EFFECTS OF DEPOT MEDROXYPROGESTERONE ACETATE ON CERVICOVAGINAL LAVAGE PROINFLAMMATORY CYTOKINES IN ANTIRETROVIRAL TREATMENT NAÏVE HIV POSITIVE WOMEN ATTENDING A COMPREHENSIVE CARE CLINIC IN KISUMU, KENYA

A dissertation submitted in part fulfillment for the Masters in Medicine (MMed) degree in Pathology in the Department of Human Pathology at the University of Nairobi.
INVESTIGATOR:

DR WALONG EDWIN OWINO OLOO
MBChB (University of Nairobi)

Registrar and Tutorial Fellow,
Department of Human Pathology,
School of Medicine,
College of Health Sciences,
University of Nairobi.
P.O Box 19676 00202,
Nairobi, Kenya.

E mail: edwin.owino@students.uonbi.ac.ke

Signature: ______________________________
SUPERVISORS:

1. Prof. Walter Jaoko MB;ChB, MTropMed, PhD

   Professor,
   Department of Medical Microbiology
   University of Nairobi
   P.O Box 19676-00202
   Nairobi, Kenya.
   E mail: wjaoko@kaviuon.org

   Signature: _____________________________________

2. Dr. Elizabeth Bukusi MB;ChB, MMed (Obs/Gyn), MPH, PhD.

   Consultant Obstetrician and Gynaecologist,
   Honorary Lecturer,
   Department of Obstetrics and Gynaecology,
   University of Nairobi
   P.O Box 19676-00202
   Nairobi, Kenya.

   Co-Director, Research Care and Training Program, Centre for Microbiology Research
   Deputy Director (Research and Training), Kenya Medical Research Institute
   P.O Box 614 40100
   Kisumu, Kenya.
   E mail: ebukusi@rctp.or.ke or ebukusi@kemri.org

   Signature: _____________________________________
3. Dr. Charles Stuart Gontier  BSc, MB;ChB, MMed.

Consultant Pathologist,
Lecturer and Unit Head,
Immunology Thematic Unit, Department of Human Pathology,
University of Nairobi,
P.O Box 19676-00202
Nairobi, Kenya.
Email: christopher.gontier@uonbi.ac.ke

Signature: _____________________________________
DECLARATION:

This is a dissertation for fulfil part requirements for the Masters in Medicine (Pathology) degree program and has not been submitted for any other degree course.
DEDICATION

To my family, my wife Priscah, my daughter Adrielle, my parents Francis and Merab Oloo, my siblings and colleagues for their support and encouragement.

To the entire community of persons living with or affected by HIV/AIDS, my desire is that the work presented here may serve as a catalyst for greater understanding.
ACKNOWLEDGEMENT

I would like to acknowledge my supervisors Prof. Walter Jaoko, Dr. Chris Gontier and Dr. Elizabeth Bukusi for the mentorship support, encouragement and for the painstaking hours put into the design, implementation and write up of this project; my study assistants Alrica Akinyi and Dr. Walter Akello, Dr. Megan Huchko, May Maloba and Cirilus Ogolla of the Cervical Cancer Screening Program for the logistical support, Kevin Owuor for the study design and statistical analysis the Research Care and Training Project for providing funding and material support for the study; the entire The Family AIDS Care and Education Service Staff led by Dr. Patrick Oyaro, Rosemary Shikari, Caroline Dande and Noel Odhiambo for their caring attitudes and support, the Nyanza Reproductive Forum staff, Prof. RC Bailey, Dr. June Odoyo, Edith Nyangaya, Lawrence Agunda, Ruth Murugu for the logistical support, The Kenya AIDS Vaccine Initiative (KAVI) led by Prof. O. Anzala, Mr Bashir and Obila Onyango, The Department of Human Pathology academic staff led by the Chair Prof. W. Mwanda, Dr. Jessie Githanga, Dr. Nyagol, fellow residents Dr. Anne Barasa, Dr. Ayub Gitaka, Dr. Germaine Makory and Dr. Scolastica Kimani for reviewing the manuscript, and most of all, the participants of the study.
TABLE OF CONTENTS

TITLE: ............................................................ Error! Bookmark not defined.
INVESTIGATORS: ................................................................................................................ ii
DECLARATION: ........................................................................................................................ v
DEDICATION ............................................................................................................................ vi
ACKNOWLEDGEMENT ............................................................................................................. vii
TABLE OF CONTENTS .......................................................................................................... viii
LIST OF ABBREVIATIONS .................................................................................................... xii
LIST OF ABBREVIATIONS ..................................................................................................... xvii
ABSTRACT: ............................................................................................................................... xviii
CHAPTR 1 - INTRODUCTION ............................................................................................... 1
CHAPTER 2 - REVIEW OF LITERATURE: ............................................................................. 5
  2.1. CONTRACEPTIVE USE AND HIV TRANSMISSION ......................................................... 5
  2.2 EFFECTS OF DMPA ON FEMALE GENITAL TRACT MUCOSAL IMMUNITY .......... 7
  2.3 EVALUATION OF FEMALE GENITAL TRACT MUCOSAL IMMUNITY ................. 11
     2.3.1 SPECIMENS AND ANALYTIC TECHNIQUES ......................................................... 11
     2.3.1.1 BIOPSY SPECIMENS ......................................................................................... 12
     2.3.1.2 CYTOLOGY SPECIMENS ................................................................................ 12
3.6.2: CONTROLS ........................................................................................................ 26

3.7 SAMPLE SIZE ....................................................................................................... 27

3.8 SPECIMEN COLLECTION ..................................................................................... 27

3.9 LABORATORY TESTS ............................................................................................. 27

3.9.1. Neisseria gonorrhoeae .................................................................................... 27

3.9.2. Bacterial vaginosis: ....................................................................................... 27

3.9.3. Trichomonas vaginalis: .................................................................................. 27

3.9.4. Herpes Simplex Virus type 2 ....................................................................... 27

3.9.5. Cervical smears: ............................................................................................ 27

3.9.6. Cytokine level determination: ....................................................................... 27

3.11 QUALITY ASSURANCE ..................................................................................... 31

3.12 ETHICAL CONSIDERATION ............................................................................. 31

3.14 DATA MANAGEMENT ......................................................................................... 32

CHAPTER 4 - RESULTS .............................................................................................. 33

CHAPTER 5 DISCUSSION ............................................................................................. 45

CHAPTER 6. CONCLUSION. RECOMMENDATIONS AND LIMITATIONS .............. 49

6.1 CONCLUSION ....................................................................................................... 49

6.2 RECOMMENDATIONS .......................................................................................... 49

6.3 LIMITATIONS ....................................................................................................... 49

REFERENCES ............................................................................................................ 50
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGUS</td>
<td>Atypical Glandular Cells of Undetermined Significance</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>BD</td>
<td>Beckton Dickinson</td>
</tr>
<tr>
<td>BV</td>
<td>Bacterial Vaginosis</td>
</tr>
<tr>
<td>CBA</td>
<td>Cytometric Bead Array</td>
</tr>
<tr>
<td>CCR</td>
<td>Cysteine-Cysteine Chemokine Receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CIS</td>
<td>Carcinoma In Situ</td>
</tr>
<tr>
<td>CSW</td>
<td>Commercial Sex Worker</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>CVL</td>
<td>Cervicovaginal Lavage</td>
</tr>
<tr>
<td>DMPA</td>
<td>Depot Medroxy-Progesterone Acetate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ERC</td>
<td>Ethical Research Committee</td>
</tr>
<tr>
<td>FACES</td>
<td>Family AIDS Care and Education Services</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorter</td>
</tr>
<tr>
<td>FGT</td>
<td>Female Genital Tract</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescence Iso-Thio-Cyanate</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly Active Anti-Retroviral Therapy</td>
</tr>
<tr>
<td>HCG</td>
<td>Human Chorionic Gonadotropin</td>
</tr>
<tr>
<td>HERS</td>
<td>Human Immunodeficiency Virus Epidemiology Research Group</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HPV</td>
<td>Human Papilloma Virus</td>
</tr>
<tr>
<td>HSIL</td>
<td>High-Grade Squamous Intraepithelial Lesion</td>
</tr>
<tr>
<td>HSV-2</td>
<td>Herpes Simplex Virus Type 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ICF</td>
<td>Informed Consent Form</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KEMRI</td>
<td>Kenya Medical Research Institute</td>
</tr>
<tr>
<td>KNH</td>
<td>Kenyatta National Hospital</td>
</tr>
<tr>
<td>LMP</td>
<td>Last Menstrual Period</td>
</tr>
<tr>
<td>LTR</td>
<td>Long Terminal Repeats</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensities</td>
</tr>
<tr>
<td>NF-Kb</td>
<td>Nuclear Factor kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer cells</td>
</tr>
<tr>
<td>Pap</td>
<td>Papanicolau</td>
</tr>
<tr>
<td>PE</td>
<td>Phyco-erythrin</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PI</td>
<td>Principal Investigator</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate Specific Antigen</td>
</tr>
<tr>
<td>QA</td>
<td>Quality Assurance</td>
</tr>
<tr>
<td>QC</td>
<td>Quality Control</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated And Normal T cells Expressed and Secreted</td>
</tr>
<tr>
<td>RCTP</td>
<td>Research Care and Training Program</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SHIV</td>
<td>Simian-Human Immunodeficiency Viruses</td>
</tr>
<tr>
<td>SLPI</td>
<td>Secretory Leukocyte Protease Inhibitor</td>
</tr>
<tr>
<td>SOPS</td>
<td>Standard Operating Procedure</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for Social Sciences</td>
</tr>
<tr>
<td>STI</td>
<td>Sexually Transmitted Infection</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor Necrosis Factor Receptor</td>
</tr>
</tbody>
</table>
Th  T helper

TNF  Tumor Necrosis Factor

TLR  Toll-Like Receptor

TPHA  *Treponema pallidum* Hemagglutination Assay

UoN  University of Nairobi

VDRL  Venereal Disease Research Laboratory
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ART Naïve</td>
<td>Refers to antiretroviral therapy inexperienced persons</td>
</tr>
<tr>
<td>Cervicovaginal Lavage</td>
<td>Specimen consisting of washings from the female genital tract</td>
</tr>
<tr>
<td>Cytokines</td>
<td>Peptides produced by cells for intercellular communication</td>
</tr>
<tr>
<td>Cytometric Bead Array</td>
<td>Immunoassay for qualitative and quantitative assays in which coloured microspheres serve as the solid phase of an antibody-antigen reaction and is detectable by laser</td>
</tr>
<tr>
<td>Depot Medroxyprogesterone Acetate</td>
<td>An injectable progestin contraceptive administered 3 monthly</td>
</tr>
<tr>
<td>Host-Virus Mutualism</td>
<td>Potentially beneficial characteristics resulting from viral infections</td>
</tr>
<tr>
<td>Immune Activation</td>
<td>Stimulation of innate, cellular and humoral immunity resulting from cellular pattern response receptor stimulation by cognate ligands</td>
</tr>
<tr>
<td>Pro-inflammatory cytokines</td>
<td>Cytokines that mediate immune activation</td>
</tr>
</tbody>
</table>
ABSTRACT:

Background: Sub-Saharan Africa bears 75% of the global HIV burden with an average prevalence of 6%, and here women are disproportionately affected as they constitute over 60% of HIV infected persons. Heterosexual transmission is the most significant mode of transmission in this part of the world. Use of the progestin contraceptive depot-medroxyprogesterone acetate (DMPA) has been shown to increase the risk of sexual transmission of HIV and other sexually transmitted infections. The relative risk of female to male HIV transmission is higher among HIV positive women on DMPA. These effects could be as a result of suppression of genital mucosal immune activation due to reduced proinflammatory cytokine production by epithelial and immune cells on exposure to mucosal antigens and suppression of reduction of T helper 1 lymphocyte associated cytokines. In contrast, DMPA increases T helper 2 cytokine production resulting in increased humoral immunity. These observations have been made in murine studies and in-vitro models using pure cell lines and do not incorporate HSV 2 co-infection. The rationale of the present study was to determine the effect of DMPA on genital mucosal immune activation among HIV women.

Objective: To evaluate mucosal immune activation by measuring the levels of the proinflammatory cytokines IL-1β, IL-6, IL-8, IL-10, IL-12p40 and TNF-α in cervicovaginal lavage fluid of asymptomatic ART naïve HIV positive women on depot medroxyprogesterone acetate (DMPA), and compared these to levels with those in asymptomatic ART naïve HIV positive women not on DMPA.
Setting: This study was conducted in the Family AIDS Care and Education Services (FACES) Comprehensive HIV Care Clinic based in Kisumu, Kenya. FACES is an implementation science project that evaluates innovative comprehensive HIV care and is affiliated to the Kenya Medical Research Institute (KEMRI). The study took place from August 2010 to January 2011.

Participants: This was a case-control study. Cases were ART Naïve HIV positive women on three monthly injectable DMPA for contraception while controls were ART Naïve HIV positive women not on contraceptives.

Main Outcome Measures: Measurement on CVL levels of the proinflammatory cytokines IL-1β, IL-6, IL-8, IL-10, 1L-12p40 and TNF-α. Comparisons were made among the cases and controls.

Materials and Methods: Blood was drawn for total lymphocyte cell count, CD4 lymphocyte cell count evaluation and HSV 2 IgG evaluation. Study participants were screened for Neisseria gonorrhoea by cervical swabs collected and cultured on Thayer-Martin media, while Trichomonas vaginalis and candida species were screened using a wet mount microscopy of high vaginal swabs. Bacterial vaginosis was screened for using Nugent’s score. Cervical smears were collected for conventional Papanicolau staining and evaluation for dysplasia. Cervicovaginal lavage fluid was then collected, centrifuged, aliquoted and stored for cytokine evaluation. Concentrations of six proinflammatory cytokines and chemokines were measured on cervicovaginal lavage by multiplex cytometric bead array to determine mucosal immune activation.

Data analysis The software SPSS version 17 was used for data entry and statistical analysis. Dependent variables between the two groups were analysed using bivariate and multivariate...
statistical tests. To examine the association between DMPA use and cervicovaginal proinflammatory cytokine levels and after evaluation of distribution using Shapiro-O-Wilk’s test, two sided student t for normally distributed data and Mann Whitney tests for skewed data were estimated for cervicovaginal cytokine levels. Dependent variables between the two groups were analysed using bivariate and multivariate statistical tests.

**Results:** A total of 126 women were screened, and seventy (70) subjects, comprising of 35 asymptomatic ART naïve HIV positive women on DMPA were recruited as cases, while 35 age-matched asymptomatic ART naïve HIV positive women not on contraceptives were recruited as controls. The mean age was 26.8 years for cases and 30 years for controls. The median age of cases was higher than controls among screened participants (p=0.021), however, among recruited participants, there was no significant age difference between the two groups showing sufficient age mat Total lymphocyte counts and CD4 cell counts were significantly higher among women on DMPA compared to controls (p=0.02 and 0.004 respectively). HSV 2 prevalence as determined by ELISA was higher among women on DMPA compared to controls (p=0.034). The levels of the cytokines IL 1β, IL 6, IL 8, IL 12p70 and TNF α were lower among women on DMPA compared to controls but only for IL 1β was this difference statistically significant (p=0.046). IL 10 levels was higher among women on DMPA (p=0.022). On multivariate analysis, IL 1β and IL 8 concentrations were affected by the duration of DMPA use (p=0.015 and 0.041 respectively). Inclusion of HSV 2 into the multivariate models showed statistical significant effects on all cytokines measured (p=<0.001).

**Conclusion:** DMPA use is associated with type II mucosal immune activation as evidenced by elevated IL 10 and a time dependent suppression of type I mucosal immune activation. However, these changes are correlated by HSV 2 coinfection which may augment these effects.

xx
**Recommendations:** These alterations in proinflammatory cytokines could explain the mechanisms of DMPA associated increase in susceptibility to HIV infection and higher female to male transmission. HSV 2 prevalence in women on DMPA is higher and is associated with higher proinflammatory cytokines. This may be a manifestation of host-virus mutualism. Further evaluation is therefore required to assess this interaction.
Unknown in 1980, HIV has already caused an estimated 25 million deaths worldwide and has generated profound demographic changes in the most affected countries (1). By the end of 2007, 33 million persons were living with AIDS worldwide; 2.7 million new infections were reported (1). Sub-Saharan Africa, which is home to just over 10% of the world’s population, has 67% of all people living with HIV/AIDS, and an average prevalence of 6%(1). African women are more affected with HIV/AIDS, 60% of persons living with HIV/AIDS in Africa being women. Southern African countries are disproportionately affected, consisting of 35% of HIV infections and 38% of AIDS related deaths (1). HAART has converted HIV from a fatal to a chronic disease the turning point having occurred at the year 1993 (1). HIV transmission varies according to the clinical stage; heterosexual transmission becomes an increasingly important route of infection and in Sub-Saharan Africa accounts for over 90% of the total transmissions (3)/(3). Young people of the 15-24 year age group account for 45% of new infections worldwide (3). More than 80% of all women living with HIV and their partners are in their reproductive years and therefore in need of effective contraception for child spacing and to avoid unwanted pregnancy(4).

HIV positive women should be empowered to take informed choices relating to their reproductive lives, free of coercion. This involves adequate and informed access to contraceptive methods(2). According to the United Nations Department of Economic and Social Affairs, Population Division, World Contraceptive Use, sub-Saharan Africa has the lowest level of contraceptive prevalence, with only 22% of women of reproductive age who are married or in union using contraception (5). In Eastern and Southern Africa, hormonal contraceptives in form of injectables and implants are the most popular methods in use, accounting for over 40% of
overall contraceptive use, while in Kenya, 39.3% of women use contraceptives, the most popular being injectable and implantable progesterone based contraceptives which constitute 15.3% of all contraception. Condom use is low despite the impact of HIV in this region, contributing to the high HIV prevalence. Contraceptive prevalence is higher in Southern Africa compared to the rest of Africa, 57% of Southern African women use contraceptives, out of which 26.5% are on injectable and implantable forms (5). Southern Africa region exhibits high HIV prevalence rates (1). According to the Demographic and Health Study, contraceptive use in women who are sexually active between the ages of 15-24 years ranges from about 47% in Kenya to 74% in Zimbabwe (3). In Kenya, DMPA use is popular, being used by 18.9% of women in this age group (5). Contraceptive use by HIV positive women has been limited in part by its association with increased risk of HIV transmission and adverse clinical outcomes (6).

Studies have found a definite link between DMPA use and increased sexual transmission of HIV among discordant couples in Kenya (7) Effects of DMPA use on mucosal immune milieu could account for the mechanisms associated with increased HIV prevalence. Furthermore, studies on cervicovaginal lavage specimens (CVL) have shown higher leucocytes in women on DMPA when confounding variables such as sexually transmitted infections have been controlled (8). Studies of cell lines have demonstrated suppressive effects of DMPA on innate immune activation by suppressing proinflammatory cytokine production, inhibition of cell mediated immunity by suppressing T helper 1 lymphocyte (Th1) cytokine production and a stimulatory effect on humoral immunity by increased T helper 2 lymphocyte (Th2) cytokine production (9). However, these do not provide an explanation for higher inflammatory cell counts in CVL from women on DMPA. Evaluation of markers of inflammation in the CVL from women on DMPA would evaluate the effects of DMPA on mucosal immune activation. Immune mediators such as
Cytokines are sensitive immune markers because they are secreted by mucosal epithelial cells and histiocytes produced constitutively to maintain mucosal immune responses or in response to an antigenic stimulus and serve to recruit effector cells of the immune system (10). However, because cytokines have transient functions whose properties include pleiotropy, redundancy, synergism and antagonism, analysis of multiple cytokines involved in initiation of the inflammatory response would evaluate mucosal immune activation (10-11). This can be accomplished using cytometric bead array that employ multiplex analysis of cytokines involved in the proinflammatory response (11).

Proinflammmatory cytokine assays on serum samples have been shown to correlate with mucosal immune activation (11). IL 6 has been evaluated as a prognostic factor in HIV positive women, with elevated levels associated with poorer prognosis (33). Female genital mucosal proinflammatory cytokine assays have been done to determine the risk of HIV transmission in microbicides studies. Cytokines exhibit synergy, antagonism, pleiotropy and redundancy, because of these properties, measuring multiple cytokines involved in specific effector functions is an accurate evaluation of the specific effector functions. Similarly, evaluation of the cytokines IL 1β, IL 6, IL 8, IL 10, IL 12p40 and TNF α in CVL accurately assesses mucosal immune activation (10).

This study aims at providing data on in vivo effects of progesterone on female genital tract mucosal immune activation. A greater understanding of the effects of DMPA on mucosal immune activation is relevant clinically as it has been postulated as contributing to increased transmission of HIV and other viral infections such as HSV 2.
1.1 HYPOTHESIS

There is no increase in proinflammatory cytokine levels in the cervicovaginal lavage fluid levels due to use of DMPA.

1.2 OBJECTIVES

1.2.1 BROAD OBJECTIVE

To determine the proinflammatory cytokine profile of asymptomatic HIV positive women on depot medroxyprogesterone acetate (DMPA) and compare these with the profile of asymptomatic HIV positive women not on DMPA.

1.2.2 SPECIFIC OBJECTIVES

1. To determine the concentrations of interleukin 1 Beta, interleukin 6, interleukin 8, interleukin 10, interleukin 12p70 and Tumour Necrosis Factor (TNF) cytokines in cervicovaginal lavage from a population of HIV positive women.

2. To compare the concentrations of interleukin 1 Beta, interleukin 6, interleukin 8, interleukin 10, interleukin 12p40 and Tumour Necrosis Factor cytokine in cervicovaginal lavage of HIV positive women on DMPA with those not on DMPA.

Comment [15]: Is it 12p70 or 12p40?
CHAPTER 2 - REVIEW OF LITERATURE:

2.1. CONTRACEPTIVE USE AND HIV TRANSMISSION

The relationship between hormonal contraceptive use and HIV transmission remains inconclusive and requires further evaluation (6). Some countries in sub-Saharan Africa that have among the highest HIV prevalence rates of above 30% in the world also have high prevalence of hormonal contraception use of more than 40% (1, 5). In particular, young women in Southern and Eastern Africa often use DMPA and have high rates of HIV prevalence. Although the use of DMPA among 15 to 24 year old women is not higher than in many Latin American countries, where HIV prevalence is much lower, it might still be a risk factor for HIV acquisition(5). There have been conflicting reports from studies examining whether there is an increased risk of HIV shedding among HIV infected women using progesterone based contraceptives, and in particular injections of DMPA (1).

First observations made in the mid 1990s suggested an increased risk of HIV transmission associated with hormonal contraception. In Thailand, a prospective study conducted among CSWs found a high risk ratio for male to female HIV transmission due to the use of DMPA (12). In Kenya, a similar prospective study among CSWs found a high risk ratio associated with oral contraceptives (13). However, studies done in Kenya and Uganda found an increased risk of HIV transmission due to DMPA use but not to oral contraceptive use (14). A meta-analysis study conducted from results in 28 countries suggested a 20% increased risk of HIV infection associated with injectable contraception(6). Higher risks were found in Africa, with this risk being more pronounced among high risk women(15). In Kenya, a case control study evaluating
the risk of HIV transmission among family planning clinic attendees in Kenya determined an Odd’s ratio of 3.99 (16).

There are several possible explanations for the association between progesterone based hormonal contraceptive use and heterosexual transmission of HIV (6).

First, hormonal contraceptives may cause thinning of the vaginal mucosa resulting in transmission of HIV across the barrier on exposure as has been shown to be the case in experimental animals (16-18). Marx and colleagues demonstrated that progesterone treatment of female macaques increases susceptibility to vaginal inoculation with Simian Immunodeficiency Virus (16). These effects have been found to be reversible with oestrogen pre-treatment (17). However, this observation has not been found in women, where DMPA use has not led to thinning of the mucosa to the extent seen in non human primate studies (18).

Second, hormonal contraceptives may induce cervical ectopy and to exposure of endocervical mucosa is rich in lymphocytes which may serve as a focus of initial infection and thereby predisposing to increased transmission of HIV and other sexually transmitted infections (19).

Third, hormonal contraceptives may put the women at increased risk of HIV by decreasing vaginal colonization by hydrogen peroxide producing lactobacillus species which are protective against HIV 1 infection (14). This is likely due to mucosal immunoregulation resulting from commensal bacterial-epithelial cell crosstalk (19).

Fourth, at the cellular level, hormonal contraceptives have been associated suppressed systemic proinflammatory responses (9). However, studies on cervicovaginal lavage fluid from women on DMPA shows increased cervical inflammatory cells (8). DMPA use has been found to increase
genital tract expression of HIV-1 co receptor CCR5 (19). Human Herpes Virus 2 (HSV 2) infection has been shown to have a partial protective effect against sexual transmission of HIV (20). No study has evaluated the effects of DMPA on mucosal immune activation.

Evaluation of the effect of DMPA on mucosal immune responses, particularly mucosal activation and inflammation is required in light of contradictory findings (8). This is especially important in areas with high HIV prevalence where heterosexual transmission is significant and among vulnerable population groups such as women have high HIV infection rates (7).

2.2 EFFECTS OF DMPA ON FEMALE GENITAL TRACT MUCOSAL IMMUNITY

The mucosa provides immunity towards infections, including the HIV virus (22). The probability of HIV transmission varies between 1:2000 and 1:200 heterosexual encounters (3). Understanding the mechanisms by which the mucosa achieves this is important for public health interventions (22).

The mucosa presents a physical barrier to infection (24). The lower genital tract, where initial exposure to heterosexual infection occurs, consists of the vulva, vagina and uterine cervix. This region has stratified non keratinized squamous epithelium (24). The cervical mucus layer produced by endocervical cells which also presents a barrier to infection (24). Cervical ectopy may occur in a proportion of women. However, exposure of the one cell thick endocervical epithelium would consist of less than 5% of female genital tract epithelium (24).

The mucosal surface has secretions that have antimicrobial properties (25). These include β defensins, trappin 2/elafin, lactoferrin and secretor of leukocyte protease inhibitor which
have in vitro activity against HIV and other bacterial and viral infections. In addition, these secretions have potent anti-inflammatory properties (24, 25). Immunoglobulins, especially IgA, are also present on the mucosal surface, produced by plasma cells in the upper female genital tract (25).

Commensal flora found in the lower female genital tract plays a role in mucosal immunity (25, 26). They include hydrogen peroxide producing lactobacilli which ensure maintenance of a low pH, creating an acidic environment which is hostile to bacteria and viruses (26). The organisms that constitute the normal flora do not induce an inflammatory response (24). This is largely due to the presence of cross talk between these organisms and the genital epithelial cells (24). The detection of commensals by luminal genital epithelial cell pattern recognition receptors lead to reduced production of inflammatory mediators (26). In their absence, in the presence of non-commensal bacteria or recognition of bacteria within the basolateral surface of the epithelium, epithelial cells increase proinflammatory cytokine production (24, 25). Genital epithelial cells do not only constitute a formidable barrier against infection, but are also capable of mucosal immune activation. This is due to their ability to recognize pathogens and respond by cytokine production (25). Other cells found within the epithelium include inflammatory cells of the myeloid and lymphoid lineages. Epithelial and inflammatory cells express pattern recognition receptors (PRR), which recognize pathogen associated molecular patterns (PAMP) (24). The most studied of the PRRs are the Toll like receptors (TLR), which are germ line encoded receptors (27). PRR recognize conserved microbial molecules such as peptidoglycans, lipopolysaccharides, single stranded DNA, single and double stranded RNA and unmethylated CpG motifs (24). PRRs are highly specific to particular molecular patterns. Certain PRRs are constitutively
expressed on the cytoplasmic membranes (27). PRRs such as TLR 3, TLR 7, TLR 9 and RIG 1 are located within intracellular excretory pathways where they may recognize intracellular viral molecular patterns (27, 28). Recognition of PAMPs by PRRs activates intracellular signals which lead to cytokine production (28). These cytokines mediate induced mucosal activation grouped as type 1 and type 2 (28). Type 1 responses are mediated by interferon α and interferon β which signal through the intracellular signalling molecule Interferon Response Factor 1 (IRF 1), which are antiviral and are protective against HIV (28). Type 2 mucosal innate responses are mediated by proinflammatory cytokines such as interleukin 1 beta (IL 1β), interleukin 4 (IL 4), interleukin 6 (IL 6), interleukin 8 (IL 8), interleukin 10 (IL 10), interleukin 13 (IL 13) and tumour necrosis factor alpha (TNF-α) which signal through Nuclear Factor kappa B (NF kB) and Interferon Response Factor 2 (IRF 2) (28). These responses constitute proinflammatory responses, leading to recruitment of inflammatory cells and induction of adaptive cellular and humoral immunity and may promote HIV transmission. (26). This is more likely in women of African descent and may predispose to higher susceptibility to HIV infection (27).

The submucosa consists of connective tissue stroma, blood vessels and lymphatics. When an infection traverses the mucosa and infiltrates the submucosa, a systemic infection may be established (28). Inflammatory responses in this strata mirrors those found in systemic circulation (28).

There exists a hormonal mediated cyclic effect on mucosal and systemic immune responses. (3). Progesterone suppresses proinflammatory cytokine production by macrophages, and reduced cytokine signalling by nuclear factor kappa B (NF kB) and
elevation of Suppressor of Cytokine Signalling (SOCS) protein(9). This results in suppression of antigen presentation and Th1 function(9). Progesterone leads to an increase in Th2 cytokine expression such as IL 4, IL 10 and IL 13 which may augment antibody production(9).

Several types of progesterone receptors have been identified(9). These include membrane serpentine G protein linked receptors which signal through the intracellular mediator cyclic adenosine monophosphate (cAMP), membrane progesterone receptors signalling through the intracellular mediator to mitogen associated protein kinase (MAPK), progesterone gated calcium ion channels and intranuclear progesterone receptor coupled to progesterone response elements. Immediate cellular response to progesterone is mediated largely by cell membrane receptors coupled to G proteins, signalling through the cyclic adenosine monophosphate intracellular signalling pathways(9). The most studied is the intracellular progesterone receptor which activate progesterone response elements which leads to DNA translation and synthesis of messenger RNA and protein synthesis. Progesterone induced blocking factor (PIBF) is among the proteins synthesized(9). PIBF is responsible for the effects of progesterone on cells of the monocyte lineage and are abrogated by the progesterone antagonist RU486 (9).

The contraceptive DMPA is an agonist of nuclear corticosteroid receptors (CR) which acts through membrane and nuclear progesterone receptors (9). Studies examining its direct effects on mucosal immunity are scant. From animal and in vitro studies on monocyte and lymphoid cell lines, DMPA exerts a potent dose dependent suppression of cellular immunity and despite elevation of Th2 cytokine profiles. This may result in suppression of humoral immunity due to reduced cognate T cell dependent B cell activation (9). Indeed,
among HIV positive women with advanced HIV infection, DMPA use has been associated with poor immunologic recovery despite virologic suppression (28). DMPA has been associated with increased female to male transmission of HIV, and a less significant increase in male to female transmission of HIV (7).

DMPA use is associated with higher prevalence of sexually transmitted infections including Chlamydia and HSV 2 infection (13). However, DMPA associated susceptibility to HIV was reduced in women infected by HSV 2 (29). This paradoxical association is surprising because HSV 2 infection has been shown to increase susceptibility to HIV infection by inducing type 2 mucosal immune activation (29). However, a study published in 2007 by Barton et al, reported symbiotic host-virus mutualism, that murine models infected with murine gamma herpesviruses developed resistance against *Listeria monocytogenes* infection and elevated TNF α and IFN λ concentrations (31). This effect was later discovered to be transient (32). It is likely that symbiotic host-virus mutualism may play a greater role in HIV pathophysiology in women using the contraceptive DMPA.

These factors may be evaluated in lavage specimens from the female genital tract. Cervicovaginal lavage specimens are suitable for microbial, virologic, immunologic, chemical and cellular evaluation associated with female genital tract immunology (33).

### 2.3 EVALUATION OF FEMALE GENITAL TRACT MUCOSAL IMMUNITY

#### 2.3.1 SPECIMENS AND ANALYTIC TECHNIQUES

Various techniques may be employed in the evaluation of female genital tract mucosal immunity (30). These techniques examine the relationships between immune cells and other
cells in the female genital tract, the functions of each individual cell type in mucosal immunity and the cumulative function of the female genital tract(30). The effects of drugs, infections, neoplasms and metabolic lesions on mucosal immunity may also be determined.

2.3.1.1 BIOPSY SPECIMENS

Examination of biopsy specimens from the genital tract fixed, sectioned and stained with haematoxylin and eosin shows proportions and relationships between lymphoid cells, epithelial and other cells(18) and is useful in diagnosis of lesions. Special stains, phenotyping and genotyping may be employed. Fresh biopsy specimens derived from surgery or autopsy may be cultured and used as explant tissue to study immunologic phenomena (34). Inflammatory, stromal or epithelial cell lines may be cultured and maintained for long periods of time. These methods are limited by their invasive nature, prohibitive cost, tedious processes and limited clinical application.

2.3.1.2 CYTOLOGY SPECIMENS

Evaluation of female genital tract cells using cytologic techniques may be employed for immunologic analysis (30). These may be obtained by exfoliative techniques, such as use of a spatula or cytobrush. These cells may then be evaluated by conventional cytology techniques, flow cytometry, genotypic studies or cell culture (30). However, these methods are traumatic leading to mucosal immune activation which may interfere with the required data.

2.3.1.3 FEMALE GENITAL TRACT SECRETIONS

Cervical or vaginal secretions may be collected using sponges or wicks. These have the advantage of collection of predetermined volumes and concentrations of analytes can be easily
determined (33). The sponges and wicks have the disadvantage that they collect relatively low volumes of analytes, may adsorb analytes under investigation and may not be particularly useful in specimen collection for cell analysis

2.3.1.4 CERVICOVAGINAL LAVAGE FLUID

Cervicovaginal lavage (CVL) is a relatively simple technique which involves introducing sterile saline which may be buffered into the genital tract. This technique is preferred in mucosal immunity studies has been widely used to collect specimens for examination cells, microorganisms and to determine concentration of numerous analytes which include cytokines, electrolytes, drugs for pharmacokinetic studies, applied microbicides, hormones and enzymes (33) The advantage of cervicovaginal lavage specimens is that relatively abundant volumes may be collected and stored, may also be used both soluble and relatively insoluble analytes. Cells are also present within these specimens which may be easily separated and analysed separately. It is safe and easy to perform. No adverse effects have been reported associated with cervicovaginal lavage collection (33). Although collection is relatively easy to standardize, the main disadvantage is the presence of a dilution effect which may reduce the sensitivity (33). To mitigate against this, the lavage fluid should have contact with the mucosal surface for approximately 20-30 seconds. Some authors recommend total protein assay in these samples, and specific analyte concentrations presented as a ratio to total protein concentrations (33).

Analytic techniques that may be applied on cervicovaginal lavage samples include cell analysis by microscopy, immunophenotyping or genotyping (11). Soluble mediators of inflammation may be evaluated using chemical or immunoassay techniques on these specimens whose advantage include multiplex analysis of multiple analytes in solution (11).
2.3.2 CYTOKINE MEDIATORS OF MUCOSAL IMMUNE ACTIVATION

Cytokines are peptides whose functions are intercellular communication (36). Their functions are broad. Cytokines may act as trophic factors essential for cell survival; they may confer death signals, proliferation and chemotaxis. They act autocrine, paracrine and endocrine communication in a wide variety of cell types, frequently through cell membrane receptors activating second messengers with subsequent cytoplasmic and nuclear cellular effects. Cytokines were discovered in the 1950s through initial studies on cellular-protein markers. In the 1970s, the term ‘cytokine’ was first published by Cohen et al to describe these proteins, due to their perceived proliferative effects on cell growth and development (37). Individual cytokines were discovered in the 1980s and 1990s with the improvement of molecular biology techniques and transgenic mice. Initially discovered as mediators of immunologic function, they were later discovered in a wide array of cells and tissue. Key cytokine characteristics include pleiotropy, where individual cytokines may have different functions within different cells or even the same cell despite binding to similar receptors, and redundancy, where different cytokines have similar functions or even bind to similar receptors. These confer the property of synergy and antagonism. It is therefore difficult to assess individual functions of cytokines. The prevailing hypothesis shows that multiple cytokines act in a network with common effector functions while regulating other associated processes. These biological networks are dependent on the function of each cytokine on the synthesis of other cytokines and thus may exhibit synergy, enhancing function, and antagonism, suppressing the function of other cytokines within these networks.

Cytokines involved in immunological processes were initially classified as into functional groups such as lymphocyte derived or lymphokines, antiviral interferons, haematopoietic growth factors and non haematopoietic growth factors. Because of the interactions associated with pleiotropy,
redundancy, synergy and antagonism, classification of cytokines takes into account biological functions and networks, family groups, physiochemical characteristics and cellular receptors (36).

Cytokines may be classified on the basis on function. This classification takes into account in vivo cytokine function in biological networks and is the most widely used classification. The classification is relatively simple, studies based on this classification display higher reproducibility (36).

- Cytokines that mediate natural immunity. Examples include the type 1 interferons, IL 1, IL 6, TNF-α and chemokines.
- Cytokines that regulate lymphocyte activation, growth and differentiation. Examples include IL 2, IL 4 and TGF β
- Cytokines that regulate the immune mediated inflammation. Examples include type 2 interferons, TNF β (also known as lymphotoxin), IL 5, IL 10 and IL 12.
- Cytokines that stimulate haematopoiesis. Examples include c-kit ligand, IL 3, IL 4, GM CSF, M-CSF and C-CSF.

Cytokines may also be classified based on family groups. Elgert’s classification takes into account cytokine family groups based on the cytokine receptors, physiology and chemistry. The main limitation occurred that as cytokines functions were defined with greater accuracy, considerable overlap occurred between different groups. The Elgert’s classification took this into account and is summarised as follows:

- Interleukins: Examples include IL 1, IL 2, IL 4 and IL 5.
• Chemokines: Examples include IL 8 and Macrophage Chemotactic Factor (MCP)

• Interferons: Examples include IFN α, IFN β and IFN λ

• Lymphotoxins or immunomodulatory cytokines: Examples include IL 3, TNF α and TGF-β

Chemical classifications were based on biochemical structures. Cytokines are proteins with quartenary structures consisting of alpha and beta subunits. Biochemical structures may not predict biological cytokine networks. The classification is as follows:

• Short chain alpha and beta subunits: these include:
  
  o Epidermal Growth Factor subfamily: two antiparallel beta subunits.

  o Chemokine family: open faced beta sandwich with conserved cystein rich loci.

  o Insulin related subfamily: short alpha subunits with three disulphide bonds.

• Long Beta chains

  o Tumour Necrosis Factor subfamily: Examples include TNF α, TNF β, CD 27 and the Fas Ligand.

  o Interleukin 1 and Fibroblast growth factor subfamily: These have characteristic trefoil folds with 12 strands of eta sheets forming six hairpin loops.

• Platelet Derived Growth Factor and Transforming Growth Factor subfamily: These have beta sheets with structures consisting of cystein knots and interlocking sets of disulphide bridges.
• Mosaic Structures: These have heterogenous structures that do not fit into the above categories. These include IL 12, Hepatocytes Growth Factor (HGF), Glial Growth Factor (GGF), Heregulins and neuregulins.

Cytokines function through cellular receptors that Classification of cytokine receptors is important as each tend to activate similar second messengers and eventual biologic functions. These include:

• Immunoglobulin superfamily: Examples include IL 1, M-CSF and C kit receptors.

• Class 1 cytokine receptors: examples include IL 2, IL 3, IL 4, G-GSFand GM CSF receptors.

• Class 2 cytokine receptors: examples include IFN α, IFN β and IFN λ receptors.

• Tumour Necrosis Factor superfamily receptors: examples include TNF α, TNF β, CD 40 and FAS.

• Chemokine receptors: these are G protein coupled serpentine membrane receptors whose second messenger is cyclic adenosine monophosphate.

Specific examples of cytokines involved in natural immunity in particular immune activation are summarised below:

2.3.2.1 Interleukin 1β: this is a predominantly macrophage produced cytokine subunit of interleukin 1 found in plasma whose primary role is inflammatory response and tissue repair, acts as a mediator of local inflammatory reaction including a cascade of secondary cytokines in a
large number of cells, enhancing leukocytes and endothelial cell adhesion, inducing CD4 T cell proliferation B cell growth and differentiation.

2.3.2.2 Interleukin 6: this is a cytokine produced by antigen or mitogen activated T cells, fibroblasts, macrophages and other cells that serves as a differentiation factor for B cells and thymocytes and stimulates immunoglobulin production by B cells. It also induces hepatocytes to synthesize various plasma proteins involved in the acute phase response and is a cofactor in initiation of the cell cycle in primitive hematopoietic cells in vitro.

2.3.2.3 Interleukin 8: this is a chemokine produced by monocytes, endothelial cells and other cells that acts as a chemotactic and activator for neutrophils and also plays a role in the extravasation of neutrophils in inflammation.

2.3.2.4 Interleukin 10: this is a cytokine produced by activated macrophages, certain lymphocytes and other cells that decreases both innate and T cell mediated immune inflammation. It inhibits the production of cytokines by activated T cells, plays a role in B cell activation, and inhibits production of interferon gamma and blocks antigen presentation and macrophage formation of interleukin 1, interleukin 6 and tumour necrosis factor.

2.3.2.5 Interleukin 12p70: This is a heterodimeric cytokine produced by phagocytic cells, B cells and other antigen presenting cells. It is a potent inducer of cytokine production, causes T and NK cells to secrete IFN-γ, is a growth factor for pre-activated T and NK cells and enhances cytotoxic activity in CD8 positive T cells and NK cells. It also has a role in the generation of T helper type 1 cells and in the differentiation of cytotoxic T lymphocytes.
2.3.2.6 Tumour Necrosis Factor alpha: This is a cytokine produced mainly by macrophages and other antigen presenting cells as part of the acute phase reaction. It has a wide array of systemic and cellular effects and an important mediator of inflammation.

2.3.3 INDICATIONS FOR CLINICAL DETERMINATION OF CYTOKINES

Cytokines are directly implicated in various pathophysiological conditions in humans increased production is responsible for elevated levels in body fluids such as blood, synovial fluid, cerebrospinal fluid and lavage specimens such as bronchioalveolar lavage and cervicovaginal lavage. Cytokine concentrations frequently correlate with severity of disease. They are surrogate markers of immunologic function such as immune activation (11).

Clinical trials involving mucosal microbicides, anti-inflammatory cytokines or their inhibitors require monitoring of cytokines during this treatment. Clinical trials evaluating various immunologic phenomena evaluate cytokines to gain a deeper understanding of immunologic functions.

Kits for cytokine quantification are available, therefore determination is feasible.

2.3.4 PREANALYTICAL CONSIDERATIONS FOR CYTOKINE ANALYSIS

Cytokines are soluble and may be analysed from cell culture supernatants, body fluids such as blood, cerebrospinal fluid or synovial fluid. Cytokines may also be evaluated in lavage specimens such as bronchioalveolar lavage, washes from wounds, ulcers, mucosal surfaces and skin. Cervicovaginal lavage is an important specimen for cytokine analysis (34).

Cytoplasmic cytokines and cell membrane bound cytokines may be analysed in peripheral blood mononuclear cells, cytology and histological specimens.
Soluble cytokines should be measured within six hours of specimen collection. If this is not possible, prompt cell separation and storage should be done (10). Cell separation should be accomplished by centrifugation at speeds of approximately 1000 rpm for a maximum of ten minutes. Aliquots of the supernatant and cell suspensions should be prepared. These aliquots may be frozen at -20°C to -196°C in pyrogen free tubes to avoid in-vitro activation. Freeze thaw cycles should be avoided. The assay may be done after thawing to room temperature and thorough mixing.

2.3.5 ANALYTICAL TECHNIQUES FOR CYTOKINE ANALYSIS

2.3.5.1 BIOASSAYS.

Cytokines were first discovered due to their biological activity on specific cells and on a specific biological network (36). These biological activities can be utilized as surrogate markers in cytokine bioassays. Quantitative evaluation can be accomplished by establishment of a standard curve extrapolated from exposure of cell lines to standard concentrations of cytokines. Three types of bioassays are recognized:

- **Proliferation tests:** this evaluates the cytokines by their effects of cytokines on proliferation. An example being the evaluation of IL-6 on B-9 lymphocyte cell lines.

- **Tests for cytotoxicity:** this evaluates the cytokines by their cytotoxic effects. An example is the cytotoxic activity of TNF-α on WEH 164 cell lines.

- **Chemotactic activity:** migration of cells on exposure to chemokines such as IL-8.

The advantages of bioassays include their high sensitivity and low detection limits. They may detect cytokine concentrations less than 0.1ng/ml. The disadvantages include poor specificity.
with coefficients of variation (CV) varying from 15-100%, poor sensitivity – particularly when cells require these cytokines for survival and long analysis times.

2.3.5.1 IMMUNOASSAYS

This involves the use of cytokine specific monoclonal, oligoclonal or polyclonal antibodies for qualitative or quantitative analysis. These methods are practical and may be performed in a wide variety of laboratories (11).

Immunoassays such as ELISA where the solid phase of the antibody-cytokine interaction occurs in coated wells. These have high analytical performance, frequently with CV below 6%. These tests are currently regarded as the gold standard for cytokine analysis.

Multiplex analysis where the solid phase of the antibody-cytokine interaction occurring on coloured microspheres are currently preferred. These have the capability of simultaneous quantitative measurements of as many as 70 cytokines. Multiplex microsphere based immunoassays integrates laser optics, fluidics and advanced digital signal processing. Although the detection limits are higher than bioassays. Their analytical performance are good, with CV ranging from 5-15%. They can be automated.

Qualitative and quantitative cytokine assays may be determined from intracellular compartments. Cell membrane bound cytokines may also be determined. This is accomplished using flow cytometry employing 3 to 4 colour FL 1, FL 2, FL 3 or FL 4.

Cytokine expression by evaluation of specific messenger RNA may be done to determine cellular responses and may be accomplished and expressed as qualitative, semi quantitative or quantitative assays.
CHAPTER 3 MATERIALS AND METHODS

3.1 STUDY DESIGN

This was a case-control study.

3.2 STUDY AREA

The study was conducted at the Research Training and Care Program/Family Aids Care and Education Services (RCTP/FACES) comprehensive care clinic in Lumumba Health Centre, in Kisumu, Kenya’s third largest city, whose HIV prevalence of 15% is twice the national average. The clinic serves as a referral centre for HIV management with special emphasis on the family. Clinical services are provided as outpatient basis, these include clinical monitoring prophylaxis of opportunistic infections, cervical cancer screening, treatment of opportunistic infections and antiretroviral therapy. Family planning is offered and family planning goals defined and implemented. Currently, over 15000 HIV positive patients are enrolled in clinical support cohort, with approximately 7000 being women.

3.3 STUDY POPULATION

Asymptomatic HIV positive ART naïve women attending the Research Training and Care Program/Family Aids Care and Education Services (RCTP/FACES) comprehensive care clinic in Lumumba Health Centre located in the city of Kisumu. From the study population, participants were recruited into the following groups:

1. Cases: On DMPA.
2. Controls: Not on hormonal contraceptives but with normal menstrual cycle patterns.

3.4 RECRUITMENT

All participants were counselled and provided written informed consent signed for participation. Benefits and risks of participating in the study were communicated to the participants. A standard questionnaire was used to collect socio-demographic data. The Principal Investigator was responsible for the recruitment of study participants, specimen collection, processing and analysis. Screening of study participants was undertaken prior to selection of the most appropriate subjects for cytokine analysis.

3.5 INCLUSION CRITERIA:

3.5.1: CASES

1. HIV positive women, aged between 18-45 years.

2. Consent to participate in the study.

3. CD4 cell counts above 350/uL.

4. Asymptomatic

5. Having received injectable DMPA contraceptives within the previous 11 weeks.

3.5.2: CONTROLS

1. HIV positive women, aged between 18-45 years.

2. Consent to participate in the study.
3. CD4 cell counts above 350/μL

4. asymptomatic

5. Having regular menses, and in the proliferative menstrual phase at recruitment.

3.6 EXCLUSION CRITERIA

3.6.1: CASES:

1. Below 18 years and above 45 years

2. Clinical HIV WHO stage 3 or 4

3. On treatment for tuberculosis

4. On Antiretroviral therapy

5. Acutely ill women,

6. Women with history of uterine cervical dysplasia


8. Lactating.

9. Presence of cervical inflammatory lesions including candidiasis, trichomoniasis, bacterial vaginosis, genital ulcer disease, and gonococcal infection.

10. Where consent is not issued.
3.6.2: CONTROLS

1. Below 18 years and above 45 years.

2. On any form of hormonal contraception

3. On treatment for tuberculosis.

4. On antiretroviral therapy

5. Acutely ill women.


8. Pregnant.

9. Lactating.

10. Participants who report absence of menses for over 30 days.

11. Presence of cervical inflammatory lesions including candidiasis, trichomoniasis, bacterial vaginosis, genital ulcer disease, and gonococcal infection,

12. Where consent is not issued

13. Women on other forms of hormonal contraceptives.
3.7 SPECIMEN COLLECTION

A general physical and gynaecological examination was performed for each study participants. In order to avoid semen contamination which may present a confounding factor, study participants who reported recent sexual intercourse had specimen collection deferred. Microscopic examination of vaginal wet mount specimens was done to aid in detecting semen contamination.

After insertion of a sterile speculum, Specimens were collected as follows:

1. Cervical swab and vaginal swab specimens were collected for testing for, *Neisseria gonorrhoeae*, agents of bacterial vaginosis, Candidiasis, and *Trichomonas vaginalis*.

2. Cervical Smears: Using a cervix brush, cervical exfoliative cytology samples were collected for Papanicolau staining. The brush was placed on the ectocervix with the longer inner bristles placed into the cervical os. Ten clockwise rotations were then made, and the material on the brush smeared on to a microscope slide, fixed by flooding with 95% ethanol, transported to the laboratory for Papanicolaou staining and evaluation for cervical dysplasia.

3. Cervicovaginal lavage fluid was collected as follows: The cervix was bathed with 10 ml of sterile saline, which was allowed to pool in the posterior fornix, where it was then aspirated by plastic bulb pipette. Fluid was dispensed into a sterile container. Cells within the cervicovaginal lavage fluid were separated by centrifugation at 1000 rpm for 10 minutes at 4°C. The following samples in 1 ml aliquots were then stored: one with the
cell pellet and 9 supernatant and stored at -80° for analysis and future research in innate immunity.

Blood was collected in EDTA and in plain bottles for CD4 determination and Herpes Simplex Virus type 2 IgG immunoassays respectively. Plasma was stored for further studies in mucosal immunity on this population to determine factors associated with use of DMPA in this population.

3.8 LABORATORY TESTS

Laboratory analysis was conducted at the RCTP/FACES laboratories in Kisumu and at the Immunology laboratory at the Kenya Aids Vaccine Initiative (KAVI), School of Medicine, University of Nairobi.

3.8.1. *Neisseria gonorrhoeae*:

Cervical swab specimens were taken for culture for *Neisseria gonorrhoeae*. These samples were streaked on Thayer-Martin culture plates, placed in a candle extinction jar and transported to the laboratory within four hours, where they were incubated at 37°C under microaerophilic conditions for a minimum of 24 hours. Presence of characteristic oxidase positive tiny grey colonies consisting of gram negative diplococci was diagnostic of *Neisseria gonorrhoeae*. Absence of these typical colonies 72 hours after incubation was categorized as negative.

3.8.2. Bacterial vaginosis:

High vaginal swab samples were transported to the laboratory within 3 hours. Smears were made on glass slides, air dried, heat fixed and Gram stained. Microscopy was performed under oil
immersion. Identification of bacterial types and scoring using the Nugent score was done. Nugent scores of 7-10 were diagnostic of bacterial vaginosis.

3.8.3. *Trichomonas vaginalis*:

High vaginal swab samples obtained using cotton tipped swabs were collected and transported to the laboratory within 3 hours. Using phosphate buffered saline, wet mount preparations were made. Microscopy performed under objective lens 10x. Presence of motile pear shaped trichomonads was diagnostic of *Trichomonas vaginalis* infection.

3.8.4. Herpes Simplex Virus type 2.

One (1) ml of serum was separated within 2 hours, transferred to cryovials and stored at -20°C. These samples were then assayed after one month in batches for HSV 2 specific glycoprotein G antibodies. This was done using HSV 2 IgG specific Enzyme Immunoassay kit, manufactured by Kalon Diagnostics, United Kingdom. Optical densities above a cut off point calculated by values determined positive and negative controls were reported as positive.

3.8.5. Cervical smears:

Papanicolaou staining was then performed, microscopy done by a cytotechnologist and Pathologist. Reporting was done in accordance with The Bethesda System (2001) for evaluation of cervical smear specimens.

3.8.6. Cytokine level determination:

The CVL supernatant was tested for the cytokines IL 1β, IL 6, IL 8, IL 10, IL 12p70 and TNF-α. These assays were conducted using multiplexed cytometric bead array. as described in appendix
6. Analysis was done using Beckton-Dickinson Flow Assisted Cell Sorter Calibur (BD-FACS Calibur) as per the manufacturing instructions, and analyzed using array analysis software. Detailed test principles and standard operating procedures are in Appendix 4. Briefly …

3.9 SAMPLE SIZE

The study conducted in Nairobi by Sinei et al (year) determined the Odds of HIV acquisition by women on hormonal contraceptives to be 3.99 more than those not on hormonal contraceptives (36). Using this data, the sample size for the present study was calculated by the case control formula described by DuPont and Plummer (Reference):

\[
n = \left( \frac{r + 1}{r} \right) \frac{(\bar{p})(1-\bar{p})(Z_\beta + Z_{\alpha/2})^2}{(p_1 - p_2)^2}
\]

Where \( n \) minimum sample size

- \( Z_\beta \) Represents the desired power which is .84 for 80% power

- \( Z_{\alpha/2} \) Level of statistical significance of 0.05 which is 1.96

\((p_1 - p_2)^2\) The difference in proportions (effect size) which is represented here by the odds ratio of 3.99

\((\bar{p})(1-\bar{p})\) Measure of variability (standard Deviation)

\( r=1 \) equal number of cases and controls

Comment [I7]: You have to describe the methodology here. One cannot keep checking the appendix except for details of the procedure

Comment [AS8]: What does this mean? An odds ratio of what?
\[ \frac{r+1}{r} \] Ratio of controls and cases

\( p_1 \): The estimated proportion of opportunistic infection among the cases at 50\% (assumed as the highest since there is no available data on the control group).

\( p_2 \): The proportion within the control group at 40\%

The sample size of 70 was determined consisting of 35 cases and 35 controls.

3.11 QUALITY ASSURANCE

Strict adherence to standard operating procedures during sample collection and processing was ensured. All tests were done in accordance to manufacturers’ recommendations and with assistance of experienced personnel.

3.12 ETHICAL CONSIDERATION

Approval for the study protocol was obtained from the Kenyatta National Hospital- University of Nairobi Ethics and Research Committee (KNH-UON ERC) prior to commencement of the study. Informed consent to participate in the study was obtained from each study participant. A copy of the approval letter is attached in appendix 6.

Sample collection was carried out according to standard procedure. Confidentiality is maintained and was communicated to individual participants.

Participants in whom disease was diagnosed were recalled, counselled, provided with treatment, and followed-up. Participants in whom repeat visits were required had their travel to and from the hospital reimbursed.
Safety protocols were adhered to in all specimen collection, handling and analysis. Use of personal protective equipment was ensured. Best laboratory practices in disposal of specimens and decontamination of infectious surfaces was done. Good clinical laboratory practice guidelines were adhered to.

### 3.14 DATA MANAGEMENT

The collected data was entered into the Statistical Package for Social Sciences version 17 (SPSS version 17). The data was cleaned for errors and inconsistent or conflicting answers, missing entries and duplicate entries to ensure high quality data. Descriptive statistics on Socio-demographic characteristics was used to characterize the study participants. Depending on the type of a variable, summary statistics such as mean, standard deviation, median, and interquartile range using appropriate for the measurement scale were used to describe distribution of these variables. Statistics include mean for continuous variables such as age and proportions for categorical variables such as gender, education level etc. These statistics are presented in tables. For the formal statistical inference, a comparison of the mean and median cytokine levels of the study and control groups was accomplished using the student t and Mann-Whitney tests of association, respectively. The decision on what test to use was based on the Shapiro-O-Wilk’s test for linearity. Multivariate Multiple Regression Model was applied for assessment of confounding factors and multiple relationships. For all the analyses, two-sided tests was used, with p-values of <0.05 being statistically significant.
CHAPTER 4 - RESULTS

The study was conducted from August 2010 to January 2011. A total of 125 participants were screened, 52 cases and 73 controls. Out of these, 70 participants met the inclusion criteria and were then recruited in the study, 35 recruited as cases, and 35 recruited as controls.
<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>CASE (n=55)</th>
<th>CONTROL (n=70)</th>
<th>STATISTIC</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Age in years</td>
<td>26 (25)</td>
<td>28 (23)</td>
<td>Mann Whitney (U)</td>
<td>0.021</td>
</tr>
<tr>
<td>(median)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marital Status</td>
<td></td>
<td></td>
<td>Fishers Exact</td>
<td>0.345</td>
</tr>
<tr>
<td>Single</td>
<td>8</td>
<td>17</td>
<td>Test</td>
<td></td>
</tr>
<tr>
<td>Married</td>
<td>39</td>
<td>39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Separated</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Divorced</td>
<td>3</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Widowed</td>
<td>6</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
<td>Fishers Exact</td>
<td>0.095</td>
</tr>
<tr>
<td>Primary</td>
<td>37</td>
<td>38</td>
<td>Test</td>
<td></td>
</tr>
<tr>
<td>Secondary</td>
<td>16</td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>College</td>
<td>1</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>University</td>
<td>3</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
These were the characteristics of the screened study participants. In this population, the median age of women on DMPA was higher than women not on contraception. This however did not affect the age matching designed using 5 year age intervals. No significant differences in social and demographic characteristics were found between the two groups.

**TABLE 2: LYMPHOCYTE COUNTS AND HSV 2 ELISA ASSESSMENT**

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>CASE (n=55)</th>
<th>CONTROL (n=70)</th>
<th>STATISTIC</th>
<th>P VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean CD 4 (median)</td>
<td>649 (474),</td>
<td>573 (524)</td>
<td>Mann Whitney U test</td>
<td>0.02</td>
</tr>
<tr>
<td>Mean Total Lymphocyte Counts (median)</td>
<td>1935 (1271)</td>
<td>1603 (1628)</td>
<td>Mann Whitney U test</td>
<td>0.004</td>
</tr>
<tr>
<td>HSV 2 Positive (percentage)</td>
<td>49 (94%)</td>
<td>55 (83%)</td>
<td>χ²</td>
<td>0.034</td>
</tr>
<tr>
<td>Mean HSV 2 Index Values (median)</td>
<td>3.43 (3.60)</td>
<td>2.47 (3.08)</td>
<td>ANOVA</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Despite higher mean total lymphocyte and CD 4 lymphocyte cell counts in women on DMPA, the non linear distribution of the data necessitated comparison of median values, which were significantly lower in cases compared to controls. The prevalence of HSV 2 infections with higher optical values was higher in cases compared to controls.
<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>CASE</th>
<th>CONTROL</th>
<th>STATISTIC</th>
<th>P VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida</td>
<td>3</td>
<td>3</td>
<td>Fisher’s exact</td>
<td>1</td>
</tr>
<tr>
<td>Bacterial Vaginosis</td>
<td>11</td>
<td>15</td>
<td></td>
<td>0.979</td>
</tr>
<tr>
<td>Trichomonas Vaginalis</td>
<td>0</td>
<td>1</td>
<td>Fisher’s exact</td>
<td>1</td>
</tr>
<tr>
<td>Neisseria gonorrhoeae</td>
<td>0</td>
<td>1</td>
<td>Fisher’s exact</td>
<td>1</td>
</tr>
<tr>
<td>Abnormal Cervical Cytology</td>
<td>13</td>
<td>3</td>
<td>Fisher’s exact</td>
<td>0.005</td>
</tr>
<tr>
<td>Participants (controls) not in proliferative cycle phase</td>
<td>N/A</td>
<td>14</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Participants screened but not included in the study due to diagnosis of inflammatory lesions of the cervix. Women on DMPA were more likely to have abnormal cervical cytology.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases (n=35)</th>
<th>Controls (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Age</td>
<td>26.7</td>
<td>30.09</td>
</tr>
<tr>
<td>Median Age</td>
<td>26.0</td>
<td>28.0</td>
</tr>
<tr>
<td>95% C.I. for age</td>
<td>24.8-28.9</td>
<td>27.6-32.5</td>
</tr>
<tr>
<td>SD of Age</td>
<td>5.9</td>
<td>7.04</td>
</tr>
<tr>
<td><strong>Marital Status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Married</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td>Single</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Separated</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Widowed</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td><strong>Highest Educational Level Attained</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary School</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td>Secondary school</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Middle level college</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>University</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><strong>Occupation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Professional</td>
<td>7</td>
<td>4</td>
</tr>
</tbody>
</table>
There were no significant differences in demographic characteristics among cases and controls recruited into the study.

### Table 4: Table showing test of homogeneity of variance for age

<table>
<thead>
<tr>
<th>Participant’s age in years</th>
<th>Levene’s Statistic</th>
<th>df1</th>
<th>df2</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Based on Mean</td>
<td>1.823</td>
<td>1</td>
<td>68</td>
<td>0.181</td>
</tr>
<tr>
<td>Based on Median</td>
<td>0.667</td>
<td>1</td>
<td>68</td>
<td>0.417</td>
</tr>
<tr>
<td>Based on median and adjusted df</td>
<td>0.667</td>
<td>1</td>
<td>65.478</td>
<td>0.417</td>
</tr>
<tr>
<td>Based on Trimmed mean</td>
<td>1.591</td>
<td>1</td>
<td>68</td>
<td>0.211</td>
</tr>
</tbody>
</table>

Levene’s test of Homogeneity of Variance shows no significant differences among cases and controls. Therefore, sufficient age matching was achieved.
TABLE 4: CONCENTRATIONS OF CERVICOVAGINAL LAVAGE FLUID PROINFLAMMATORY CYTOKINES IN ng/ml.

<table>
<thead>
<tr>
<th>CYTOKINE</th>
<th>CASE (n=35)</th>
<th>CONTROL (n=35)</th>
<th>STATISTIC</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean IL 10 (SD)</td>
<td>12.5 (6.5)</td>
<td>9 (6.5)</td>
<td>Paired student t test</td>
<td>0.022</td>
</tr>
<tr>
<td>Mean IL 1β (median, IQR)</td>
<td>38.3 (16.8, 28.9)</td>
<td>59.9 (28.1, 215.4)</td>
<td>Mann Whitney (U) test</td>
<td>0.046</td>
</tr>
<tr>
<td>Mean IL 6 (median, IQR)</td>
<td>21.3 (16.8, 28.9)</td>
<td>21.7 (18.1, 35.8)</td>
<td>Mann Whitney (U) test</td>
<td>0.438</td>
</tr>
<tr>
<td>Mean IL 8 (median, IQR)</td>
<td>690 (97.2, 18708)</td>
<td>10969 (148.3, 20.2)</td>
<td>Mann Whitney (U) test</td>
<td>0.456</td>
</tr>
<tr>
<td>IL 12p70</td>
<td>11.7 (7.3, 16.9)</td>
<td>9.1 (7.6, 14.5)</td>
<td>Mann Whitney (U) test</td>
<td>0.846</td>
</tr>
<tr>
<td>TNF α</td>
<td>15.1 (9.8, 16.9)</td>
<td>13.6 (4, 19.1)</td>
<td>Mann Whitney (U) test</td>
<td>0.715</td>
</tr>
</tbody>
</table>

Cytokine assays done on cervicovaginal lavage fluid, compared between the two groups, Mann-Whitney statistical test applied in skewed data while student t test applied in normally distributed data. The concentrations of IL 10 are significantly raised in women on DMPA. There is a statistically significant reduction in IL 1β concentrations in women on DMPA. The concentrations of IL 6, IL 8, IL 12p70 and TNF α in cases show a non statistically significant reduction in women on DMPA.
Participants recruited as cases had used DMPA for a mean duration of 23 months, median duration of 12 months, mode duration of 12 months. The interquartile range was 14 months.
FIGURE 1: SCATTER PLOT SHOWING CORRELATION BETWEEN IL 1 BETA

CHART 2: EFFECT OF DURATION OF DMPA USE ON CVL IL 1 BETA

P Value: 0.015

This chart shows a scatter plot representing multivariate multiple regression model, showing the effect of duration of DMPA use on the concentration of IL 1β in cervicovaginal lavage fluid. This shows that prolonged use of DMPA is associated with a time dependent reduction in IL 1β concentrations.
FIGURE 2: SCATTER PLOT SHOWING CORRELATION BETWEEN IL 8 CONCENTRATIONS (ng/ml) AND DURATION OF DMPA USE

P value: 0.041

Multivariate multiple regression models to determine effects of duration of DMPA use on cytokine levels did not show significant differences in IL 1β, IL 6, IL 10, IL 12p70 and TNF α. However, as illustrated in the chart above, duration of DMPA use had a time dependent reduction in IL 8 concentration.
TABLE 4: MULTIVARIATE MULTIPLE REGRESSION ANALYSIS OF THE EFFECT OF HSV 2 ELISA INDEX VALUES ON CVL CYTOKINE CONCENTRATIONS

<table>
<thead>
<tr>
<th>CYTOKINE</th>
<th>COEFFICIENT OF HSV 2 IgG</th>
<th>95% Confidence Interval</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF α</td>
<td>20.29</td>
<td>12.9-27.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL 1β</td>
<td>11.44</td>
<td>7.7-15.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL 6</td>
<td>46.1</td>
<td>21.6-70.7</td>
<td>0.001</td>
</tr>
<tr>
<td>IL 8</td>
<td>198.5</td>
<td>84.4-312.6</td>
<td>0.002</td>
</tr>
<tr>
<td>IL 10</td>
<td>15.6</td>
<td>8.8-22.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL 12p70</td>
<td>3.4</td>
<td>1.7-5.2</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Represented on these tables are multivariate regression models evaluating the effect of the HSV 2 index values as determined by ELISA on CVL cytokine concentrations. This shows a strong association between HSV 2 and the cytokines measured in cervicovaginal fluid and as such presents a significant confounding factor. A higher HSV 2 index value is associated with elevation of all cytokines measured.
CHAPTER 5 DISCUSSION

To the best of our knowledge, this is the first study to have evaluated \textit{in vivo} effects of DMPA on mucosal innate immune activation in HIV positive women drawn from a population with high HIV prevalence. It draws comparisons from animal models and in vitro studies on pure cell lines.

We found that DMPA is associated with reduction in the CVL proinflammatory cytokine IL $1\beta$ and elevation of the cytokine IL 10 which has regulatory inflammatory activity. There was a non statistically significant reduction of the proinflammatory cytokines TNF $\alpha$, IL 6, IL 8 and IL 12 p 40. Furthermore, women on DMPA have higher HSV 2 prevalence as determined by ELISA with high index values. These women were found to have lower total and CD 4 Lymphocyte counts.

We found elevated anti-inflammatory IL 10, and mild reduction of proinflammatory cytokines IL 1$\beta$, TNF $\alpha$, IL 6, IL 8, IL 12 p 40, of which only IL 1$\beta$ is statistically significant. \textbf{DMPA use was therefore not associated with increased inflammation} and therefore the findings of a study by Ghanem et al, which found elevated inflammatory cells in CVL of women on DMPA, may be \textit{unrelated} to type I mucosal immune activation but evidence of type II mucosal immune activation (8). We also did not show significant suppression of proinflammatory cytokines and hence mucosal innate immune activation, as had been shown in studies on monocytoid cell lines (9). However, this may provide an explanation for the association of DMPA with HIV and HSV 2 transmission (7). DMPA associated reduction in proinflammatory response then suppresses viral antigen detection and mucosal innate and adaptive immune activation. This may lead to
greater viral susceptibility (36). However, latent viral infections, in particular latent HSV 2, diminishes DMPA associated suppression of proinflammatory cytokines (32).

We also evaluated proinflammatory cytokines in HIV positive women not on DMPA. The cytokine profiles in this population were higher than those found in HIV negative HSV 2 negative populations, probably due to persistent antigenic challenge or systemic immune activation (32).

In our study, DMPA was not associated with significant reduction in proinflammatory cytokine levels, significant reduction found in the cytokine IL 1β, unlike previous studies that found a significant dose dependent DMPA induced reduction of the proinflammatory cytokines IL 1, IL 6, IL 8, IL 12 and IL 18 by monocyte cell lines on activation by bacterial lipopolysaccharides and unmethylated CpG (10). There was a significant dose dependent DMPA induced elevation of Th2 cytokines IL 4, IL 10, IL 13 and TGF β. DMPA leads to Th2 mediated immune cytokine production on activation of monocytoid cell lines. This difference may be unique in this population which consists of HIV positive women, and exhibit high HSV 2 prevalence. We hypothesize that elevation of IL 10 may signify Th2 cytokine expression is due to DMPA effect on activated of macrophages. Reduction of IL 1β levels in this study suggests suppression of proinflammatory responses. IL 10 inhibits macrophage activation and T cell proliferation. However, it may act in synergy with IL 4 and IL 13 promoting B cell differentiation into plasma cells, and isotype switching, further promoting immunoglobulin hypermutation and antibody avidity. DMPA has been associated in increased mucosal IgG and IgA levels, but lower systemic antibody levels as a result of loss of T cell dependent antibody responses (9). This has been a challenge on vaccine studies as DMPA has been shown to abrogate vaccine induced protection to HIV (17).
Our study found elevated IL 10 in women on DMPA. The effects of these changes on mucosal immunity include polarization to type 2 innate immune responses which may lead to susceptibility to HIV and HSV 2 infection. Previous studies have shown in vitro inhibition of interferon α, and inhibition of antiviral type 1 innate immune responses(38). In HIV positive women, increased susceptibility may lead to increased risk of hyper infection by other strains of HIV.

We found a higher prevalence of HSV 2 in women on DMPA. HSV 2 positive women on DMPA exhibited higher ELISA index values, showing a likely increase in HSV 2 specific antibodies. There is scant information evaluating the effects of DMPA on HSV 2 acquisition or HSV 2 and HIV co-infection. From a recent study, it was found that among HSV 2 positive women, DMPA did not seem to increase the risk of HIV acquisition, whereas among HSV 2 negative participants, the risk of HIV acquisition was much higher(37). HSV 2 specific antibodies may signify increased HSV 2 reactivation (39). However, correlation between these antibodies and HSV 2 reactivation is poor (40). Mechanisms of HSV 2 latency may present persistent immune activation leading to elevated cytokines. It is likely that these antigens may have a potent effect on professional phagocytes leading to a mild difference in IL 1β, IL 6, IL 8, IL 12 and TNF α and significant IL 10 elevation. Studies have shown that gamma-herpesvirus infection may have a protective effect against bacterial infections by establishing a level of innate immunity that is maintained as the host tries to prevent HSV-2 emergence from latency as a manifestation of beneficial virus-host mutualism (32, 33).

We found that CD 4 lymphocyte and total lymphocyte counts are lower in women on DMPA compared to those not on contraceptives. This finding concurs with a study which found poor immunologic response of severely immunosuppressed HIV positive women on DMPA to ART
despite adequate viral suppression (29). Other studies found that DMPA use leads to an increase in predominantly CD4 positive CCR 5 positive cells(21). DMPA use may select for the less pathogenic non syncitial forming R5 trophic HIV rather than the syncitium forming X4 tropic HIV, resulting in poorer progress to AIDS (21). It is likely HIV positive women on DMPA may require earlier initiation of antiretroviral therapy (ART) because of lower systemic CD 4 counts although this require correlation with systemic viral load assessment. However, it is also likely that progestins such as DMPA inhibit HIV replication by inhibition of immune activation. Progestins may also reduce infection of permissive cells by reducing antigen presentation therefore HIV positive women on DMPA may have a better prognosis, which may further be improved by early initiation of highly effective anti retroviral therapy(29).

These changes in mucosal and systemic immune responses may be due to unique attributes of this population. Studies have shown differences in mucosal immune activation in women of African descent compared to other population groups. African women have higher levels of activated lymphocytes which are permissive to HIV, and due to their activation status may have a higher predisposition to HIV and HSV 2 infection (28). DMPA may contribute to an increased risk in this population through polarization to type 2 mucosal immune responses.
6.1 CONCLUSION

The use of DMPA in HIV positive women is associated with type II mucosal immune activation as evidenced by elevated IL 10 and a time dependent suppression of type I mucosal immune activation as evidenced by a time dependent reduction in IL 1β and IL 8 concentrations. DMPA use is also associated with lower median CD4 cell and total lymphocyte counts. These may be due to the confounding effects of HSV 2 infection whose prevalence is higher in women on DMPA.

6.2 RECOMMENDATIONS

Use of DMPA is associated with type II immune activation which may be responsible for increased female to male transmission of HIV, it is the recommendation of this study that HIV viral shedding be evaluated in light of these findings. HSV 2 prevalence in women on DMPA is higher and is associated with higher proinflammatory cytokines. This may be a manifestation of host-virus mutualism. Further evaluation is therefore required to assess this interaction.

6.3 LIMITATIONS

1. Due to the high prevalence of Herpes Simplex Virus infection, it was impossible to exclude from the study all participants who test positive for this infection.

2. Due to logistical limitations, total protein levels were not measured in CVL to act as a determinant of dilution associated with specimen collection.
REFERENCES


APPENDICES

APPENDIX 1: FLOW CHART

This flow chart summarises participant recruitment and laboratory testing.

Prior to enrolment

Obtain Screening informed consent. Complete questionnaire, pregnancy testing, PAP, HIV and STI testing to determine enrolment eligibility.

Select participants. Those on DMPA, not on contraception. After taking a menstrual history, determine cycle phase.

Participants on DMPA

Participants not on contraception

Day 0-10 Proliferative phase: Clinical assessment, pelvic exam, completion of questionnaire, specimen collection, processing and storage
Laboratory assessment of proinflammatory cytokine profiles by cytometric bead array.
Please take as much time as you want to read this form, ask questions, and talk about this project with family or friends.

What is this project about?

This project aims to investigate immune system factors in the mucosa (lining) of the lower female genital tract to determine whether there are factors, such as inflammation which is a process by which the body reacts to pathogens and other potentially threatening process and results in healing and repair. We would like to investigate whether progesterone based contraceptives contributes to this process in HIV positive women.

Why are we doing this project?

Cytokines are protein compounds which are important for intercellular communications between cells of the immune system. They have various functions; those which mediate the inflammatory process are known as the proinflammatory cytokines.

The proinflammatory cytokine levels may give us an understanding of the factors that may influence the risk of HIV spread in women using depot medroxyprogesterone acetate contraceptives. It also helps scientists to understand the between HIV positive and negative persons concerning genital tract immunology factors and the influence of contraceptives. This
information is lacking particularly in Sub-Saharan Africa.

**What are the benefits to the study participants and the community?**

We will undertake diagnosis for infectious diseases of the genital tract and on making a diagnosis treatment will be provided. Cervical smears will be done and findings communicated to the study participants.

Scientific information derived from this study will be useful in understanding immune factors of the genital tract, will be of benefit for the scientific community as a whole particularly in the field of reproductive health.

**How will the samples be used?**

Over the next two months the samples will undergo screening for infectious disease involving the genital tract. Proinflammatory cytokine profiles will then be established to determine mucosal immune factors which will give us an insight in the role of depot medroxyprogesterone acetate in HIV and AIDS. Samples will be retained for a period of 5 years for future studies in mucosal immunity.

**What will happen if I decide to give a sample?**

We will request you to abstain from sexual activity or vaginal douching for at least 48 hours prior to sample collection. However, if you choose not to abstain then you may use a condom during sexual activity.

We will ask you about your demographic information and menstrual history for the past six months in order to establish your menstrual cycles.
We will use all this information to decide whether you can give a sample, but we will not keep any of this information or put it in the database. We will then draw about [4 ml] of blood from your arm. A gynaecologic exam will then be done and a cervical smear, swab and cervicovaginal lavage fluid will be collected. All of this will take about 1 hour.

We will send your sample to the FACES laboratory for preliminary analysis and the Immunology Laboratory, School of Medicine, University of Nairobi for analysis. Samples collected will be stored for a period of 5 years to aid in validation of the test.

The institution may send the samples to other researchers for use in future mucosal immunity studies as described in this form. The researchers will have to follow all U.S. and international laws and guidelines that apply to research. All studies using the cell lines will have to be approved by the institutional Ethical Review Board. An ERB is a committee similar to the one that approved this project to make sure that your rights were protected. Also, a Community Advisory Group will be set up for each community that takes part in this project. This group will include people from your community and will make sure that future studies using your community’s samples are similar to ones described in this form. This group will also suggest ways to do those studies to limit any possible harm to your community.

**Will there be any costs or payments?**

It will not cost you anything to be a part of this project. We will give you KSh 300 for your time, travel, and inconvenience if you come in to give us a sample.

The institution does not let anyone sell material from samples or cell lines. Also, because the cell lines will not have names on them, neither the researchers nor anyone at the Repository
would know if your sample was even used. So you will not get any additional payment if you take part in this project.

**How will you protect my privacy?**

We will protect your privacy in several ways. While FACES will keep your signed consent form, nobody else will see it. We will not keep your name with your sample or give your sample a code number that could identify you. So nobody at the Repository or who studies your sample will know that it came from you.

Also, we will collect more samples than we will use. This way nobody, not even you or us, will know if your sample was used or if any information in the database came from you. (Samples that are not used will be disposed of in standard ways).

**What are the benefits of giving a sample?**

Your samples will be screened for infectious disease and when diagnosed, treatment will be offered in line with institutional guidelines. Researchers will study these samples for many years to learn about health and disease. This research will eventually benefit the health of people around the world.

**What are the risks of giving a sample?**

Drawing blood has very minor risks. These include brief pain, slight bruising, dizziness or fainting, and (very rarely) infection where the needle goes in. Lower genital tract sampling may be uncomfortable but has minor risks.

**Are there any risks to my community or group?**
Information on the demographic characteristics of participants will be included with the samples, in the database, and in the published report. In future studies, researchers may find that certain mucosal immunity variations appear more often in people from your group than in people from other groups, and that these variations are more common in people with a certain disease. This may make some people look down on your group unfairly.

Some people may use the information from the cervicovaginal lavage cytokine profile study or from future studies using the published to exaggerated differences between groups for prejudiced or other bad reasons. Others may use the information to downplay differences between groups, to say that all people’s mucosal immune characteristics are about the same, so we don’t need to respect the special concerns of different groups. Biology does not provide a reason for prejudice, but discrimination does exist.

We will work to make sure that the ethnic or geographic identity of your community is described as carefully as possible--in the sample collection, in the database, in and in any articles researchers write about the trial.

**Can I change my mind after I give a sample?**

Giving a sample is completely up to you. You will not lose any benefits if you choose not to give a sample. However, after you give a sample you cannot take it back or take any information out of the database. However, in this case your sample and results will not be included in the analysis.
How will I find out what happens with this project?

Because your sample will not have your name on it, we will not be able to give you individual results from this research. However, we will update your community through FACES Nyanza programme on how researchers are using the cervicovaginal lavage samples and your community’s samples and what they are learning about health and disease.

The results of the infection screen will be communicated to your attending physician for clinical care.

The results will also be analyzed and published in a journal and on the FACES-Nyanza web site.

A dissertation will also be written and will be kept in the University of Nairobi- School of Medicine library, and at the Department of Human Pathology library.
COMPARATIVE STUDY OF PROINFLAMMATORY CYTOKINE PROFILE OF HIV POSITIVE WOMEN ON DEPOT MEDROXYPROGESTERONE ACETATE ATTENDING A COMPREHENSIVE CARE CENTRE IN KISUMU, KENYA.

Consent Form

Who can I talk to if I have questions or problems?

If you have questions about this sample collection, contact:

(PI) DR WALONG EDWIN OLOO (phone) +254738590623, Email: edwin.owino@students.uonbi.ac.ke.

If you have questions about your rights as part of this research project, contact:

(ERC) KENYATTA NATIONAL HOSPITAL-UNIVERSITY OF NAIROBI ETHICAL REVIEW COMMITTEE. PO BOX 20723 KNH-NAIROBI. (Phone) (+254)20 726300. E-Mail: KHHplan@Ken.Healthnet.org
CONSENT AND SIGNATURE

Please read the paragraph below, think about your choice, and sign if you agree:

I agree to participate and undergo a medical examination and allow female genital tract sampling and blood for researchers to use for the proinflammatory cytokine profile study and in other approved studies of the type described in the form, and other studies in mucosal immunity. I have read or listened to the information, I have asked any questions I had, and all my questions were answered. I know that giving a sample is my choice.

Your Signature ____________________________

Date _____________

I agree to the storage of these samples for a period of not more than five (5) years, and I understand that any study conducted on these samples must undergo review and ethical approval from an ethical review committee.

Your Signature_____________________________________

Date______________

Copy given to participant: _____Yes
APPENDIX 3: QUESTIONNAIRE

RECRUITMENT PHASE

SECTION ONE – SOCIODEMOGRAPHIC DATA

STUDY NO: ________________

FACES ID: ________________

DATE: ________________

NAME OF STUDY PARTICIPANT: ________________

AGE:

1. 18-20 □
2. 21-25 □
3. 26-30 □
4. 31-35 □
5. 36-40 □
6. 41-45 □

MARITAL STATUS (circle 1):

1. SINGLE: □
□
□
□
□
□

66
2. MARRIED:

3. SEPARATED:

4. DIVORCED:

5. WIDOWED:

6. OTHER:
### HIGHEST EDUCATIONAL LEVEL ATTAINED (circle 1):

1. NONE: □
2. PRIMARY: □
3. SECONDARY: □
4. COLLEGE: □
5. UNIVERSITY: □
6. ADULT EDUCATION: □
7. OTHER: □

### OCCUPATION (circle 1):

1. HOUSEWIFE: □
2. FARMER: □
3. ARTISAN: □
4. UNEMPLOYED: □
5. PROFESSIONAL: □
6. OTHER: □

### SECTION 2: DRUG USE (circle 1):

1. ALCOHOL USE: □
   □ 68
2. CIGARETTE/TOBACCO USE:

3. OTHER:

SECTION 3: OBSTETRIC AND GYNAECOLOGY HISTORY

a) AGE AT MENARCHE (COMPLETED YEARS).

1. 10-15  
   
2. 16-20  
   
3. <10  
   
4. >20  

b) LAST MENSTRUAL PERIOD (DATE ON FIRST DAY OF LAST PERIOD).

c) DEGREE OF BLOOD LOSS:

1. LIGHT (1 PAD/24 HOURS):  
   
2. MODERATE (2-3 PADS/24 HOURS):  
   
3. HEAVY: (>3 PADS/24 HOURS):  

69
d) HAVE YOU EVER BEEN PREGNANT?

1. YES: □
   □
2. NO:

e) NUMBER OF LIVE BIRTHS:

1. 1-3 □
2. 4-6 □
3. ≥7 □

f) NUMBER OF STILL BIRTHS:

1. 1-3 □
2. 4-6 □
3. ≥7

g) NUMBER OF ABORTIONS:

1. 1-3 □
2. 4-7 □
3. >7
h) DATE OF LAST DELIVERY:

SECTION 4: CONTRACEPTION:

a) CURRENT FAMILY PLANNING METHOD:

1. NATURAL: □

2. MALE/FEMALE CONDOMS: □

3. SPERMICIDAL:

4. ORAL CONTRACEPTIVE PILLS: □

5. INJECTABLE: □

6. INTRAUTERINE DEVICES: □

7. OTHERS:

b) FAMILY PLANNING METHODS IN THE PAST SIX MONTHS:

1. NATURAL:

2. MALE/FEMALE CONDOMS: □

3. SPERMICIDES: □

4. ORAL CONTRACEPTIVE PILLS: □

5. INJECTABLE: □

6. INTRAUTERINE DEVICES:

71
7. OTHERS:
c) DURATION OF CONTRACEPTIVE USE (FOR THOSE ON DEPOT MEDROXYPROGESTERONE ACETATE):

1. <3 months
2. 3-6 months
3. 7-12 months
4. >12 months

SECTION 5: LOWER GENITAL TRACT INFECTIONS:

a) IS THERE HISTORY OF A LOWER GENITAL TRACT INFECTION?

1. YES:
2. NO:

b) IF YES, WHAT WERE THE SYMPTOMS:

1. VAGINAL DISCHARGE:
2. ULCER:
3. PELVIC PAIN:
4. RASH/ERUPTIONS:

c) DID YOU SEEK TREATMENT AT A HEALTH CARE PROVIDER?

1. YES:
2. NO:

d) WAS TREATMENT SUCCESSFUL?

1. YES: □

2. NO:

e) IF YOU ARE CURRENTLY SUFFERING FROM A LOWER GENITAL TRACT INFECTION WHAT ARE THE SYMPTOMS?

1. VAGINAL DISCHARGE: □

2. ULCER: □

3. PELVIC PAIN:

4. RASH/ERUPTIONS:

f) HISTORY OF GYNAECOLOGIC SURGERY

1. YES: □

2. NO
SECTION 6: PHYSICAL EXAMINATION

1. GENERAL EXAMINATION:

   a. PALLOR: 1-PRESENT. □ 2-ABSENT. □
   b. JAUNDICE: 1-PRESENT. 2-ABSENT.
   c. OEDEMA: 1-PRESENT. 2-ABSENT.
   d. FEVER: 1-PRESENT 2-ABSENT.
   e. LYMPHADENOPATHY: 1-PRESENT. 2-ABSENT.

2. SYSTEMIC EXAMINATION:

   a. CNS: 1-NORMAL. □ 2-ABNORMAL. □
   b. RESPIRATORY SYSTEM: 1-NORMAL. □ 2-ABNORMAL. □
   c. CARDIOVASCULAR SYSTEM: 1-NORMAL. 2-ABNORMAL.
   d. GASTROINTESTINAL SYSTEM: 1-NORMAL. 2-ABNORMAL.
   e. UROGENITAL SYSTEM: 1-NORMAL. □ 2-ABNORMAL.
i. INSPECTION: 1-NORMAL □ 2-ABNORMAL. □

ii. MONS PUBIS: 1-NORMAL □ 2-ABNORMAL. □

iii. VULVA: 1-NORMAL □ 2-ABNORMAL. □

iv. PERIANAL REGION: 1-NORMAL 2-ABNORMAL.

v. VAGINA: 1-NORMAL □ 2-ABNORMAL.

vi. CERVIX: 1-NORMAL □ 2-ABNORMAL.

vii. GENITAL ULCERS: 1-PRESENT 2-ABSENT.

SPECIMEN COLLECTION:

DAY 1:

a) 4ML VENOUS BLOOD ON EDTA BOTTLE FOR CD 4 COUNT DETERMINATION.

I. CD4 COUNT: _______________

II. CD8 COUNT: _______________

III. TOTAL LYMPHOCYTE COUNT: _____________

b) 4ML VENOUS BLOOD IN PLAIN BOTTLE FOR VDRL, HSV 2 IGM.

I. VDRL: 1-Positive. □ 2-Negative □

II. HSV 2 IgM: 1-Positive. 2-Negative □

III. HSV 2 IgG: 1-Positive. 2-Negative
c) 5ML URINE FOR PREGNANCY TEST.

I. PREGNANCY TEST RESULT: 1-Positive □ 2-Negative □
a) CERVICAL SMEAR FOR PAPANICOLAU STAINING AND CYTOLOGICAL ANALYSIS REPORTED BY BETHESDA CATEGORIZATION:

1. NORMAL: 

2. REACTIVE: 

3. LOW GRADE INTRAEPITHELIAL NEOPLASIA: 

4. HIGH GRADE INTRAEPITHELIAL NEOPLASIA: 

5. INVASIVE CARCINOMA: 

b) CERVICOVAGINAL LAVAGE:

1. FOLLICULAR PHASE: 
   i. VOLUME IN MLS: ________________
   
   ii. MACROSCOPIC APPEARANCE:
   
      1. CLEAR: 
      
      2. BLOOD STAINED: 
      
      3. OTHER:

2. PERIOVULATORY PHASE: 
   i. VOLUME IN MLS: ________________
ii. MACROSCOPIC APPEARANCE:

1. CLEAR: ❑
   ❑
2. BLOOD STAINED: ❑

3. OTHER:

3. SECRETORY PHASE:

i. VOLUME IN MLS: ______________________

ii. MACROSCOPIC APPEARANCE:

1. CLEAR: ❑
   ❑
2. BLOOD STAINED: ❑

3. OTHER:

CERVICOVAGINAL LAVAGE CYTOKINE ANALYSIS: PROLIFERATIVE PHASE SAMPLES:

1. TUMOR NECROSIS FACTOR α: ___________________

2. INTERLEUKIN 1 β: __________________

3. INTERLEUKIN 6: __________________

4. INTERLEUKIN 8: __________________

5. INTERLEUKIN 10: __________________
6. INTERLEUKIN 12 p 40: ____________________
APPENDIX 4: TEST PRINCIPLES AND PROCEDURES.

Multiplex assays arose from the need to analyze multiple analytes in small samples of specimen, and correlates with traditional immunoassays due to the similarity of the methods used. Multiplex assays use cytometric beads which are coated with the primary monoclonal antibody specific to the cytokines under study. A second antibody coated with a fluorochrome is then added, these serve as markers. The cumulative incubation period for samples lasts 3 hours. These are then assessed by a flow cytometer, preferably a multiple channel cytometer with cell sorting ability such as the BD FACS. This is then plotted on a scatter plots and assayed against a standard curve, therefore detecting the cytokine molecules. These assays have been applied to studies on growth regulating molecules and paracrine signalling molecules. The advantages include the ability to carry out multiple analyses in small samples, relatively cheap compared to the ELISA procedure, accurate and reproducible results when compared to ELISA as this method eliminates a predilution/ pipetting stage.

TEST PRINCIPLES AND PROCEDURES

1. BD CBA kits containing:

   a. Antibody-conjugated capture beads (for each cytokine there is one vial of beads)

   b. Cytometer Setup Beads.

   c. PE-detection reagent.
d. Standard recombinant proteins (one single standard mixture is provided to generate standard curves for all the analytes tested). Each kit contains two vials.

e. PE-positive control detector.

f. FITC-positive control detector.

g. Wash buffer.

h. Assay diluents.

2. A flow cytometer equipped with a 488-nm laser capable of detecting and distinguishing fluorescence emissions at 576 and 670 nm. BD FACS Calibur (BD Biosciences) and BD Cell Quest Software.

**BEAD ARRAY PROTOCOL**

1. The Instruction Manual provided with the BD CBA kit gives excellent step-by-step instructions. Briefly:

   2. Reconstitute a vial of lyophilized standard with 0.2 ml of Assay Diluent and allow it to equilibrate for at least 15 min. Please note: following reconstitution, use the Standard within 12h.

   3. Prepare serial standard dilutions according to the manufacturer’s recommendations.

   4. Pool the individual capture beads immediately before use by vortexing and mixing an aliquot of 10 µl of each capture bead suspension for each test sample and standard sample.

   5. Transfer 50 µl of mixed beads to each assay tube.
6. Prepare test samples; depending on the cytokine levels in the test samples, it may be necessary to dilute test samples with Assay Diluent to ensure that fluorescence intensity falls within the range of the standard curve.

7. Add 50 µl standard and test samples to the tubes containing mixed Capture beads.

8. Add 50 µl of PE-detection antibodies.

9. Incubate the tubes for 2 h at RT protected from light.

10. Meanwhile, perform the Cytometer Setup procedure:

(b) Add 50 µl Cytometer Setup beads to setup tubes A, B, and C.

(c) Add 50 µl of FITC-positive control to tube B and 50 µl of PE-positive control to tube C.

(d) Incubate tubes A, B, and C for 30 min at RT protected from light.

(e) Add 450 µl of Wash Buffer to tube A, and 400 µl of Wash Buffer to tubes B and C. Proceed with the Standard and Test samples:

11. Add 1 ml of Wash buffer to each assay tube and centrifuge at 200 × g for 5 min.

12. Discard the supernatant.

13. Resuspend the bead pellet in 300 µl Wash buffer. The samples are now ready for analysis on a flow cytometer. For Cytometer Setup and Data acquisition, we refer the reader to the instruction manual of the BD CBA kit.
Data analysis

BD CBA Software is essential for the data analysis. In general, the Mean Fluorescence intensities (MFIs) of the serially diluted standard samples are calculated by the software and used to generate the standard curves of each cytokine. The standard curves model the protein concentration as a function of the MFI. Before proceeding to analysis of the unknown samples, make sure the unknowns fall within the range of the standard curve. If the MFI is above the standard curve, the experiment has to be repeated with more dilute samples. Conversely, if the MFI is below the standard curve, the test has to be repeated with more concentrated samples. Any individual value that appears to be a clear outlier may be excluded and tested again in a following experiment. The software automatically calculates cytokine concentrations present in the test samples, using the corresponding standard curves and dilution factors. Sufficient replication within and across experiments is important for making precise estimates of both concentrations and errors.
APPENDIX 5: ROLE OF THE INVESTIGATOR(S)

The study investigator is responsible for:

1. Administration of the study.

2. Ensuring Pre and post test counselling of study participants.

3. Consent administration to study participants.

4. Administration of questionnaires to study participants.

5. Collection of biometric data, mapping and geographical profiling, physical addresses.

6. Contact, setting appointments and location follow-up.

7. Will liaise with the study participants’ practitioners for follow-up.

8. Ensure a physical and gynaecologic examination is conducted.

9. Sample collection and pre analytical processing as set up in the study algorithm. Will ensure safety of study participants and investigators.

10. Sample processing and adherence to standard operating procedures.

11. Ensure safe sample storage.

12. Data storage and analysis will ensure confidentiality is maintained at all levels.
APPENDIX 6: REVIEW BOARD CLEARANCE

KENYATTA NATIONAL HOSPITAL
Hospital Rd. along, Ngong Rd.
P.O. Box 29723, Nairobi.
Tel: 726300-9
Fax: 726272
Telegrams: MEDSUP, Nairobi.
Email: KNhqain@Ken-Healthnet.org

30th March 2010

Ref: KNH-ERC/ A/439

Dr. Walong Edwin O.O.
Dept. of Human Pathology
School of Medicine
University of Nairobi

Dear Dr. Walong

RESEARCH PROPOSAL: “COMPARATIVE STUDY OF CERVICOVAGINAL PROINFLAMMATORY CYTOKINE PROFILE OF HIV POSITIVE WOMEN ON DEPOT MEDROXYPROGESTERONE ACETATE ATTENDING A COMPREHENSIVE CARE CLINIC IN KISUMU, KENYA” (P328/12/2009)

This is to inform you that the KNH/UON-Ethics & Research Committee has reviewed and approved your above revised research proposal for the period 30th March 2010
29th March 2011.

You will be required to request for a renewal of the approval if you intend to continue with the study beyond the deadline given. Clearance for export of biological specimens must also be obtained from KNH/UON-Ethics & Research Committee for each batch.

On behalf of the Committee, I wish you a fruitful research and look forward to receiving a summary of the research findings upon completion of the study.

This information will form part of the database that will be consulted in future when processing related research study so as to minimize chances of study duplication.

Yours sincerely

DR. L W. MUCHIRI
AG. SECRETARY, KNH/UON-ERC

c.c. Prof. K. M. Bhatt, Chairperson, KNH/UON-ERC
The Deputy Director CS, KNH
The Dean, School of Medicine, UON
The Chairman, Dept. of Human Pathology, UON
The HOD, Records, KNH
Supervisors: Prof. Walter Jaoko, Dept. of Medical Microbiology, UON
Dr. Elizabeth Bukusi, Dept. of Obstetrics & Gynaecology, UON
The co-Director, Research Care and Training Program, Kisumu
### APPENDIX 7: STUDY BUDGET

<table>
<thead>
<tr>
<th>ITEM</th>
<th>AMOUNT IN KSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERC</td>
<td>1,000</td>
</tr>
<tr>
<td>STATIONERY, PRINTING AND BINDING</td>
<td>15,000</td>
</tr>
<tr>
<td>SWABS, MEDIA, STAINS</td>
<td>55,000</td>
</tr>
<tr>
<td>HSV 2 KALON KIT</td>
<td>60,000</td>
</tr>
<tr>
<td>CERVICAL SMEAR KITS</td>
<td>60,000</td>
</tr>
<tr>
<td>BD CBA KITS</td>
<td>220,000</td>
</tr>
<tr>
<td>COURIER</td>
<td>5,000</td>
</tr>
<tr>
<td>PARTICIPANT REIMBURSEMENT</td>
<td>21,000</td>
</tr>
<tr>
<td>STUDY ASSISTANT SALARY</td>
<td>90,000</td>
</tr>
<tr>
<td>TOTAL</td>
<td>582,000</td>
</tr>
</tbody>
</table>

Funding for this study was provided by a grant from Research Care and Training Program in partnership with the Kenya Medical Research Institute (RCTP/KEMRI)
### APPENDIX 8: STUDY TIMELINES

<table>
<thead>
<tr>
<th>MONTH</th>
<th>ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Month 1-5</td>
<td>Proposal Development</td>
</tr>
<tr>
<td>Month 6-8</td>
<td>Presentation to the department and ERC approval</td>
</tr>
<tr>
<td>Month 9-10</td>
<td>Data collection</td>
</tr>
<tr>
<td>Month 11</td>
<td>Data analysis</td>
</tr>
<tr>
<td>Month 12</td>
<td>Presentation of Results</td>
</tr>
<tr>
<td>Month 13-14</td>
<td>Final Write up and Submission of Thesi</td>
</tr>
<tr>
<td>Month 15-16</td>
<td>Manuscript writing and submission for publication</td>
</tr>
</tbody>
</table>