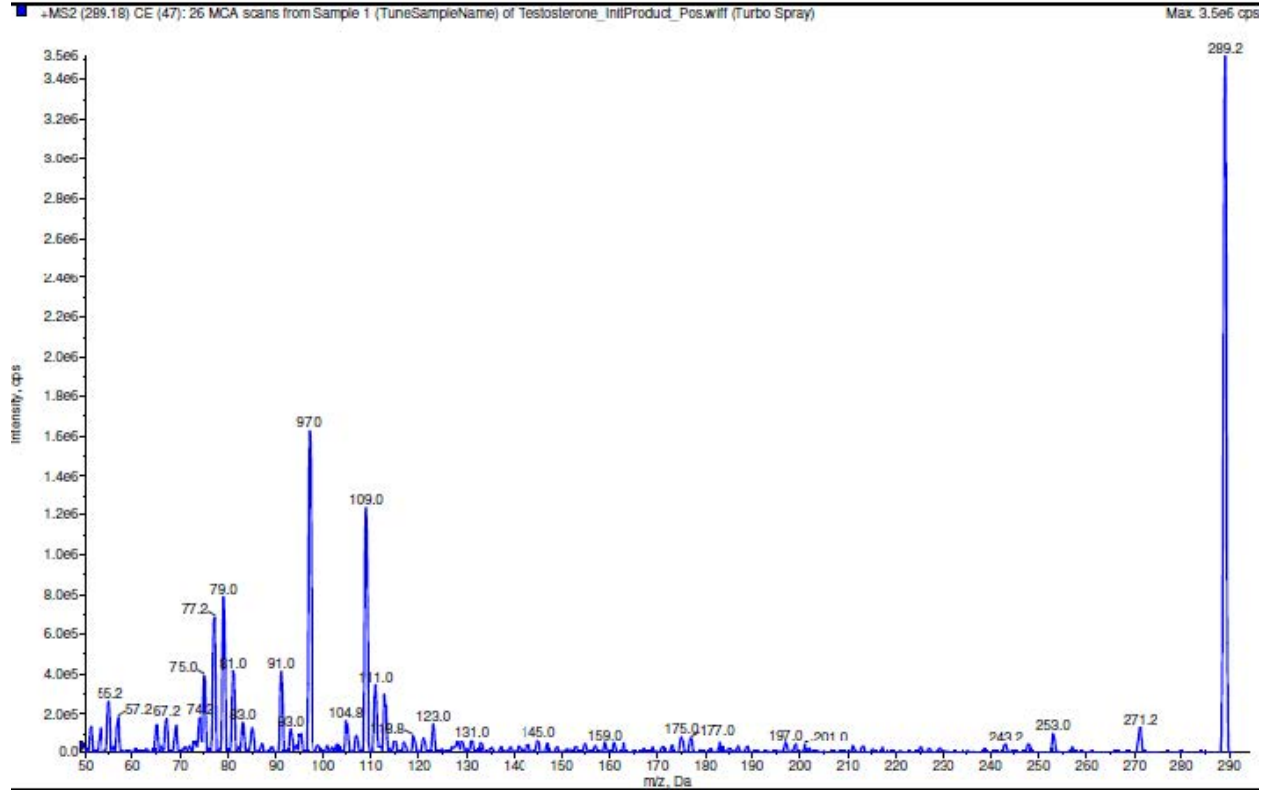
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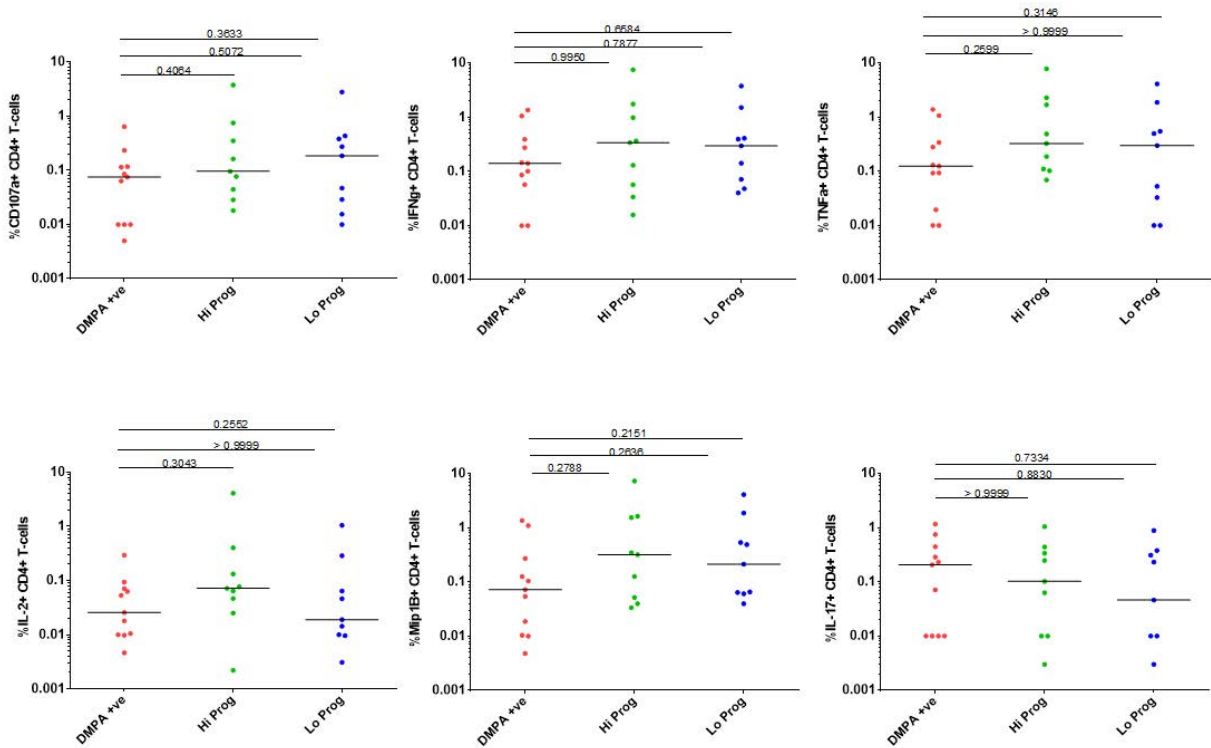
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APPENDICES

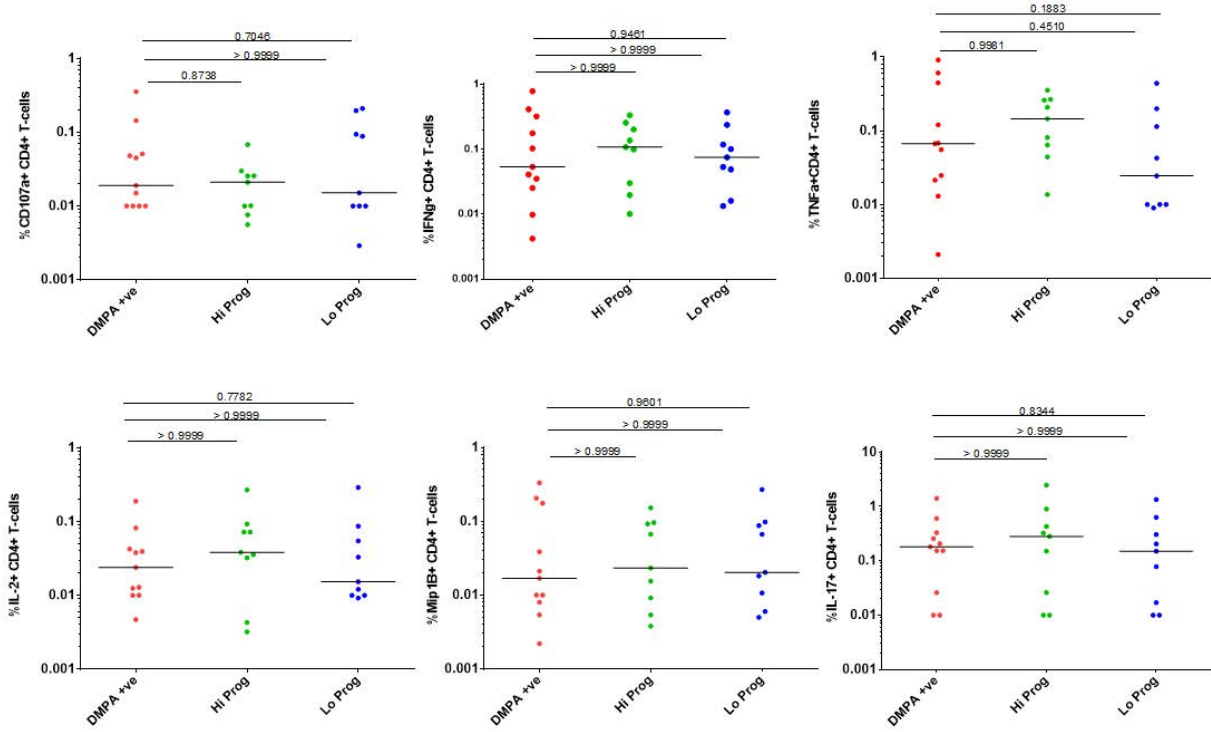
Appendix 1: mass spectrum for testosterone



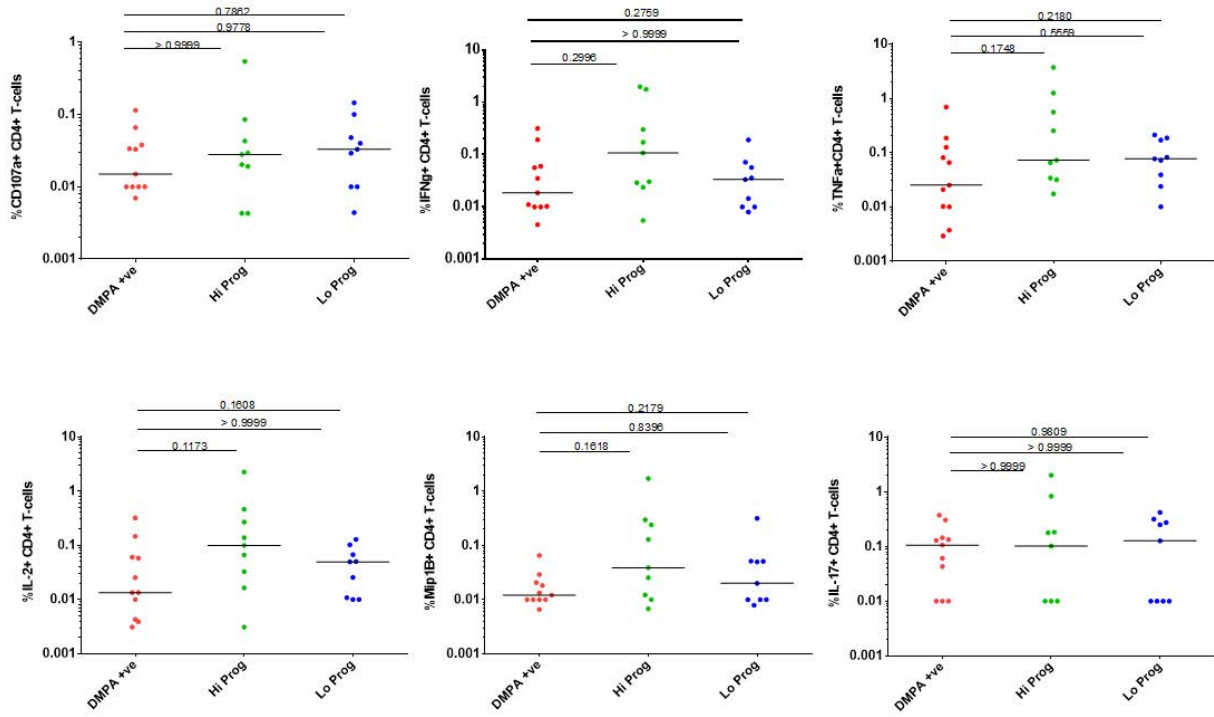
Appendix 2: Expression of $TNF\alpha$, $IFN\gamma$, IL-2, IL-17, Mip-1 β and CD107a as frequency of parent population CD4+ T cells in CMV stimulated PBMCs. Multiple comparisons using the Kruskal-Wallis test was used to compare the groups. Horizontal lines on graphs represents the median. P-values < 0.05 were considered significant.



Appendix 3: Expression of TNF α , IFN γ , IL-2, IL-17, Mip-1 β and CD107a as frequency of parent population CD4+ T cells in HIV stimulated PBMCs. Multiple comparisons using the Kruskal-Wallis test was used to compare the groups. Horizontal lines on graphs represents the median. P-values < 0.05 were considered significant.



Appendix 4: Expression of TNF α , IFN γ , IL-2, IL-17, Mip-1 β and CD107a as frequency of parent population CD4+ T cells in PPD stimulated PBMCs. Multiple comparisons using the Kruskal-Wallis test was used to compare the groups. Horizontal lines on graphs represents the median. P-values < 0.05 were considered significant.





Association between injectable progestin-only contraceptives and HIV acquisition and HIV target cell frequency in the female genital tract in South African women: a prospective cohort study

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Summary

Background The use of injectable progestin-only contraceptives has been associated with increased risk of HIV acquisition in observational studies, but the biological mechanisms of this risk remain poorly understood. We aimed to assess the effects of progestins on HIV acquisition risk and the immune environment in the female genital tract.

Methods In this prospective cohort, we enrolled HIV-negative South African women aged 18–23 years who were not pregnant and were living in Umlazi, South Africa from the Females Rising through Education, Support, and Health (FRESH) study. We tested for HIV-1 twice per week to monitor incident infection. Every 3 months, we collected demographic and behavioural data in addition to blood and cervical samples. The study objective was to characterise host immune determinants of HIV acquisition risk, including those associated with injectable progestin-only contraceptive use. Hazard ratios (HRs) were estimated using Cox proportional hazards methods.

Findings Between Nov 19, 2012, and May 31, 2015, we characterised 432 HIV-uninfected South African women from the FRESH study. In this cohort, 152 women used injectable progestin-only contraceptives, 43 used other forms of contraception, and 222 women used no method of long-term contraception. Women using injectable progestin-only contraceptives were at substantially higher risk of acquiring HIV (12·06 per 100 person-years, 95% CI 6·41–20·63) than women using no long-term contraception (3·71 per 100 person-years, 1·36–8·07; adjusted hazard ratio [aHR] 2·93, 95% CI 1·09–7·868, $p=0\cdot0326$). HIV-negative injectable progestin-only contraceptive users had 3·92 times the frequency of cervical HIV target cells (CCR5+ CD4 T cells) compared with women using no long-term contraceptive ($p=0\cdot0241$). Women using no long-term contraceptive in the luteal phase of the menstrual cycle also had a 3·25 times higher frequency of cervical target cells compared with those in the follicular phase ($p=0\cdot0488$), suggesting that a naturally high progestin state had similar immunological effects to injectable progestin-only contraceptives.

Interpretation Injectable progestin-only contraceptive use and high endogenous progesterone are both associated with increased frequency of activated HIV targets cells at the cervix, the site of initial HIV entry in most women, providing a possible biological mechanism underlying increased HIV acquisition in women with high progestin exposure.

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Introduction

Hormonal contraceptives have empowered women to prevent unwanted pregnancies since being introduced in the 1960s. Unlike condoms, hormonal contraceptives do not need the cooperation of the male partner, can be taken discreetly, and are available in longacting formulations that are more than 99% effective. Injectable progestin-only contraceptives, including depot medroxyprogesterone acetate (DMPA) and norethindrone enanthate (NET-EN), are the favoured form of contraception in sub-Saharan Africa, where they are used by more than 8 million women.¹ Unfortunately, injectable progestin-only contraceptives do not provide protection against sexually transmitted infections and have been associated with a substantially increased risk of acquiring HIV in most but not all studies.^{2–5}

In areas with both widespread injectable progestin-only contraceptive use and high HIV incidence,

including many regions in sub-Saharan Africa, the questions of whether and how injectable progestin-only contraceptives affect HIV acquisition risk are of utmost importance. Decisions regarding the use of injectable progestin-only contraceptives must carefully balance the risk of HIV acquisition with that of unwanted pregnancy. The epidemiological studies driving these decisions are complicated by behavioural and demographic confounders that might mask the biological effect of injectable progestin-only contraceptives on HIV acquisition.⁶ Studies of the association between HIV acquisition and injectable progestin-only contraceptives use have not reached consistent conclusions, and although progestins have been shown to have a multitude of effects on immune function *in vitro*,^{7–10} whether these effects are manifest *in vivo* is unclear.^{11,12}

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Research in context

Evidence before this study

We searched PubMed for articles published before July 7, 2015, using the search terms “depo provera” OR “dmpa” OR “nuroxerone” OR “net-en” AND “HIV” and read those written in English. We examined relevant citations from the results of our search and identified studies that examined HIV acquisition and contraceptive use from an epidemiological perspective, with some, but not all, reporting a significant association. We also identified studies that assessed the effect of progestins on the function of immune cells *in vitro*, in animal models, in *ex-vivo* tissue, or in blood or assessed cohorts not at high risk of HIV acquisition. Some studies examined the biological associations with contraceptive use in high-risk cohorts but limited their observations to soluble immune mediators and did not find a concomitant increase in HIV acquisition risk.

Added value of this study

Previous studies examining the epidemiological risk of injectable progestin-only contraceptive use and HIV acquisition have variably been confounded by differences in behavioural risk factors reported between those using no long-term contraceptive and injectable progestin-only contraceptive users. These confounders might have masked potential biological effects of injectable progestin-only contraceptive use. Our study characterises a unique cohort of young women in which profiles of HIV risk factors had similar proportions across the cohort, so women using injectable progestin-only contraceptives and women using no hormonal contraception were similar in terms of HIV risk factors.

In this study, we identified an increased frequency of HIV target cells at the cervix of HIV-negative women using injectable progestin-only contraceptives and, in the same group of women, noted a significantly higher incidence of HIV acquisition in injectable progestin-only contraceptive users. We noted a similar pattern in HIV target cell frequency in the naturally high progestin state of the luteal phase in women not using long-term contraceptive, suggesting that progestins, both endogenous and exogenous, might be key mediators of target cell availability. Our work links epidemiological observations of HIV infection to the immunological effects of injectable progestin-only contraceptive use in the female genital tract and provides new mechanistic insight into the role of progestins in HIV acquisition risk in women.

Implications of all the available evidence

Our results, taken together with other studies, have cast further doubt about the safety of injectable progestin-only contraceptive in areas of high HIV incidence. Moreover, by concluding that a high progestin concentration, both in naturally cycling women and in injectable progestin-only contraceptive users, correlates with increased target cell availability at the cervix, we suggest that progestins might induce increased cervical HIV target cell frequency. This work provides new insight into the role of progestins in the female genital tract and might inform the development of biological prophylactics to reduce HIV acquisition for women.

We designed a prospective study and secondarily assessed the effects of progestins on HIV acquisition risk and the immune environment in the female genital tract, hypothesising that there is a biological mediator of the increased risk of HIV acquisition often observed in injectable progestin-only contraceptive users.

Methods

Study cohort and participants

HIV-negative women aged 18–23 years were recruited for the Females Rising through Education, Support, and Health (FRESH) study,¹³ an observational, prospective cohort study in Umlazi, South Africa (appendix). Participants were recruited by referral from three to four community organisations in Umlazi, South Africa, and community outreach events. We included women aged 18–23 years residing in Umlazi, South Africa, who were able to understand the information and consent forms, willing to adhere to study requirements, and willing to give voluntary consent for participation. We excluded individuals with unknown HIV status and unwilling to have HIV tests done; unwilling to consent to blood storage; enrolled in any other study that involves frequent blood sampling or might otherwise interfere with this study; with any conditions or conflict that is likely to

prevent adherence to the study protocol and that might interfere with the outcome of the study, particularly adherence to the follow-up schedule, such as low likelihood to stay in or near Umlazi in the next 2–4 years or a chronic condition that requires regular blood sampling; pregnancy; and haemoglobin concentrations of less than 10 g/dL. The study protocol was approved by all local regulatory bodies (appendix). All participants provided written informed consent.

Procedures

Twice per week, participants attended classes focused on personal empowerment, job skills training, and HIV prevention, and had a finger prick blood draw for HIV RNA testing. Participants had a pelvic exam, phlebotomy, and completed a counsellor-administered HIV risk questionnaire every 3 months. The questionnaire asked participants whether they were using a family planning method, which method they were using, for how long they had been using that method, and whether they had a change in family planning method in the past 3 months. The questionnaire also addressed condom use and sexual behaviour. Study follow-up ranged from 3 months to 24 months. One nurse did all of the pelvic exams, during which endocervical cells were collected using a cytobrush

See [Online](#) for appendix

(appendix). Samples were processed as described previously.¹³ Peripheral blood mononuclear cells were isolated from blood using a Ficoll gradient.

Consensus injectable progestin-only contraceptive use (appendix) was the primary covariate on which the primary analyses were based. Patient characteristics, high risk behaviour, and sexually transmitted infection status, assessed at the most recent follow-up, were also taken into account.

To detect sexually transmitted infections, HIV screening was done with HIV RNA PCR (Global Laboratories, Durban, South Africa), and positive results were repeated for confirmation. We tested for *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, HSV-1, and HSV-2 with a posterior fornix swab (appendix).

Plasma progesterone and oestradiol concentrations were measured by the Massachusetts General Hospital Clinical Laboratory Research Core using chemiluminescent microparticle immunoassays (Abbott Laboratories, Diagnostics Division, IL, USA). Progesterone values below the limit of detection (0.1 ng/mL) were read as half the minimum limit of detection (ie, 0.05 ng/mL). Oestradiol values below the limit of detection (10 pg/mL) were read as half the minimum limit of detection (ie, 5 pg/mL). Naturally cycling women were determined to be in the follicular or luteal phase of the menstrual cycle on the basis of their plasma progesterone concentration (follicular: progesterone ≤ 0.3 ng/mL or luteal: progesterone ≥ 1.2 ng/mL).

Cervical and peripheral blood cells were stained with titrated monoclonal antibodies (appendix) and analysed with FACS ARIA III or LSRFortessa (BD Biosciences, San Jose, CA, USA) and FlowJo software (TreeStar, Ashland, OR, USA), as described previously.¹³

An important determinant of HIV acquisition risk is the frequency of HIV target cells at the mucosal site of exposure in the female genital tract.^{14–16} To test the hypothesis that injectable progestin-only contraceptives increase target cell frequency at the superficial layers of the cervix, we did flow cytometric analysis on freshly collected matched cervical cells and peripheral blood. Because injectable progestin-only contraceptives affect endogenous hormone concentrations (appendix) and endogenous progesterone might affect immune function, we controlled for plasma progesterone concentrations; we analysed samples in which plasma progesterone concentration was at most 0.3 ng/mL. We assessed the concentration of 14 soluble cytokines in cervicovaginal lavage and the cervicovaginal microbiome, as described previously.¹³ Medroxyprogesterone acetate concentration was measured in plasma as described previously¹⁷ and in the appendix.

Outcomes

The aims of our primary cohort study were identification and characterisation of host immune response and viral dynamics in acute HIV-1 infections. The secondary aims

of our study included assessment of biological and behavioural correlates of HIV acquisition. Time to HIV-1 infection was defined as the time from enrolment to the first confirmed positive HIV RNA PCR result, or censored at the last day of contact. Assessment of HIV status was made at study entry and twice per week during follow-up with a rapid screening HIV RNA PCR assay followed by a confirmatory assay (ie, to confirm an initially positive test). Incidence rates are expressed as the number of HIV acquisition events per 100 person-years of follow-up.

Statistical analysis

This analysis was done using study records and samples from Nov 19, 2012, through to May 31, 2015. Descriptive measures were used to summarise the data. Continuous variables were summarised using median and interquartile range; categorical variables were summarised using frequency and percentage. Wilcoxon rank sum was used to compare continuous variables and Fisher's exact test was used to compare categorical variables between groups. The HIV positivity (time to HIV-1 infection) data were analysed with the Kaplan-Meier method and the significance was tested by log-rank tests. Cox's proportional hazards models were used to estimate hazard ratios (HRs) and their corresponding 95% CI. Two-sided p values are reported for all the statistical tests used in the analysis and $p=0.05$ was used as the cutoff for significance. R version 3.1.0 and Prism version 6 were used for the statistical analysis.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Between Nov 19, 2012, to May 31, 2015, we characterised 432 HIV-uninfected South African women from the FRESH study. In this cohort, 152 (35%) women used an injectable progestin-only contraceptive (DMPA or NET-EN), 43 women used another form of contraception, 15 women switched contraceptive methods during the study and were not included in the analyses comparing injectable progestin-only contraception users to women using no long-term contraception, and 222 (51%) women used no method of long-term contraception. Of the women using an injectable progestin-only contraceptive, 116 (76%) used DMPA and the remaining 36 (24%) used NET-EN. Use of injectable progestin-only contraceptives was self-reported but verified by measuring plasma progesterone and oestradiol concentrations in a randomly selected subset of 222 women and by directly measuring plasma medroxyprogesterone acetate concentrations in a subset of 44 self-reported DMPA users (appendix). There was 97% and 93% concordance

between reported DMPA use and measured use by progesterone and medroxyprogesterone acetate quantification, respectively. Of the women who were using no long-term contraceptive, 129 were sampled during the follicular phase and 77 were sampled during the luteal phase, as determined by plasma progesterone concentration; the other 16 women were inconclusive based on progesterone measurements.

Despite the narrow age range and residency requirement dictated by study enrolment criteria, a few small demographic differences remained between women using injectable progestin-only contraceptives and women using no long-term contraceptive. Injectable

progestin-only contraceptive users were 1 year older on average ($p=0.022$) and the age difference between the participant and her current sexual partner was 1 year greater for injectable progestin-only contraceptive users than for those using no long-term contraception ($p=0.00036$; table). No differences were seen in condom use, anal sex frequency, casual partner count, or bacterial vaginosis frequency. Injectable progestin-only contraceptive use was not associated with increased likelihood of having any sexually transmitted infections (table).

Despite intensive counselling and education, the HIV incidence in the cohort was 7.43 per 100 person-years

	Overall participants*	Participants receiving DMPA or NET-EN*	Participants receiving no long-term contraception*	Other forms of contraception	p value of injectable progestin-only contraceptives vs no long-term contraception*
Sexual demographics					
Median age at enrolment	21 (20–22)	22 (20–23)	21 (20–22)	21 (21–22)	0.02235
Median age difference between participant and current sexual partner	3 (1–5)	4 (2–6)	3 (1–4)	4 (1–6)	0.00036
Sexual behaviour†					
Condom use in past 30 days					0.1539
Always	61/419 (15%)	18/141 (12%)	38/212 (18%)	4/43 (9%)	..
Sometimes	141/419 (34%)	52/141 (35%)	65/212 (31%)	16/43 (37%)	..
Never	102/419 (24%)	41/141 (28%)	44/212 (21%)	16/43 (37%)	..
Not applicable (no sex)	115/419 (27%)	38/141 (25%)	65/212 (30%)	7/43 (16%)	..
Vaginal sex in past 7 days					0.2504
0 times	240/421 (57%)	80/151 (53%)	132/214 (62%)	20/41 (49%)	..
1 time	128/421 (30%)	50/151 (33%)	57/214 (26%)	18/41 (44%)	..
2 or more times	53/421 (12%)	21/151 (14%)	25/214 (12%)	3/41 (7%)	..
Vaginal sex episodes in past 30 days					0.5212
0 times	115/420 (27%)	38/151 (25%)	65/213 (31%)	7/41 (17%)	..
1–5 times	278/420 (66%)	104/151 (69%)	135/213 (63%)	30/41 (73%)	..
More than 5 times	27/420 (6%)	9/151 (6%)	13/213 (6%)	4/41 (10%)	..
Vaginal drying agent usage					0.4194
Always	5/385 (1%)	2/142 (1%)	1/190 (<1%)	2/40 (5%)	..
Sometimes	50/385 (13%)	20/142 (14%)	20/190 (11%)	8/40 (20%)	..
Never	330/385 (86%)	120/142 (85%)	169/190 (89%)	30/40 (75%)	..
Anal sex in past 30 days	6/417 (1%)	1/151 (<1%)	5/210 (2%)	0/41	0.2077
Casual or new sex partner in past 30 days	28/419 (7%)	7/151 (5%)	14/212 (7%)	5/41 (12%)	0.4286
Sexual health					
<i>Neisseria gonorrhoeae</i> infection	24/394 (6%)	8/151 (5%)	12/190 (6%)	4/40 (10%)	0.8179
<i>Chlamydia trachomatis</i> infection	53/394 (13%)	13/151 (9%)	29/190 (15%)	7/40 (17%)	0.0695
<i>Mycoplasma genitalium</i> infection	30/394 (8%)	8/151 (5%)	17/190 (9%)	5/40 (13%)	0.2170
<i>Trichomonas vaginalis</i> infection	34/394 (11%)	12/151 (8%)	12/190 (6%)	9/40 (22%)	0.6709
HSV-1 infection	4/394 (1%)	2/151 (1%)	2/190 (<1%)	0/40	1.0000
HSV-2 infection	12/394 (3%)	6/151 (4%)	5/190 (3%)	1/40 (3%)	0.5468
Bacterial vaginosis infection (Nugent score ≥ 7)	102/394 (26%)	33/151 (22%)	54/190 (28%)	12/40 (30%)	0.1719

Data are median (IQR) or n/N (%), unless otherwise specified. p values are determined by Fisher's exact test for categorical data and by Mann-Whitney for continuous data. These results represent the data collected at the most recent visit at which mucosal samples were collected, except when a woman became HIV positive before data was collected, in which case data is from the first HIV-positive visit (within a week of becoming infected). Age, age of sexual debut, and age of first partner were collected at the initial study visit. DMPA= Depot medroxyprogesterone acetate. *15 participants switched contraceptive during the study so were not included in columns where participants were analysed by contraceptive method. †Sexual behaviour information from one participant using NET-EN and ten participants using no long-term contraception was not available.

Table: Baseline characteristics

(95% CI 4.59–11.36), with 24 women becoming infected by the time of analysis. In our study, the HIV incidence in injectable progestin-only contraceptive users (12.06 per 100 person-years, 6.41–20.63) was significantly higher

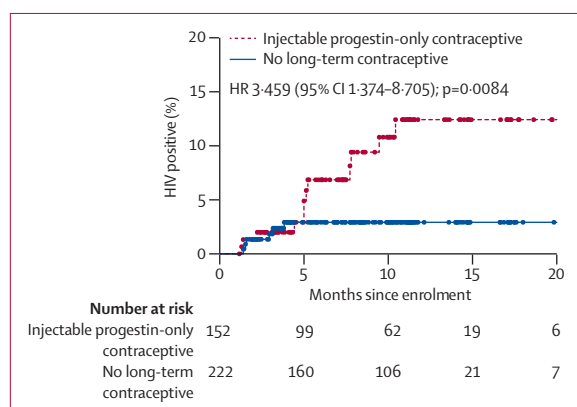


Figure 1: Time to infection stratified by contraceptive usage
Kaplan-Meier curve assessing HIV acquisition in women using injectable progestin-only contraceptives compared with those on no long-term contraceptive. p value determined by a log-rank test.

than women using no long-term contraception (3.71 per 100 person-years, 1.36–8.07; HR 3.46, 95% CI 1.37–8.71, $p=0.0084$; figure 1, appendix). Demographic and behavioural differences between injectable progestin-only contraceptive users and non-users could not explain this increased risk; injectable progestin-only contraceptive use remained a significant predictor of HIV acquisition even after including the age difference between the participant and the partner as a covariate in a Cox proportional hazards model (adjusted hazard ratio [aHR] 2.93, 95% CI 1.09–7.86, $p=0.0326$).

Having established the association between injectable progestin-only contraceptive use and HIV acquisition in this cohort, we hypothesised that biological correlates of injectable progestin-only contraceptive use could help to explain this epidemiological association. Hormonal contraceptive use has been shown to be associated with specific genital inflammatory cytokines¹⁸ and a lower prevalence of bacterial vaginosis,¹⁹ both of which might affect HIV acquisition risk.

After controlling for low progesterone and adjusting for multiple comparisons, no significant differences were identified in cytokine concentration in injectable

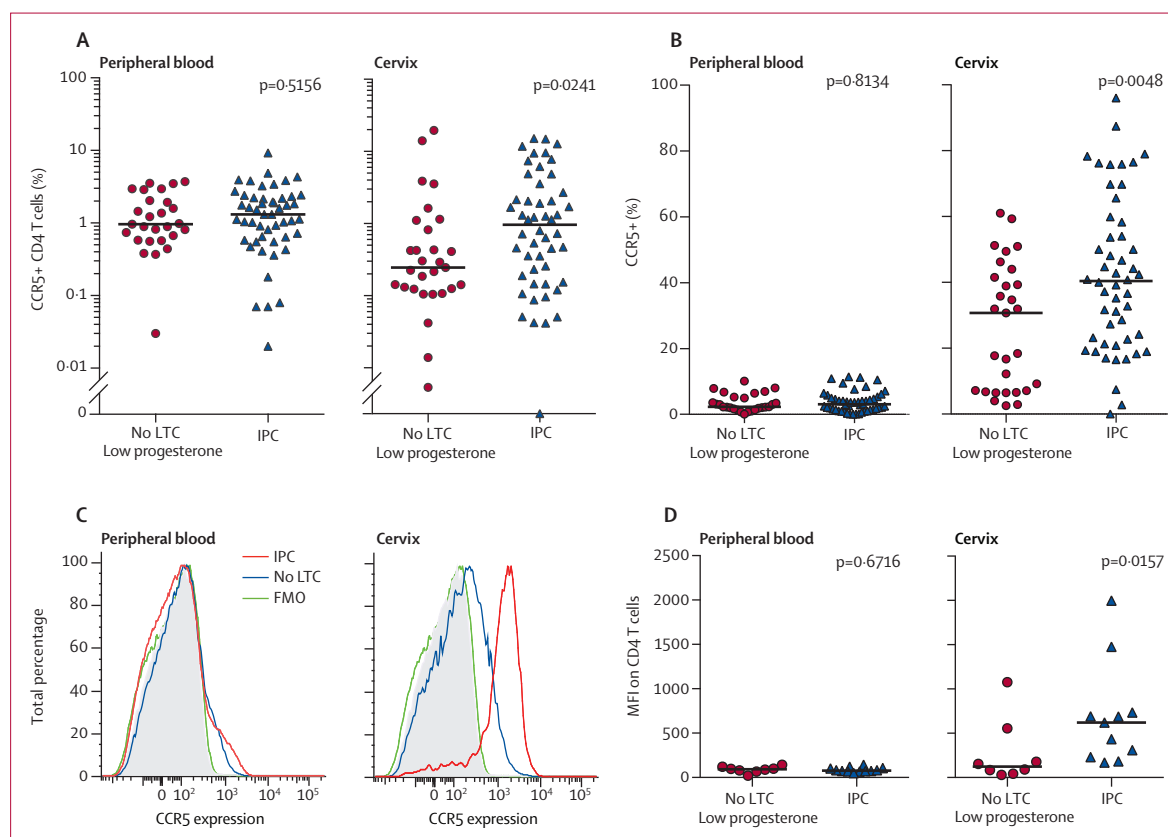


Figure 2: HIV target cells in women using no LTC compared with women using an IPC

CCR5+ CD4 T cells as a percentage of live CD45+ cells in the blood and cervix (A) and CCR5+ expression as a percentage of CD4 T cells in the blood and cervix (B). CCR5 expression levels on CD4 T cells as shown by a representative fluorescence-activated cell sorting histogram (C) and within all patients analysed (D). All participants had a plasma progesterone concentration of 0.3 ng/mL or less. p values were determined by the Mann-Whitney test. LTC=long-term contraception. IPC=injectable progestin-only contraception. FMO=Fluorescence minus one. MFI=Median fluorescence intensity.

progestin-only contraceptive users compared with those with no long-term contraception, by contrast with previous reports¹⁵ (appendix). In view of reports¹³ implicating the genital microbiome in inflammation and HIV acquisition risk, we also did bacterial 16S rRNA gene sequencing of nucleic acid isolated from cervical swabs. We identified no correlation between injectable progestin-only contraceptive use and bacterial populations in the female genital tract (appendix).

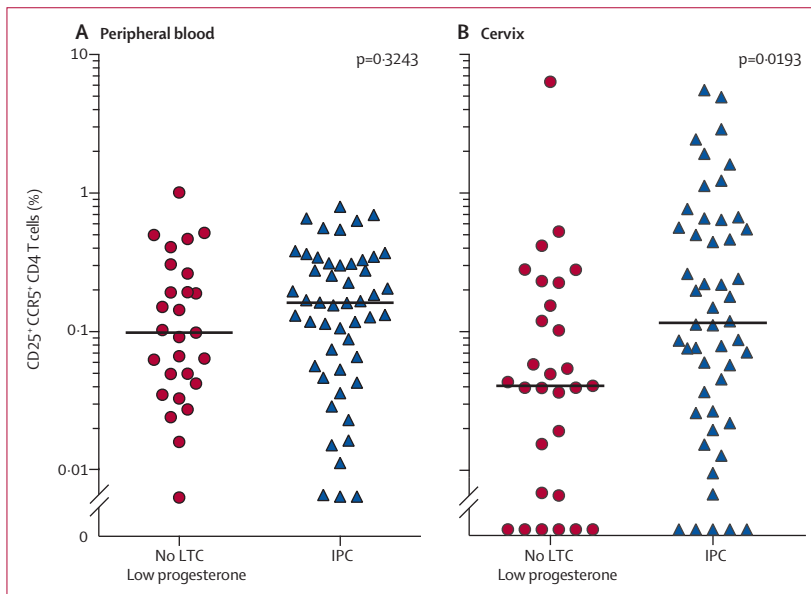


Figure 3: Frequency of activated HIV target cells in women using no LTC compared with women using an IPC. HIV target cell activation as measured by CD25+CCR5+CD4 T cells as a percentage of live CD45+ cells in the peripheral blood (A) and the cervix (B). All participants had plasma progesterone concentrations of 0.3 ng/mL or less. p values were determined by the Mann-Whitney test. LTC=long-term contraception. IPC=injectable progestin-only contraceptive.

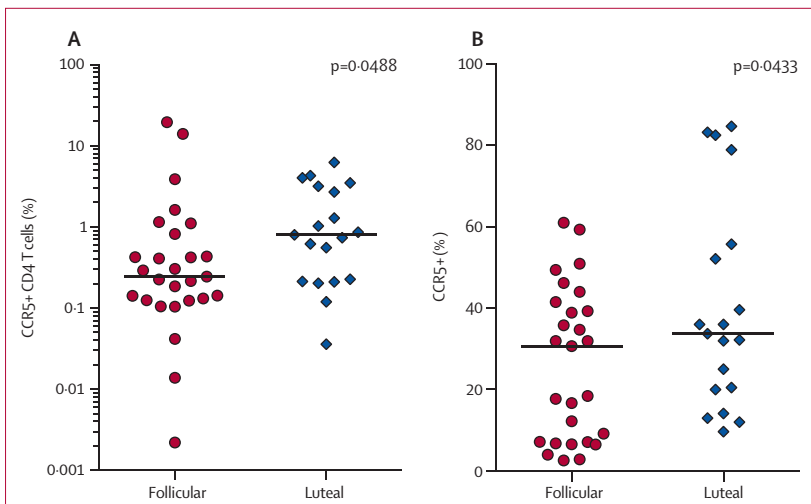


Figure 4: Cervical HIV target cell frequency in the follicular and luteal phases of the menstrual cycle. Frequency of cervical CCR5+CD4 T cells of live CD45+ T cells (A) and CCR5 expression on cervical CD4 T cells (B) in women using no long-term contraceptive in the follicular versus luteal phases of the menstrual cycle. The follicular phase was defined as plasma progesterone concentration of 0.3 ng/mL or less; the luteal phase was defined as a plasma progesterone concentration of 1.2 ng/mL or above. p values were determined by the Mann-Whitney test.

We identified significantly higher frequencies of HIV target cells (CCR5+ CD4 T cells) as a percentage of live CD45+ cells in the female genital tract of women using injectable progestin-only contraceptive compared with those using no long-term contraception (3.92 times higher, $p=0.0241$). We did not find this difference in peripheral blood cells in the same individuals (figure 2). We identified no difference in the number of live cervical CD45+ cells in the two groups. Cervical CD4 T cells of injectable progestin-only contraceptive users had higher CCR5 expression on the cell surface, as measured by the proportion of CD4 T cells expressing CCR5 (1.32 times higher, $p=0.0048$; figure 2), and higher median fluorescent intensity of CCR5 staining ($p=0.0157$; figure 2). Analysis of peripheral blood CD4 T cells in the same individuals did not show differences (figure 2). Oestradiol concentration did not correlate with CCR5+CD4 T cell frequency or CCR5 expression (appendix).

Because activated target cells produce more viral particles per cell than resting target cells,^{20,21} which might accelerate viral dissemination,²² we next assessed target cell activation using the cell surface markers IL2R α chain (CD25), HLA-DR, and CD38. Women using injectable progestin-only contraceptives had no difference in CD25+ target cells in the blood (figure 3) but had a significantly higher frequency of these cells in the female genital tract ($p=0.0193$; figure 3). Cervical target cells from injectable progestin-only contraceptive users did not co-express HLA-DR and CD38 at higher levels (appendix). Thus, injectable progestin-only contraceptive users had not only a higher frequency of cervical target cells but also more activated CD25+ target cells.

In view of the increased HIV target cells seen with exogenous progestin administration, we next examined whether natural states of high progestin, occurring during the luteal phase of the menstrual cycle, also exhibit female genital tract-specific changes in HIV target cells. Women in the luteal phase had a higher cervical target cell frequency (3.25 times higher, $p=0.0488$; figure 4) and more CCR5 expressing CD4 T cells (1.10 times higher, $p=0.0433$; figure 4) than women in the follicular phase.

Discussion

Defining the role of injectable progestin-only contraceptives in HIV acquisition is of utmost relevance, particularly in regions of the world where HIV transmission is highest. By prospectively following HIV-negative women in an area of high incidence, we first establish that injectable progestin-only contraceptives users in this cohort had significantly increased rates of HIV acquisition, an observation that has been shown previously in many²⁻⁴ but not all^{23,24} large cohorts. More importantly, we show that injectable progestin-only contraceptive use is associated with higher levels of HIV target cells in the cervix, providing a biological explanation for the reported increase in acquisition risk. We further

show that a high level of endogenous progesterone is similarly associated with a higher frequency of cervical HIV target cells, supporting the conclusion that this cellular pattern is a biological effect of a high progestin state rather than an artifact of behavioural or demographic confounders associated with injectable progestin-only contraceptive use. These findings support a role for both endogenous and exogenous progestins in modulating the frequency of cervical target cells.

Behavioural and demographic confounders have previously obscured definitive analysis of an association between injectable progestin-only contraceptive use and HIV acquisition risk.^{2,6} Because participants were from a narrow age range and a single community, our cohort allowed pointed assessment of the link between injectable progestin-only contraceptive use and HIV acquisition. Of the potential confounders that we assessed, only age and age difference between a woman and her partner were significantly different between groups. These differences, although statistically significant, were very small in magnitude and of uncertain biological significance. In a Cox proportional hazard model, injectable progestin-only contraceptive use remained a significant predictor of HIV acquisition.

Previous epidemiological studies⁹ concluding that hormonal contraceptives increase risk have been faulted for a lack of coordinated investigation of plausible biological causes. We pursued the hypothesis that biological factors explain the increased HIV incidence in injectable progestin-only contraceptive users. In-vitro studies^{7–10} of human cells and tissue have suggested that endogenous reproductive hormones and exogenous progestins might directly affect immune cell function. Potential in-vivo effects have been less well described. We therefore assessed the immunological environment of the female genital tract in injectable progestin-only contraceptive users in our cohort, with particular focus on the frequency of HIV target cells at the site of exposure as a potential explanation for increased HIV acquisition.^{7,22} Women using injectable progestin-only contraceptives had a significantly higher frequency of activated cervical CCR5+ CD4 T cells. Activated target cells are not only more prone to infection but also support higher degrees of viral replication than resting cells.^{20,21} The increased frequency of activated cervical target cells therefore likely accelerates viral dissemination after exposure to HIV in the female genital tract.

Although the use of injectable progestin-only contraceptives has been of particular interest with respect to increased HIV acquisition risk, the luteal phase of the menstrual cycle, characterised by high endogenous progesterone, has also been described through model systems as a time of increased risk of HIV acquisition.^{25,26} We therefore examined cervical immune cells in women in different stages of the menstrual cycle. With the use of plasma progesterone concentrations to define follicular and luteal phases, we identified significantly higher HIV

target cell frequency in the luteal phase. We conclude that women in any high progestin state—because of injectable progestin-only contraceptive use or naturally high endogenous progesterone—had increased HIV target cell frequency in the cervix compared with women in a low progestin state. No difference was reported in cervical target cell frequency between high progestin states (DMPA use, NET-EN use, and naturally high endogenous progesterone), suggesting a common immunomodulatory effect.

Our study had several limitations. The sample size was much smaller than previously published epidemiological studies examining the link between injectable progestin-only contraceptive use and HIV acquisition, which limited our ability to investigate the potential differential effects of DMPA and NET-EN use. Also, our analysis of immunological changes in the cervix during the follicular and luteal phase would have ideally been done by high frequency longitudinal sampling.

By showing the effect of injectable progestin-only contraceptives on HIV target cells in vivo, we provide a plausible biological mechanism for the significantly increased risk of HIV acquisition in women using injectable progestin-only contraceptives. We also noted similar immunological changes in the female genital tract of women in naturally high progestin states, which suggests that increased HIV acquisition risk is likely driven by both exogenous and endogenous progestin exposure. The significance of this analysis is two-fold. First, our findings in naturally cycling women further support that notion that differences in cellular phenotype and frequency are due to a biological effect, rather than to behavioural or demographic confounding. Second, if the target cell effect is truly mediated by both exogenous and endogenous progestins, then increased HIV acquisition risk might affect not only women using injectable progestin-only contraceptives but also naturally cycling women in the luteal phase. We also importantly identified that injectable progestin-only contraceptive use was not associated with increased genital inflammation or changes in bacterial communities, both of which have been associated with increased HIV acquisition in women, which suggests that the mechanism of increased HIV risk mediated by injectable progestin-only contraceptive is distinct from that of these other biological mediators, although these risk factors might converge on a common pathway of inducing increased HIV target cells in the female genital tract.

Our results suggest that a specific biological mechanism underlies the association between progestin exposure, particularly in the form of injectable progestin-only contraceptive use, and increased HIV acquisition risk in women. The effect of these findings on recommendations for women living in regions with high HIV incidence remains unclear. The potential risks of injectable progestin-only contraceptives will need to be

weighed against those of other contraceptive alternatives and communicated clearly to women facing these choices. Together, our findings show new mechanistic insight into the role of progestins in HIV acquisition risk and suggest that efforts aimed at decreasing genital target cell availability in high progestin states might aid in the development of prophylactic strategies to improve prevention of HIV infection in women.

Contributors

KEC, NP, MNA, and GSO did the flow cytometry. KEC, AMo, EHB, and GSO collected behavioural data, NI processed and managed clinical samples, MSG advised about statistical analyses. AMA and AL did mass spectrometry measurements of plasma DMPA. MNA, EHB, and BAB did the nucleic acid extraction and bacterial sequencing. MNA did the cervicovaginal lavage cytokine measurements. TN, KLD, BDW, and DSK designed and managed the clinical study. EHB, MNA, and DSK did the data analysis and prepared the manuscript. All authors discussed the results and commented on the report.

Declaration of interests

We declared no competing interests.

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