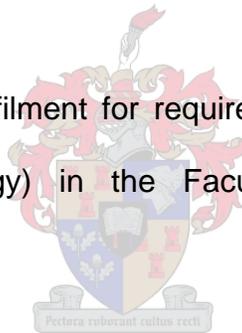


**AN *IN VITRO* INVESTIGATION OF THE ANTI-INFLAMMATORY AND
IMMUNOSUPPRESSIVE EFFECTS OF THE SYNTHETIC CONTRACEPTIVES
MEDROXYPROGESTERONE ACETATE (MPA) AND NORETHISTERONE
ACETATE (NET-A).**

By

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A thesis presented in partial fulfilment for requirement for the degree Magister in
Scientiae (Medical Microbiology) in the Faculty of Medicine, University of
Stellenbosch.



Promoter: Professor PJD Bouic

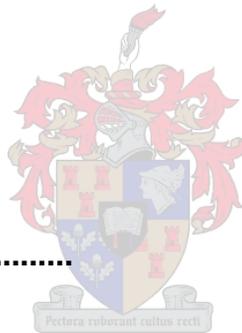
Co-Promoter: Professor JP Hapgood

April 2005

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously, in its entirety or in part, submitted it at any university for a degree.

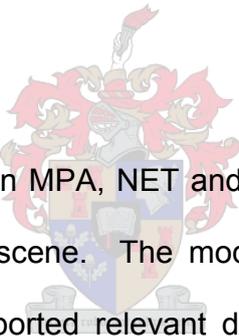
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SUMMARY

The aim of this study was to investigate the anti-inflammatory and immunosuppressive effects of the synthetic progestins, MPA and NET-A on human cells *in vitro*. These injectable contraceptives are used extensively throughout the world, including Africa. The potential of these two synthetic hormones to have certain immunosuppressive and GC properties have previously been shown. Therefore, it was of concern to us to investigate whether these two hormones could possibly demonstrate any of these GC-like properties at contraceptive doses. This was achieved by determining the effects of these two synthetic hormones *in vitro* on certain immunologic parameters.

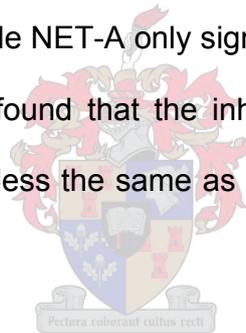


Chapter 1 is a literature review on MPA, NET and GCs. This chapter starts with a short introduction that sets the scene. The mode of action, effectiveness, side-effects as well as previously reported relevant data on both MPA and NET-A is portrayed in this review. Research on the known GC, Dex, is also included in the section dealing with GCs, because this synthetic hormone was used as a comparative GC in all our experiments. This chapter soon makes the reader realize how much evidence exists that indicate the possible immunosuppressive effects these two contraceptive hormones, in particular MPA, could have.

The possible anti-inflammatory or pro-inflammatory effects of MPA and NET-A are investigated in Chapter 2. This was done *in vitro* by measuring the effects of these two synthetic hormones on the inflammatory markers, IL-6 and TNF α , by means of

ELISA. In this chapter we demonstrate that MPA, even at contraceptive doses, exhibits significant anti-inflammatory properties on both cytokines tested, while NET-A displayed considerably less anti-inflammatory tendencies. In its true anti-inflammatory manner, we found that Dex significantly inhibited the release of both inflammatory markers from human monocytes.

In Chapter 3, we investigated the effects of MPA and NET-A on the activation of human lymphocytes. This was achieved by flow cytometric measurement of the expression of the activation membrane marker CD69 by CD4 and CD8 T cells. Here we discovered that MPA had a very significant inhibitory effect on the activation of both CD4⁺ and CD8⁺ T cells, while NET-A only significantly inhibited the activation of CD8⁺ T cells. In addition, we found that the inhibition of CD4⁺ and CD8⁺ T cell activation by MPA was more or less the same as the known GC, Dex, and in some cases even more potent.



Chapter 4 consists of an investigation of the effects of MPA and NET-A on the cytokines belonging to T_H1 and T_H2 subsets of CD4 T cells. This was achieved by determining whether MPA and/or NET-A targeted specific subsets of T helper cells by measuring the distinct regulatory cytokines, IFN γ and IL-4. The mechanism and role of the T helper subsets are discussed in the introduction of this chapter. Our results were portrayed as a ratio of T_H2: T_H1 on which the statistical analysis was done. In addition to the analysis done on the ratio, we analyzed the helper subsets separately in order to determine which subset(s) were influenced. The results of this

chapter showed that neither MPA nor NET-A significantly affected either one of the helper subsets, while Dex significantly decreased this ratio.

After our observed effects of MPA and NET-A on CD8 T cells, it became of interest in Chapter 5 to investigate the effects of these two synthetic hormones on the CD8 T cell-specific chemokine, RANTES. This was achieved by measuring the effects MPA and NET-A had on RANTES production *in vitro* by means of ELISA. Surprisingly, we discovered in this chapter that MPA and NET-A enhanced RANTES production before and after activation of CD8 T cells. We also found that Dex had the same effect on RANTES production, but to a lesser degree.

Finally, a general conclusion depicting the significance and implications of our results as well as possible future research that is required is presented in Chapter 6. It was of great importance to discuss and interpret the magnitude of data generated out of all our experiments to the utmost of our capabilities. We found that MPA, even at contraceptive doses, displayed significant immunosuppressive as well as anti-inflammatory properties. NET-A, on the other hand, demonstrated weaker immunosuppressive properties in our research and no significant anti-inflammatory properties. These findings could have clinical implications in females being treated with these synthetic contraceptives. We also demonstrated significant variation found amongst genders in response to MPA, NET-A and Dex.

OPSOMMING

Die doel van hierdie studie was om die anti-inflammatoriese en immuun onderdrukkende eienskappe van die sintetiese voorbehoedmiddels, MPA en NET-A, op menslike selle *in vitro* te ondersoek. Hierdie inspuitbare voorbehoedmiddels word op groot skaal gebruik reg deur die wêreld, insluitende Afrika. Die potensiaal van die twee sintetiese hormone om immuun onderdrukkende en glukokortikoïede (GC) eienskappe te toon is al voorheen bewys. Daarom was dit van groot belang om te ondersoek of hierdie twee hormone enige van hierdie eienskappe kan toon by konsentrasies wat bereik word gedurende voorbehoeding. Ons het dit bereik deur die uitwerking van hierdie twee sintetiese hormone op sekere immunologiese parameters *in vitro* te bepaal.



Hoofstuk 1 bestaan uit 'n literatuur studie wat handel oor MPA, NET en GCs. Hierdie hoofstuk begin met 'n kort inleiding wat die scenario vir die leser skep. Die meganisme van werking, doeltreffendheid en newe-effekte asook vorige gerapporteerde toepaslike data oor MPA en NET word in hierdie hersiening beskryf. Navorsing oor die bekende GC, dexametasoon (Dex), word ook ingesluit by die deel wat oor GCs handel, omdat hierdie hormoon in al ons eksperimente gebruik was as 'n vergelykbare GC. Hierdie hoofstuk laat die leser beseft hoeveel bewyse daar is wat dui op die moontlike immuun onderdrukkende eienskappe van beide die twee voorbehoedings hormone, en spesifiek van MPA.

Die moontlike anti-inflammatoriese of pro-inflammatoriese uitwerking van MPA en NET-A word ondersoek in Hoofstuk 2. Dit was bereik deur die *in vitro* uitwerking van die sintetiese hormone op die inflammatoriese merkers, IL-6 en TNF α , te bepaal deur middel van 'n ELISA. In die hoofstuk demonstreer ons dat MPA, selfs by konsentrasies bereik gedurende voorbehoeding, anti-inflammatoriese eienskappe besit deur dat dit 'n beduidende uitwerking op altwee sitokienes gehad het. NET-A het aansienlik minder anti-inflammatoriese neigings getoon. Die ware anti-inflammatoriese wyse van Dex was weerspieël deur die feit dat dit die vrystelling van beide inflammatoriese merkers vanaf monosiete beduidend geïnhibeer het.

In Hoofstuk 3 ondersoek ons die uitwerking van MPA en NET-A op die aktivering van menslike limfosiete. Ons het dit bereik deur die uitdrukking van die aktiverings membraan merker CD69 deur CD4 en CD8 T limfosiete te meet deur middel van vloei sitometrie. Hier het ons ontdek dat MPA 'n baie beduidende inhiberende uitwerking gehad het op die aktivering van beide CD4⁺ en CD8⁺ T limfosiete, terwyl NET-A slegs 'n beduidende inhiberende uitwerking gehad het op CD8⁺ T limfosiete. Ons het ook ondervind dat die inhiberende aksie wat MPA op die aktivering van CD4⁺ en CD8⁺ T limfosiete het min of meer dieselfde was as die aksie van Dex, en in sommige gevalle selfs sterker.

Hoofstuk 4 bestaan uit 'n ondersoek van die moontlike uitwerking wat MPA en NET-A kan hê op die sitokinie wat behoort aan die substelle van CD4 T limfosiete. Dit was bereik deur middel van die meting van die onderskeie regulatoriese sitokiene, IFN γ en IL-4, om te bepaal of MPA en/of NET-A spesifieke substelle van T helper

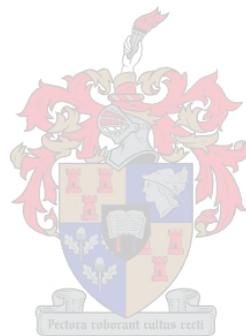
selle teiken. Die meganisme en rol van die T helper sell word in die inleiding van die hoofstuk bespreek. Ons resultate waarop die statistiese analiese gedoen is word as 'n verhouding van T_H2 : T_H1 weergegee. Ons het ook die helper substelle apart geanaliseer om te bepaal watter een van hulle beïnvloed was. Die resultate van die hoofstuk het gewys dat nie MPA of NET-A 'n beduidende effek gehad het op enige van die twee helper substelle nie, terwyl Dex die verhouding beduidend verlaag het.

Na die waargenome effek wat MPA en NET-A gehad het op CD8 T limfosiete, het ons besluit om in Hoofstuk 5 die effek van die twee sintetiese hormone op die spesifieke CD8 T limfosiet chemokien, RANTES, te bepaal. Dit was bereik deur die uitwerking wat MPA en NET-A het op RANTES produksie *in vitro* te bepaal deur middel van 'n ELISA. Tot ons verbasing het ons ontdek dat MPA en NET-A die produksie van RANTES verhoog het voor en na die aktivering van CD8 T limfosiete. Ons het ook gevind dat Dex dieselfde uitwerking gehad het, maar in 'n mindere mate.



Om af te sluit, gee ons in Hoofstuk 6 'n algemene gevolgtrekking in die vorm van die betekenis en implikasies wat ons resultate inhou, asook moontlike toekomstige navorsing wat nodig is. Dit was van groot belang om die grootheid van data wat gegenereer is uit ons eksperimente te bespreek en vertolk tot die beste van ons vermoëns. Ons het ontdek dat MPA, selfs by konsentrasies bereik gedurende voorbehoeding, beduidende immuun onderdrukkende en anti-inflammatoriese eienskappe getoon het. In sake die effek van NET-A, het ons bevind dat NET-A swakker immuun onderdrukkende eienskappe besit en dat dit geen anti-

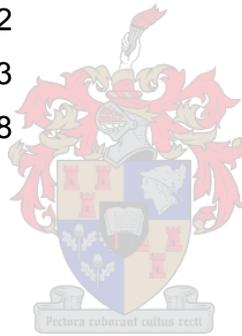
inflammatoriese eienskappe toon nie. Hierdie bevindings kan moontlik kliniese implikasies tot gevolg hê in vrouens wat hierdie sintetiese voorbehoedmiddels gebruik. Ons was ook in staat om beduidende variasies te demonstreer tussen geslagte betreffende die reaksie op MPA, NET-A en Dex.



ABBREVIATIONS

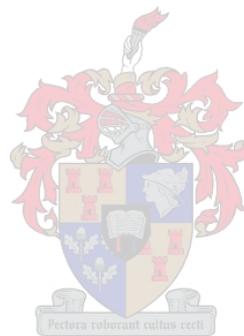
$^3\text{H-TdR}$	$^3\text{H-thymidine}$
AIDS	Acquired immunodeficiency syndrome
AP-1	Activation protein complex-1
APC	Antigen presenting cell
APR	Acute phase response
AR	Androgen receptor
BMD	Bone mineral density
CEE	Conjugated equine estrogen
CMI	Cell-mediated immunity
Con-A	Concanavalin A
CRP	C-reactive protein
CTL	Cytotoxic T lymphocyte
Dex	Dexamethasone
DFA-ECKR	Duffy blood group antigen-erythrocyte chemokine receptor
DMPA	Depo-Medroxyprogesterone acetate
DNCB	2,4-Dinitrochlorobenzene
$\text{E}_{(2)}$	Estradiol
EBV	Epstein-Barr virus
EE	Ethinyl estradiol
ELISA	Enzyme linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptor
FSH	Follicle stimulating hormone
GC	Glucocorticoid
GDP	Guanosine diphosphate
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GTP	Guanosine triphosphate
HDL	High density lipoprotein

HIV	Human immunodeficiency virus
HPA	Hypothalamic-pituitary-adrenal axis
HRP	Horseradish peroxidase
HRT	Hormone replacement therapy
HSA	Human serum albumin
HSP	Heat shock protein
HSV	Herpes simplex virus
i.m.	Intra muscular
ICAM	Intercellular adhesion molecule
IFN- γ	Interferon gamma
Ig	Immunoglobulin
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-18	Interleukin-18
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IPC	Injectable progestogen-only contraceptive
IRS	Insulin-response substrate
I- κ B	Inhibitor κ B
LDL	Low density lipoprotein
LH	Luteinizing hormone
LPS	Lipopolysaccharide
LTR	Long terminal repeat
mAbs	Monoclonal antibodies
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MHC	Major histocompatibility complex
MIP-1 α	Macrophage inflammatory protein-1 alpha



MIP-1 β	Macrophage inflammatory protein-1 beta
MMP	Matrix metalloproteinase
MPA	Medroxyprogesterone acetate
MR	Mineralocorticoid receptor
NET	Norethindrone
NET-A	Norethisterone acetate
NET-En	Norethisterone enanthate
NF-AT	Nuclear factor of activated T cells
NF- κ B	Nuclear factor-kappa B
NK	Natural killer
NKA	Neurokinin A
NPY	Neuropeptide Y
NT	Neurotensin
PAR	Proteolitically activatable thrombin receptor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PHA	Phytohemagglutinin
PLC	Phospholipase C
PR	Progesterone receptor
RA	Rheumatoid arthritis
RANTES	Regulated on activation, normal T cell expressed and secreted
RPMI 1640	Roswell Park Memorial Institute medium 1640
RT-PCR	Reverse transcriptase polymerase chain reaction
SAA	Serum amyloid A protein
SGOT	Serum glutamic oxaloacetic acid
SGPT	Serum glutamic pyruvic transaminase
SIV	Simian immunodeficiency virus
SP	Substance P
STD	Sexually transmitted disease
TCID	Tissue culture infectious dose
TCR	T cell receptor
TG	Triglyceride

TNF-RI	Tumor necrosis factor receptor
TNF α	Tumor necrosis factor-alpha
TU	Testosterone undecanoate
VCAM	Vascular cell adhesion molecule
VLDL	Very low density lipoprotein
WHO	World Health Organization



“Chance favors the prepared mind”
- Louis Pasteur



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Finally, to my family and loved ones, I thank each one of you for your presence through difficult times and for the special support each of you gave to me in your own unique way.

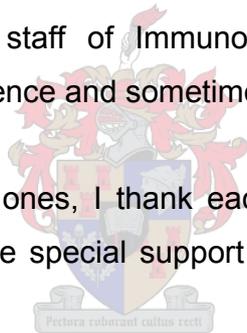
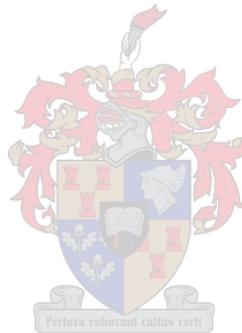


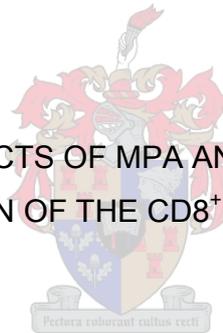
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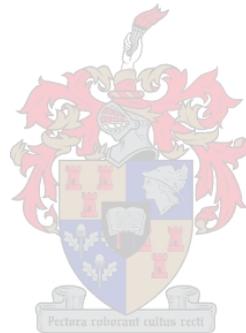
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CHAPTER 1

LITERATURE STUDY

1.1 Setting the scene

The immune system is our major defence against attack from pathogens and intruding foreign bodies. This intricate network relies on the proper interaction of various immune cells, receptors and signalling molecules. Any kind of disruption of this delicate framework would cause interference in homeostasis and could ultimately lead to severe complications. These disruptions are either caused by pathogens, self-induced states like autoimmune disorders, or due to side-effects associated with the use of drugs.

Endogenous glucocorticoids (GCs) are known as essential hormones due to the fact that without them life would not be possible. Their release and regulation is controlled by the hypothalamic-pituitary-adrenal axis (HPA) (Brooke & Sapolsky, 2000). Some of the major functions of endogenous GCs include the prevention of an excessive immune response and maintenance of certain homeostatic aspects (Chrousos, 1995; Wick *et al.*, 1993). It is this immune-modulating function of endogenous GCs that is responsible for their synthetic counterparts being used in modern clinical medicine. Dexamethasone (Dex) is the most commonly known synthetic GC and differs from the endogenous GCs in terms of regulatory mechanisms such as receptor binding and interactions with various transcription factors (Wilckens & De Rijk, 1997). GCs mediate their effect

through binding to the glucocorticoid receptor (GR) and setting either one of two events in motion, trans-activation or trans-repression.

Medroxyprogesterone acetate (MPA) and norethisterone acetate (NET-A) are the most commonly used progestins for hormone replacement therapy (HRT) (Stahlberg *et al.*, 2004). Besides their use in HRT, MPA and norethisterone enanthate (NET-En) are the major injectable contraceptives used extensively throughout Southern Africa and the rest of the world. Two studies showed that the use of Depo MPA (DMPA) increased in South Africa, while one of the studies suggested that it was due to DMPA being cheaper than other similar methods of contraception (Smit *et al.*, 2001; Margulies and Miller, 2001). Together with oral contraceptive pills, hormonal contraceptives are used by more than 100 million women worldwide (United Nations Population Division Department for Economic and Social Information and Policy Analysis, 1994) of which 20 million are current users of MPA (Affandi, 2002). The mechanisms of actions are the inhibition of ovulation, impermeability of cervical mucus and thinning of the endometrium that causes insufficient glycogen secretion to support a blastocyst entering the endometrial cavity (Mishell, 1996). Most of the various biological effects of MPA are known, but the same can not be said for Norethisterone (NET).

The potential GC activity of MPA has been documented (Bamberger *et al.*, 1999; Bamberger and Schulte, 2000), while little, if any, data exists on the GC activity of NET. Some studies found the GC effect of MPA to be at least equal to (Bamberger *et al.*, 1999) or even stronger (Bamberger and Schulte, 2000) when compared to the known GC, Dex. Bamberger *et al.* (1999) discovered that MPA can dissociate between trans-

repression and trans-activation in normal human lymphocytes and that the suppressive effects of MPA on Interleukin-2 (IL-2) gene expression were as strong as that of Dex and hydrocortisone. It was also shown that MPA can trans-repress the human IL-2 gene in normal human lymphocytes in the absence of significant trans-activation and that this effect was mediated by the GR (Bamberger *et al.*, 1999). Expression studies also revealed that the effects of MPA are mediated by the GR, but not by the progesterone or the androgen receptors (Bamberger and Schulte, 2000). Bamberger and Schulte (2000) transfected normal human lymphocytes with the IL-2 and the glucocorticoid response element (GRE) constructs and found that trans-repression of the IL-2 promoter in response to MPA was comparable to Dex, in some experiments even stronger.



Several studies on the immunosuppressive properties of MPA have been done through the past three decades, but most of these studies used supra-physiological concentrations of MPA (Jeremiah *et al.*, 1968; Brunelli *et al.*, 1996). Only a few studies exist that focus on concentrations reached from contraceptives doses (Corsini & Puppo, 1983). These immunosuppressive properties of MPA were demonstrated in various ways. This ranges from the demonstrated inhibitory effect of MPA on the blastogenic response of mitogen-activated peripheral blood lymphocytes (Corsini & Puppo, 1983) to the ability of MPA to increase the survival rate of renal allografts in dogs (Jeremiah *et al.*, 1968). Furthermore, skin allograft experiments done on rabbits showed that MPA can suppress the primary antibody response and retard rejection of the allografts (Jeremiah *et al.*, 1968). It has also been demonstrated that MPA can increase skin reactivity to recall antigens in woman using the drug as a contraceptive (Gerretsen *et*

al., 1979), while further studies showed that it had a profound effect on the efferent phase of the cell-mediated immune response (Gerretsen *et al.*, 1980).

MPA is also used in HRT and several studies have been done in this regard. Studies done on the effect of MPA in HRT on lymphocytes and granulocytes indicated that HRT selectively affects various immune cell subsets, like natural killer cells, CD4⁺CD45⁺RO and CD8⁺CD11b⁺ cells (Brunelli *et al.*, 1996). Other data *in vitro* demonstrated that progesterone functions as a potent inducer of T helper 2 (T_H2) type cytokines and therefore enhances the humoral immune response (Piccinni *et al.*, 1995).

Considerably less data exists on the possible effects NET has on the immune system. Norethisterone is also used in HRT where Lippert *et al.* (2001) demonstrated that NET did not have a significant effect on estradiol stimulated proliferation of MCF-7 cells. Experiments with skin allografts on castrated adult rabbits showed that norethindrone and norethynodrel significantly and consistently prolonged allograft survival, but that it had a variable effect on circulating antibody production (Hulka *et al.*, 1965).

Evidence exists that indicates a possible association between contraceptives, sexually transmitted diseases (STD's) and the incidence of human immunodeficiency virus type 1 (HIV-1) infection. Two cross-sectional studies (Bulterys *et al.*, 1994; Rehle *et al.*, 1992) and two prospective studies (Martin *et al.*, 1998; Ungchusak *et al.*, 1996) found a positive association between depot medroxyprogesterone acetate (DMPA) and HIV-1 infection, while some studies found no association between injectable contraceptives and the prevalence of HIV-1 infection (Mati *et al.*, 1995). Other studies linked the use of

oral or injectable hormonal contraceptives to a change in susceptibility to STD's, which in turn may influence transmission of HIV-1 (Baeten *et al.*, 2001; Cottingham & Hunter, 1992). There are other studies that found a significant association between cervical HIV-1 proviral shedding and the use of hormonal contraception, including DMPA (Mostad *et al.*, 1997; Wang *et al.*, 2004). Investigations on whether steroid hormones had a direct effect on the human immunodeficiency virus demonstrated that hormone-receptor complexes can bind to the regulatory sequence of HIV-1 and up regulate expression of the virus (Kinter *et al.*, 2001; Ghosh, 1992; Furth *et al.*, 1990; Kolesnitchenko & Snart, 1992).

Two recent studies have shed some light on the association between hormonal contraceptives and the incidence of HIV-1 infection. The alarming results of these two studies indicated that women who use progestin-containing contraceptives are at an increased risk of becoming infected with HIV (Smith *et al.*, 2000; Marx *et al.*, 1996). Marx *et al.* (1996) used the SIVmac model to show that subcutaneous progesterone implants, which could mimic hormonally-based contraceptives, thinned the vaginal epithelium and enhanced SIV vaginal transmission almost 8-fold over that observed in placebo controls. In a study examining the individual roles of progesterone and estrogen in vaginal transmission of HIV, Smith and co-workers treated ovariectomized female macaques with either progesterone or estrogen followed by intravaginal inoculation with SIVmac (the equivalent of HIV) (Smith *et al.*, 2000). None of the estrogen-treated macaques were infected, while 83% of the progesterone-treated animals became infected following intravaginal SIV inoculation. While estrogen-treatment caused vaginal epithelial thickening in the study of Smith *et al.* (2000), it is

also known to increase cervical mucus production, decrease cervical ectopy, and alter vaginal bacterial flora (Fawcett, 1986; Hillier & Lau, 1997). These combined effects of estrogen on the vagina might be responsible for the observed reduction in the probability of infection. Since women who use long-acting, progestin-based contraceptives, such as DMPA, have low levels of estrogen the possibility exists that these women are at an increased risk of HIV infection. Both these authors suggested that other progesterone-induced mechanisms could also be involved.

All of the above mentioned evidence indicates the possibility that MPA and/or NET can increase susceptibility to HIV infection through one or several of the following different mechanisms: 1.) immunosuppression through a GC effect, 2.) increase of STD's, 3.) thinning of the vaginal epithelium and 4.) up-regulation of viral replication. Not only is susceptibility increased, but infectivity is possibly also enhanced due to an increase in cervical proviral shedding. This creates great concern when it's taken into account that DMPA and NET-En are used extensively in South Africa and the rest of the developing world, where the prevalence of STD's and the frequency of sexual transmission of HIV-1 are high. It is the variety of immunosuppressive properties of MPA and NET and GC-like characteristics of MPA that inspired this research into the possible effects these widely used synthetic progestins could have on the immune system. What follows is a detailed literature review on GCs, MPA and NET as well as the relevant research that was conducted by us.

1.2 Glucocorticoids.

1.2.1 Endogenous versus synthetic.

Glucocorticoids can be divided into endogenous GCs and synthetic GCs, of which Dex is the most commonly known (Fig. 1). The differences between endogenous GCs produced by the adrenal glands, and their synthetic equivalents are in terms of their regulatory mechanisms, which are crucial for their biological actions. For example synthetic GCs differ from endogenous GCs in binding to the corticosteroid-binding globulin, the affinity for their diverse GC receptors, tissue specific metabolism and the interaction with various transcription factors (Wilckens, 1995).

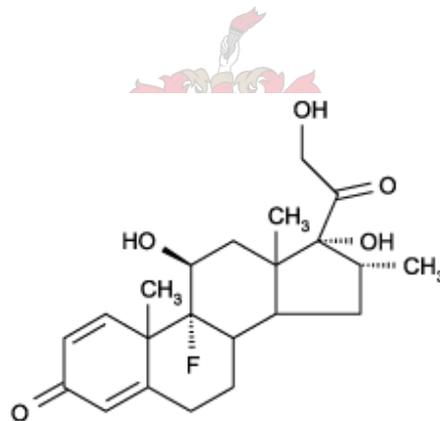


Figure 1. Structure of Dexamethasone

Endogenous GCs are essential hormones, the lack of which is incompatible with life and their release and regulation is controlled by the HPA (Brooke & Sapolsky, 2000). The major effects of endogenous GCs include the prevention of an excessive immune response and maintenance of adequate blood sugar levels and blood pressure (Chrousos, 1995; Wick *et al.*, 1993). Endogenous GCs can be beneficial to surviving a major physical stressor primarily in the peripheral system, but also in the short term.

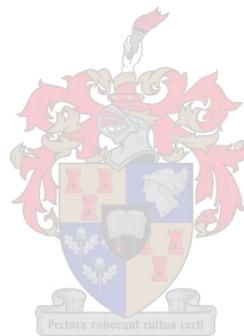
They perform all the functions necessary for an organism to cope with a stressful crisis like mobilizing energy (primarily to muscle), help increase cardiovascular tone, and enhance cognition (Brooke & Sapolsky, 2000). To conserve energy for these tasks, unessential activities such as growth, digestion, reproduction, and immunity are turned off (Brooke & Sapolsky, 2000). Evidence exists that endogenous corticosteroids may modulate the susceptibility, or the outcome, of both human (Takasu *et al.*, 1990; Chikanza *et al.*, 1992) and experimental diseases (MacPhee *et al.*, 1989; Sternberg *et al.*, 1989).

Synthetic GCs are widely used in autoimmune diseases (Ramirez *et al.*, 1996; Hirano *et al.*, 2000), inflammatory disorders (Franchimont *et al.*, 2000; Ang *et al.*, 2000; Trifilieff *et al.*, 2000), allergic diseases (Franchimont *et al.*, 2000), and suppression of inflammation associated with transplant rejection (Almawi *et al.*, 1998; Hricik *et al.*, 1994). Glucocorticoids continue to be the major immunomodulatory agents used in clinical medicine today, despite the major side effects that limit their therapeutic use. The mode of action of GCs to exert their different effects is accomplished through either one of two events, trans-activation or trans-repression. The mechanism of trans-activation is responsible for the metabolic and cardiovascular side-effects induced by GCs (Becker *et al.*, 1986; Brasier & Li, 1996), while the trans-repression by GCs of target genes not containing any GR-binding sites is accountable for the anti-inflammatory and immunosuppressive effects (Wick *et al.*, 1993; Boumpas *et al.*, 1991; Bamberger *et al.*, 1996; Bamberger *et al.*, 1997). Therefore, dissociation between these two main activities will help separate the beneficial from the deleterious effects and will ultimately be the gateway to the development of improved GC-based drugs.

1.2.2 Mechanisms at cellular level.

The theory around the interactions of GCs with their receptors is a vast field and is therefore only discussed briefly in the following section, seeing that it was not a direct component of our research. At cellular as well as molecular level the effects of GCs are mediated by the intracellular GR (Evans, 1988; Truss & Beato, 1993; Tsai & O'Malley, 1994; Beato *et al.*, 1995; Mangelsdorf *et al.*, 1995), which is part of a growing family of proteins, termed nuclear receptors (Beato, 1989). The molecular mechanism involved in the effects of GCs was originally described as the result of GC binding to their cytosolic receptor whereafter the GC-activated GR translocates to the cell nucleus to bind to the GRE, which are short upstream promoter elements situated in the promoter region of GC-responsive regulated genes (Almawi, 2001; Evans, 1988; Truss & Beato, 1993; Tsai & O'Malley, 1994; Beato *et al.*, 1995; Mangelsdorf *et al.*, 1995; Bamberger *et al.*, 1996). The DNA-bound receptor interacts with factors belonging to the basal transcription machinery to induce either positive events (trans-activation) by direct interaction with cis-acting sequences, or negative events (trans-repression) by repression of gene transcription and/or alteration of mRNA half-lives (Evans, 1988; Truss & Beato, 1993; Tsai & O'Malley, 1994; Beato *et al.*, 1995; Mangelsdorf *et al.*, 1995; Bamberger *et al.*, 1996; Krane, 1993). The predominant mechanism of the metabolic and cardiovascular side-effects induced by GCs can be described to glucocorticoid receptor-mediated trans-activation (Becker *et al.*, 1986; Brasier & Li, 1996), whereas, in contrast, the anti-inflammatory and immunosuppressive effects of GCs involve the trans-repression of target genes not containing any GR-binding sites (Wick *et al.*, 1993; Boumpas *et al.*, 1991; Bamberger *et al.*, 1996; Bamberger *et al.*, 1997).

The end result of GC-GR-GRE formation is achieved through a series of events. Firstly, glucocorticoids are able to passively diffuse through the plasma membrane due to their low molecular weight and lipophilic nature. There they bind their inactive GR located in the cytosol as an inactive complex containing two molecules of heat shock protein (HSP-90) and other cytosolic proteins, which act as molecular chaperons. Then the GC-GR complex dissociates from HSP binding and enters the nucleus where it binds to GRE sites and depending on the target gene, GR binding may either inhibit or stimulate transcription (Fig. 2) (Almawi, 2001).



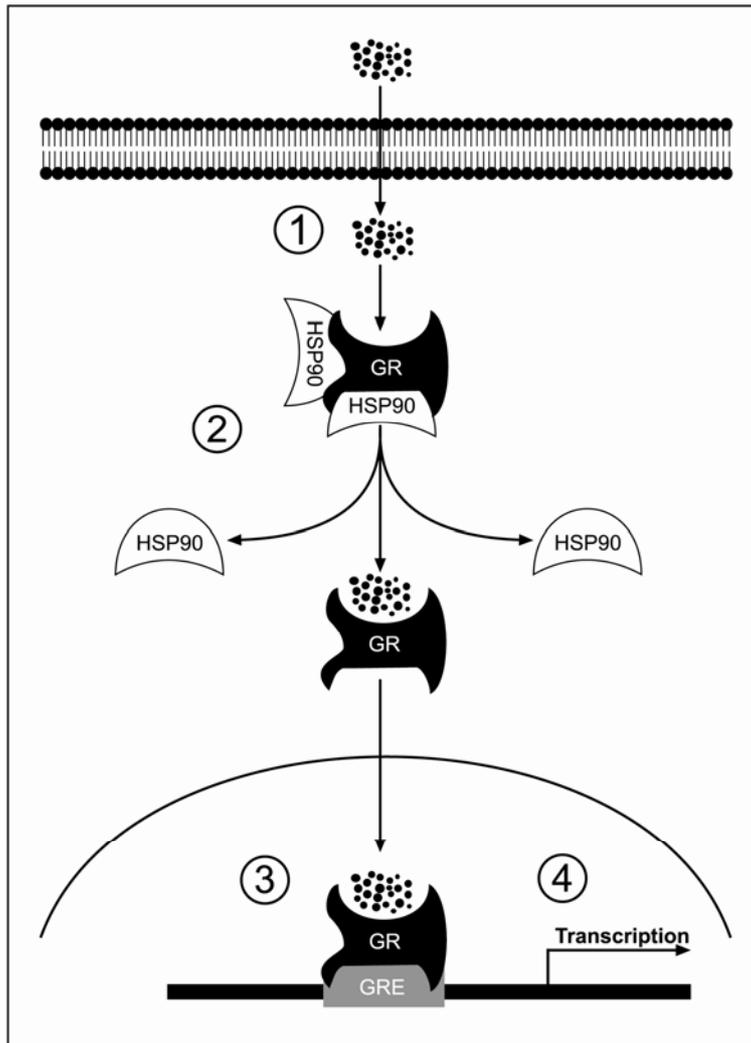


Figure 2. A model of Glucocorticoid action. GCs diffuse through the cell membrane (1), and binds to the inactive GR complexed with HSP-90 (2). The GC-GR complex then dissociates from HSP binding and enters the nucleus where it binds to GRE sites (3). Depending on the target gene, GR binding may either inhibit or stimulate transcription (4) (Almawi, 2001, p 80).

A review was published that explored some observations relating to the structure of untransformed GC and progesterone receptors and the mechanisms by which these receptors are transformed to their DNA-binding state (Pratt, 1987). Observations

discussed in this review raised the possibility that the primary, and perhaps the only, role for the hormone is to promote dissociation of the receptor- HSP-90 complex.

The results of DNA cloning have revealed the precise amino acid sequence, indicating that the GR is a member of the steroid super-family (Almawi, 2001). This family of receptors contain specific domains (N-terminal, DNA-binding, and hormone-binding domains), each of which is required for a specific function (Almawi, 2001). The DNA-binding domain is involved in trans-activation, while the GCs themselves bind to a domain located in the carboxy terminal region of the receptor (Fig. 3) (Beato, 1989).

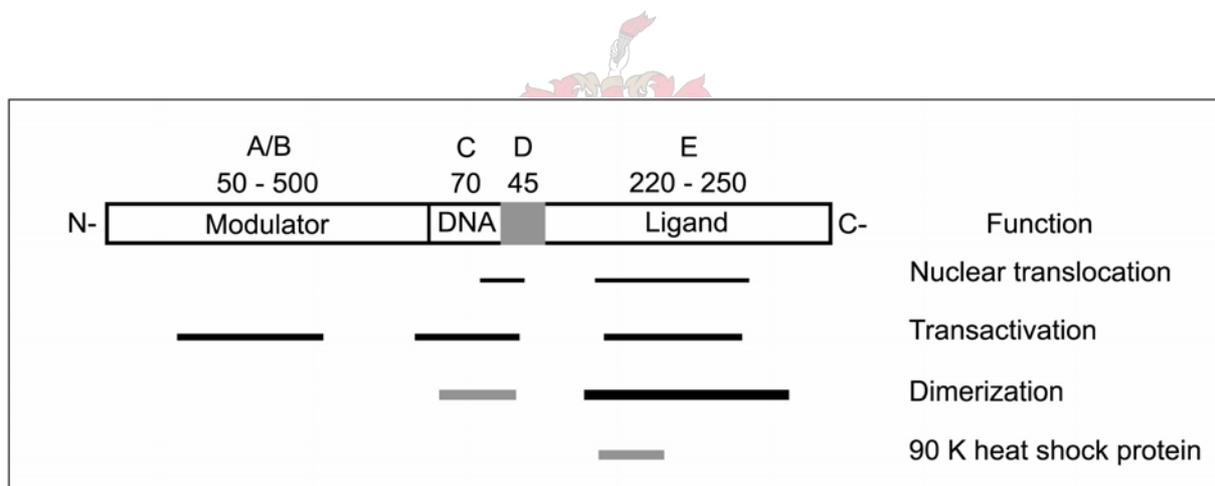


Figure 3. General function and functional organization of nuclear receptors (Beato, 1989).

Furthermore, the function controlled and induced by this binding is mostly contained in the amino terminal region, with specific contributions from the other domains (Krane, 1993). The research team of Funder was the first to suggest that antagonists do not compete directly with agonists for binding to the GR (Suthers *et al.*, 1976). Instead, a

separate site exists where the antagonists interact with the receptor and ultimately allosterically modulate binding at the agonist site. They developed a technique to measure the rate of dissociation of tritiated Dex ([³H] DM) or tritiated aldosterone ([³H] A) from the GR of rat liver or kidney cytosol. They found that progesterone and a number of other steroids consistently and significantly increased the dissociation rate of [³H] DM-receptor complexes in both liver and kidney cytosol. They postulated that binding to the agonist site allowed the receptor to assume a conformation that is biologically active, while binding to the antagonist site renders the receptor inactive (Suthers *et al.*, 1976).

This marked the beginning of various studies to determine whether this second steroid-binding site exists and where it is located (Svec *et al.* 1989; Svec *et al.* 1980; Jones & Bell, 1981; Moguilewsky & Deraedt, 1981; Bell *et al.*, 1986). Svec and Rudis (1981) used steroid specificity studies of the interaction with the second site to indicate that this site is similar to the agonist-binding site of a progesterone receptor. These studies were done in various tissues and cell types. Svec and colleagues searched for this site in various preparations of the receptor (Sves *et al.*, 1989). First they partially purified the GR whereafter they observed that the acceleration of dissociation by progesterone occurred after the receptor was purified 132.5-fold. They were aware that this was not a totally pure receptor preparation, but the documentation of dissociation suggested that, with this degree of purification, progesterone is interacting with the GR itself. The second part of their study aimed to localize this second site to a position along the receptor. By using the multimeric and monomeric forms of the GR, they determined

that the second site is within the protein chain containing the agonist-binding site (Svec *et al.*, 1989).

By creating truncations and internal deletions in the steroid-binding domain of the human GR, Hollenberg and co-workers determined sequences within this domain that are responsible for the negative influence of the domain over the rest of the GR (Hollenberg *et al.*, 1989). The results from their study supported the possibility of two distinct steroid-binding sites within the steroid-binding site. Studies show that the mero-receptor is the smallest receptor fragment that binds agonists (Sherman *et al.*, 1978; Vedeckis, 1983) and that this region is on the carboxy-terminus of the GR (Rusconi & Yamamoto, 1987). Seeing that progesterone-induced acceleration of dissociation takes place with the mero-receptor, it can be said that the second site has to be on that species. Sufficient data concerning this second site on the GR exists to safely postulate that a second binding site, which is topographically close to the agonist site, lies in a hydrophobic region of the receptor (Jones & Bell, 1980; Bell *et al.*, 1986), and that interaction with this site may determine an agonist's biological potency (Teubner *et al.*, 1988).

1.2.3 Activity of GR-GC complex at the nuclear level.

Rapid dissociation of the GR-GCs complex from the HSP-90 occurs when GCs bind to the inactive GR. The HSP-90 selectively associates with the unliganded GR and is constitutively expressed by many cells (Krane, 1993). This protein facilitates the response of the receptor to the GC and is released from the receptor after GC/GR binding, whereafter the GC-GR complex acquires the capacity to translocate to the

nucleus (Almawi, 2001). After translocation the complex binds to corresponding palindromic DNA sites, known as GRE (Berg, 1989; Miesfeld, 1990), to directly regulate gene expression. These sites are contained in the 5'-untranslated promoter region of GC-responsive genes as well as cytokine genes (Fig. 3) (Schmidt *et al.*, 1994).

An intact GRE site (Goswani *et al.*, 1994) and GR DNA binding domain (Northop *et al.* 1992) are essential for GR action. The activated GR complex binds the GRE DNA elements through zinc finger domains, resulting in downstream inhibition of gene expression in a cis-or trans-acting manner. Trans-activating events are brought about by binding of the activated GR in a cis-acting fashion, thus the GR causes a blockage of DNA sites flanking the binding sites of basal and induced transcription factors (Ray and Sehgal, 1992; Mordacq and Linzer, 1989). The induction of GC-specific inhibitor or mediator is required for inhibition in a trans-acting fashion (Almawi, 2001). Activated GR may stimulate (Auphan *et al.*, 1995; Scheinman *et al.*, 1995) or inhibit (Almawi *et al.*, 1998) gene transcription, so when dealing with this dual transcriptional modality of the GR, it is important to take the cell type studied, the GC-regulated gene under investigation, and the type of GC into consideration. Several studies exist that supports this notion. In a study investigating the effects of different GCs on the production of cytokines by peripheral T lymphocytes and the effects on the inhibition of nuclear factor-kappa B (NF- κ B) DNA binding activity by activated Jurkat cell line, Lanza *et al.* (1999) found that not all the cytokines investigated were affected and not with the same intensity. They thus concluded that GC compounds might differ in their binding and affinity properties, tissue-specific metabolism, and interaction with transcription factors. Another study measured the GC sensitivity in three target tissues, namely the

cardiovascular system, the immune system and the HPA axis (Ebrecht *et al.*, 2000). For their purpose Ebrecht *et al.* (2000) determined Dex inhibition of lipopolysaccharide-induced IL-6 and tumor necrosis factor-alpha (TNF- α) production in peripheral leukocytes, beclomethasone dipropionate-induced skin blanching, and suppression of cortisol levels (Ebrecht *et al.*, 2000). From their results they suggested that the variability in GC sensitivity is target tissue specific in healthy subjects.

In addition to the GR-GRE interaction model, several other mechanisms were postulated for GCs antagonism of transcription factor activity. These include the induction of the synthesis of I- κ B, protein-protein interaction, competition with transcription factors for nuclear coactivators, and post transcriptionally.

The signalling pathway, known as transcriptional crosstalk, utilizing the inducible transcription factor complex, NF- κ B, regulates the expression of various genes involved in inflammatory and immune responses. It is activated upon exposure of cells to the following: proinflammatory cytokines [TNF, IL-1, oxidants (ozone, H₂O₂, superoxide anions)], bacterial compounds (Lipopolysaccharide), viral products (HTLV-1 Tax protein, dsRNA), protein kinase C activators (platelet-activating factor, phorbol esters), and UV- or γ -irradiation (Vanden Berghe *et al.*, 1999). NF- κ B, a member of the mammalian rel gene family, is composed of heterodimer of RelA (p65) and NF- κ B1 (p50) where the RelA protein is responsible for the transactivation potential. In the non-activated state, NF- κ B is sequestered in the cytoplasm bound to its specific inhibitor κ B (I- κ B) protein. Activation by extracellular signals induces the phosphorylation and ubiquitination of I- κ B, allowing NF- κ B to translocate to the nucleus where it binds to its specific DNA site (κ B)

and subsequently activates transcription (Mercurio & Manning, 1999). It was demonstrated in TPA-stimulated Jurkat T cell line (Auphan *et al.*, 1995) and in tumor necrosis factor (TNF)-stimulated HeLa cells (Scheinman *et al.*, 1995) that GCs, at least in part, inhibited NF- κ B activity by inducing I- κ B synthesis. Conflicting results exist that opposes the theory that stimulation of I- κ B synthesis is the mechanism by which GCs antagonizes NF- κ B activity (Costas *et al.*, 2000; Goppelt-Struebe *et al.*, 2000).

Activated GR antagonizes transcription factors by combining with either unliganded or DNA bound transcription factors, thus inhibiting the functional capacity of this protein complex. Transcription of the two nuclear oncogenes, followed by the fusion of the JUN and FOS proteins is required for the formation of activated protein complex-1 (AP-1) (Krane, 1993). Through binding of the AP-1 by the GR, a complex is formed of which the DNA binding capacity is inhibited (Tuckermann *et al.*, 1999; Adcock *et al.*, 1995). GCs antagonism of AP-1 binding through earlier inhibition of JNK showed that GR inhibition of transcription factor binding might be caused by the suppression of a signaling enzyme or pathway necessary for optimal transcription factor activation (Gonzalez *et al.*, 2000). In addition, GR may directly interact with transcription factors, without influencing their DNA binding capacity, through protein-protein binding with the transcription factor already bound to its putative DNA site (De Bosscher *et al.*, 1997).

Co-activators are transcription factors whose specificity is conferred by the ability to bind to DNA-binding transcription factors instead of directly to DNA. These co-activator proteins form an integral part of the link between basal and induced transcription factors and are required for optimal transcription factor activity. It has been shown that, at least

in part, GR can compete with transcription factors for nuclear co-activator proteins (Freedman, 1999; Aarsinalo *et al.*, 1998), thus antagonizing transcription factors.

Glucocorticoids can perform their effects post-transcriptionally at two levels, by reducing cytokine mRNA half-life and by attenuating cytokine-mediated effects (Batuman *et al.*, 1994; Tobena *et al.*, 1996). Dayer and co-workers (1976) showed that Dex entailed a significant decrease in collagenase secretion from synovial cells isolated from Rheumatoid arthritis (RA) sufferers (Dayer *et al.*, 1976). Cytokines such as IL-1 or TNF- α , can induce collagenase synthesis and cyclohexamide inhibits this action, indicating that protein synthesis is required (Conca *et al.*, 1989). Other cytokines also affected by GCs include IL-2 (Boumpas *et al.*, 1991), IL-6 (Tobler *et al.*, 1992), IL-8 (Tobler *et al.*, 1992), and c-myc (Tosato *et al.*, 1990).

1.2.4 Immune suppression by GCs.

The immunosuppressive and anti-inflammatory effects of GCs are well documented and is the reason for their widespread use in the treatment of inflammatory and auto-immune states, interacting with virtually every step of the inflammatory and immune responses (Homo-Delarche *et al.*, 1991). It is by now clear that the mechanism of action of GCs is multi-faceted and include the inhibition of both proximal and distal events of T cells (Almawi *et al.*, 1991) and the suppression of cytokines and adhesion molecules (Haynesworth *et al.*, 1996; Munck and Náray Fejes Tóth, 1994).

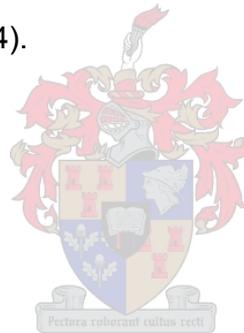
In order to determine whether GCs interfere with CD28-mediated co-stimulatory signals for T-cell activation, Fessler and co-workers (1996) transfected Jurkat T cells with a

plasmid containing the IL-2 promoter linked to the chloramphenicol acetyl transferase reporter gene (Fessler *et al.*, 1996). Upon stimulation, Dex inhibited the activity of the IL-2 promoter, however in the presence of anti-CD28 mAb, this promoter became resistant to Dex. These results suggest that accessory pathways for IL-2 production are inhibited by GCs via CD28 and that the inhibition of this pathway may be an important mechanism for the T-cell directed immunosuppressive effects of low-to-moderate doses of GCs.

Baus *et al.* (1996) determined whether Dex treatment affects the early step of T cell receptor (TCR) signal transduction in T cell hybrids and found that Dex had no effect on cell surface expression of TCR-associated structures nor did it inhibit calcium responses induced by a heterologous G protein-coupled muscarinic receptor. This suggested that the TCR signalling pathway was inhibited by Dex at a post receptor stage. Their results indicated that GCs interfere with an early step of the signal transduction cascade initiated by TCR/CD3 cross-linking, thus blocking IL-2 production in activated T cells.

The development of auto-immune disease or atopic reactions are known to be caused by a shift in the balance between T_H1- and T_H2-like cytokines. Asthma or allergies has been related to T_H2 responses, while auto-immunity has been linked to T_H1 responses (Anderson & Coyle, 1994; Mosmann & Sad, 1996). Agarwal and Marshall (2001) studied the role of cytokines previously reported to regulate the T_H1/T_H2 cytokine balance, including IL-12, interferon gamma (IFN- γ), IL-10, IL-4, and IL-13, in the GC-mediated human type1/type2 cytokine alterations. They concluded that the alteration of type1/type2 cytokines by Dex in tetanus-stimulated peripheral blood mononuclear cells

(PBMCs) is primarily the result of down-regulation of type1 cytokines, bringing about the production of type2 cytokines. Franchimont and co-workers (2000) examined the regulatory effects of GCs on key elements of IL-12 and IL-4 signalling, seeing that IL-12 promotes T_H1 cell-mediated immunity while IL-4 stimulates T_H2 humoral-mediated immunity. They concluded that GCs blocked IL-12-induced Stat4 phosphorylation, without altering IL-4-induced Stat6 phosphorylation, therefore suppressing the T_H1 cellular immune response. These findings show that GCs cause an *in vitro* shift in the type1/type2 cytokine balance of human PBMCs toward a predominant type2 response, and may therefore represent one of the most important determinating factors of the microenvironment that can ultimately contribute to the development of a specific type1/type2 cytokine pattern (Fig. 4).



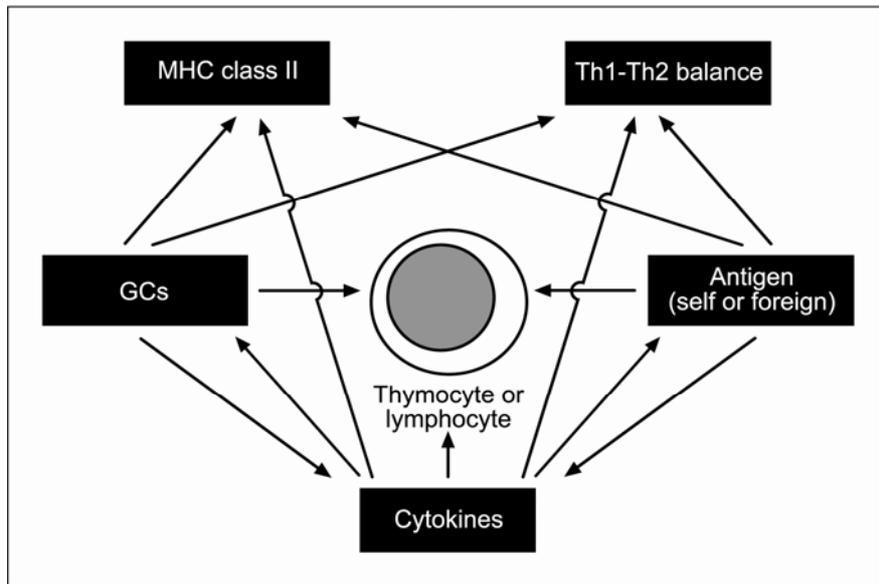


Figure 4. A model describing the interaction between GCs and factors that modulate thymocyte and lymphocyte development or function. Interactions, indicated by arrows, may be stimulatory or inhibitory, depending on the macro- and microenvironment, and the cell-cycle stage and pre-activation status of the cell. GCs up- or downregulate major histocompatibility class II depending on the presence of T_H1/T_H2 cytokines, which are in turn directly influenced by GCs; for example, interleukin 4 (IL-4), a key cytokine in isotype switching of B cells, is favoured by GCs. The nature and quantity of the antigen (self or nonself; viral or bacterial) dictates the fate of the target cell (positive or negative selection or a change of function) depending on the dynamic interactions between all the factors described. The GC-inducing cytokines IL-1, IL-6, tumor necrosis factor α (TNF- α) and others are costimulatory to the target cell (e.g. IL-6 to B cells) and their actions are modified by GCs; e.g. TNF- α is inhibited, and IL-6 is enhanced. The system is dynamic; thus, the functional antagonism or synergism between multiple signals results in a fine-tuned and specific adaptation to various conditions (Wilckens & De Rijk, 1997, p 419).

By now, the suppression of cytokine production by GCs is well known, but in contrast to this, GCs have also been shown to promote the production and release of several

cytokines. The cytokines IL-6 and TNF- α were induced by the administration of corticosterone at either basal or stress-related levels in an in situ liver perfusion system in the absence of other stimuli by Liao *et al.* (1995). However, when it was stimulated with endotoxin, the basal dose potentiated cytokine production, while the stress-related dose acted in a suppressive manner.

GCs are the foundation of asthma therapy and have long been used as the most effective agents for the treatment of other allergic diseases. The systemic reaction to tissue injury and/or infection, known as the acute phase response (APR), comprises of changes in body temperature, behaviour, and synthesis and release of cytokines and GCs (Baumann & Gauldie, 1994). The APR is considered as a crucial step in the re-establishment of homeostasis and involves both the induction and suppression of liver proteins, which are essential for tissue repair and clearance of cell debris or endotoxins, as well as other homeostatic functions. This is induced by a synergistic action of GCs and cytokines such as IL-1, IL-6 and TNF- α (Wilckens & De Rijk, 1997). The GC-IL-6-interaction plays a vital role in the protection from lethal sepsis, and one could speculate that an inadequate GC response or GC-IL-6-interaction during sepsis or septic shock might cause an insufficient APR, possibly despite high levels of IL-6.

Dissociated GCs are GCs that maintain trans-repression of immune genes in the absence of significant trans-activation of GRE-dependent promoters. Several *in vitro* and *in vivo* models were used to investigate whether dissociated GCs retained the anti-inflammatory and immunosuppressive potential of classic GCs (Vayssiere *et al.*, 1997). Secretion of the proinflammatory lymphokine IL-1 β was severely inhibited by the

dissociated GC, RU 24858, in human monocytic THP1 cells. In two *in vivo* models, this compound had an anti-inflammatory and immunosuppressive activity as potent as that of the classic GC prednisolone (Vayssiere *et al.*, 1997). In another study Vanden Berghe *et al.* (1999) demonstrated that the GC compounds studied, which included Dex and RU 24858, were able to inhibit TNF-induced IL-6 secretion in murine fibroblasts and HeLa cells (Vanden Berghe *et al.*, 1999). RU 24858 exerts strong activation protein complex-1 (AP-1) inhibition (trans-repression), but little or no trans-activation (Belvisi *et al.*, 2001). Belvisi *et al.* (2001) investigated whether this improved *in vitro* profile of RU 24858 resulted in the maintenance of anti-inflammatory activity with reduced systemic toxicity compared with standard GCs. They found that *in vitro* separation of trans-repression from trans-activation activity did not translate to an increased therapeutic ratio for GCs *in vivo* or that adverse effects are a consequence of trans-repression (Belvisi *et al.*, 2001). It was shown by Ray *et al.* (1997) that the interference of the GR with the transcriptional activation potential of DNA-bound NF- κ B complexes might contribute to mechanisms underlying the ant-inflammatory effects of GCs.

IL-5 is a selective eosinophil-activating factor produced by helper T cells. The development of chronic eosinophilic inflammation is dependent on IL-5 (Mori *et al.*, 1997). Mori *et al.* (1997) established allergen-specific CD4⁺ T cell clones from asthmatic patients to depict the regulatory mechanisms of human IL-5 synthesis. They found that GCs efficiently suppressed IL-5 synthesis of T-cell clones activated via either TCR or IL-2 receptor (IL-2R), and that Dex completely inhibited the induction of IL-5 mRNA upon TCR and IL-2R stimulation.

1.2.5 GCs and viral replication.

GC-responsive promoters and HIV-1 long terminal repeat (LTR) uses the same set of co-activators, therefore these proteins may stimulate HIV-1 LTR and GC-inducible genes simultaneously (Kino & Chrousos, 2001). Viral proliferation may be stimulated directly, while indirectly viral propagation might be enhanced due to the suppression of the host immune system through GC-mediated mechanisms. Glucocorticoids such as Dex are capable of directly upregulating HIV-1 replication in acutely infected cells and increase HIV expression from chronically infected promonocytic U1 cells stimulated with TNF- α (Kinter *et al.*, 2001). Kinter and co-workers investigated the potential effect of Dex in U1 cells stimulated with IL-6 (Kinter *et al.*, 2001). Dex and IL-6 synergistically induced HIV expression in U1 cells, while no substantial HIV RNA accumulation was demonstrated in U1 cells co-stimulated with IL-6 and Dex. The expression of monocyte chemoattractant protein-1 (MCP-1) RNA was upregulated by IL-6, while Dex inhibited this effect. Electrophoretic mobility shift assay (EMSA) revealed that Dex potentiated IL-6-induced activation of AP-1 and ERK1/2 mitogen-activated protein kinase (MAPK) phosphorylation. It was further observed that Dex potentiated the effect of HIV-1 LTR driven transcription in U1 cells stimulated with TNF- α (Kinter *et al.*, 2001). These results showed that stimulation with IL-6 and Dex, in the absence of activation of the HIV LTR or viral transcription, can induce high levels of virion production in latently infected cells. This suggests that LTR-independent pathways exist that are influenced by cytokines and GCs through which HIV can maintain substantial levels of virion production and protein expression (Kinter *et al.*, 2001).

Kino and co-workers examined the direct effect of GCs on HIV-1 LTR by measuring the ability of Dex to modulate the activity of this promoter coupled to the luciferase reporter gene in human cell lines (Kino *et al.*, 2000). They found that GCs suppress, rather than stimulate, the HIV-1 promoter, thus acting protectively for the host. They also postulated that the apparent negative clinical association of GCs with AIDS is most likely due to the immune-suppression of the host.

1.3 Medroxyprogesterone acetate

1.3.1 Use as contraceptive and pharmacological properties

The first systemic contraceptives were developed in the mid 1950s and they were short-acting progestogens administered orally (Fraser, 1981). MPA was developed by the Upjohn Company in the late 1950s (Babcock *et al.*, 1958). The Upjohn Company sponsored the first clinical trials where MPA was used as a treatment of threatened or habitual abortion and endometriosis. In 1963 the first clinical trials for its use as a contraceptive began and the first reports on the contraceptive efficacy of Depo-MPA followed in 1966 (Coutinho & De Souza, 1966; Csapo *et al.*, 1966; Zanartu & Onetto, 1965). Depo-MPA was approved as a contraceptive agent in many Western countries in the late 1960s except in the United States, where regulatory approval was stopped after tests on beagles showed that 17-acetoxy progestins was associated with an increased risk of cancerous mammary nodules (Mishell, 1996). Regulatory approval for marketing DMPA as a contraceptive was obtained in the United States after it was later

discovered that the beagle, unlike humans and other animals, uniquely metabolize 17-acetoxy progestins to estrogen, causing mammary hyperplasia.

There are at least more than 20 million current users of MPA worldwide (Affandi, 2002). The Essential Drug List include DMPA as an injectable progestogen-only contraceptive (IPC), and together with NET-En, are used extensively in more than 90 countries (Brenner, 1995; Connell, 1994; Kaunitz, 1994), including South Africa. Two studies showed that the use of DMPA increased in South Africa, while one of the studies hypothesized that it was due to DMPA being cheaper than other similar methods of contraception (Smit *et al.*, 2001; Margulies and Miller, 2001).

Medroxyprogesterone acetate (17 α -acetoxy-6 α -methyl-pregn-4-ene-3,20-dione) is a derivative of progesterone and comes in the form of a white to off-white odourless crystalline powder that is stable in air and melts between 200°C to 210°C (Upjohn, 1973) (Fig. 5). Although it is metabolised in the body, MPA itself is the major progestogenic compound responsible for its action (Stanczyk & Roy, 1990).

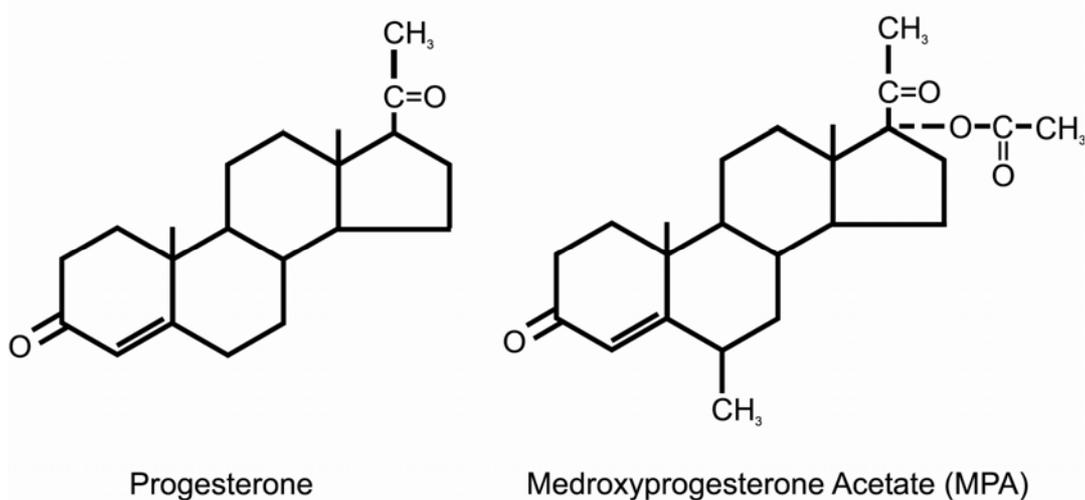


Figure 5. Comparative structures of progesterone and MPA.

Depo-MPA is a microcrystalline suspension prepared from MPA for use as a long-term contraceptive in the form of an intramuscular depot injection. The effectiveness of DMPA can be contributed to the prolonged duration of action since the progestin is slowly released after injection into the systemic circulation from the gluteal or deltoid muscle. MPA is not metabolized as rapidly as the parent compound progesterone; therefore it can be given in smaller amounts with an equal degree of progestational activity (Mishell, 1996). MPA has a higher relative affinity for the human endometrial progesterone receptor (PR) than progesterone itself (Shapiro *et al.*, 1978; Maclaughlin & Richardson, 1979) and it is suggested this affinity is due to its unusual inverted A-ring conformation (Duax *et al.*, 1978). MPA binds to progesterone receptors in the genital tract (Terenius, 1974; Haukkamaa & Luukkainen, 1974), where it acts as a potent progestogen on oestrogen-primed endometrium (Shapiro *et al.*, 1978).

Various dosage schemes have been tested, from intramuscular injections of 100mg every 3 months to 500mg every 6 months (Fraser, 1981). The standard contraceptive regimen of 150mg every 3 months appeared to be the most satisfactory and is still used today (Fraser, 1981). Final results from a large World Health Organization clinical trial on the use of DMPA demonstrated that it is an extremely effective contraceptive (WHO Special programme of Research, Development and Research Training in Human Reproduction, 1983). The pregnancy rate at one year was only 0.1%, and at two years, the cumulative rate was 0.4%. Similar results were found in other studies (Chinnatamby, 1971; Dodds, 1975; Powell & Seymour, 1971).

The serum concentration of the progestin (MPA) is much lower on a ng/mL basis in DMPA users than the serum progestin concentration in oral contraceptive users (Ortiz *et al.*, 1977). Serum levels of MPA depend on the rate of absorption from the microcrystals deep in the gluteal muscle depot, by the rate of hepatic metabolism and enterohepatic circulation, by urinary excretion of water-soluble metabolites, and ethnic differences (Fraser, 1981). MPA can be detected in the systemic circulation within 30 minutes after intramuscular injection of 150mg (Vermeulen *et al.*, 2001). Within 24 hours after injection peak serum levels ranging from 2.6nM to 7.8nM are achieved (Ortiz *et al.*, 1977), where after a plateau between 2.6nM and 3.9nM is reached for the duration of the contraceptive treatment (Fraser, 1981; Mishell, 1996). After three months there is a gradual decline to 1.3nM during the fourth and fifth months (Fraser, 1981) and in some women, MPA can be detected in the serum for as long as nine months after a single injection of 150mg (Mishell, 1996).

1.3.2 Mode of action as a contraceptive

There are three mechanisms of action involved that contribute to DMPA being the most effective reversible hormonal method of contraception. The major effect is inhibition of ovulation through elimination of the luteinizing hormone surge by suppressing follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels (Fraser, 1981; Nash, 1975; Khoiny, 1996). For several months after a single injection, MPA is released from the crystalline suspension in the muscle causing inhibition of ovulation for a prolonged and variable of time among individual women. The serum level of MPA during the first three months inhibits the midcycle gonadotropins peak, but does not alter the basal levels of FSH or LH. For the first four months after the first or subsequent injection serum estradiol levels remain in the range of values found in the early to midfollicular phase of ovulatory levels (Khoiny, 1996).

The second mechanism is by altering tubal motility and inducing shallow and atrophic endometrium lacking sufficient glycogen secretion to provide energy for the development of a blastocyst entering the endometrial cavity in the event fertilization of the egg occurs (Mishell, 1996; Hatcher *et al.*, 1994; Monier & Laird, 1989). Mishell *et al.* (1968) examined the histology of the endometrium at various intervals after initiation of DMPA use. Examination of endometrial biopsies revealed three histological types of patterns: proliferative, quiescent, and atrophic. They found no secretory endometrium and most of the women had a quiescent pattern, characterized by narrow, widely spaced glands and decidualization of the stroma. Thirdly, the prolonged elevated serum levels of MPA sustains a thick and viscous cervical mucus, making it

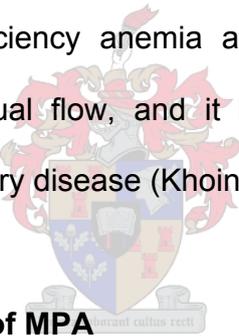
impermeable to sperm penetration and thus preventing sperm reaching the oviduct and fertilizing the egg (Mishell, 1996, Khoiny, 1996; Koryntova *et al.*, 2001).

Numerous studies have reported an unpredictable delay in the return of fertility, because of the lag time in clearing DMPA from the circulation causing a delay in the resumption of ovulation (Powell & Seymour, 1971; Mishell, 1996). Estradiol levels rise as the effect of DMPA is wearing off after 60 to 70 days. Many weeks before the resumption of ovulation the proliferative effect within the endometrium starts gradually increasing. Normal proliferative endometrium might be attained by day 90 (Roberts *et al.*, 1975), but may still possess some progestogenic effects (Jeppsson *et al.*, 1977). The endometrium may become more atrophic during prolonged DMPA administration (Mishell *et al.*, 1968), but, because the half-life of the drug is constant, the return of fertility is not related to the number of injections a woman receives. Schwallie and co-workers (Schwallie & Assenzo, 1974) found that the median time to conception varied between 9 and 12 months after the last injection but did not differ according to the number of injections or the bodyweight of the individual.

Since DMPA (e.g. Depo-Provera[®], Upjohn Company) is a progestin only contraceptive, serious complications associated with estrogen such as thrombophlebitis and pulmonary embolism are avoided (Hatcher *et al.*, 1994; Kaunitz, 1994). DMPA appears to be good and safe choice of contraceptive in breastfeeding teens, because it has no adverse effect on lactation or on the growth and development of the infant, as well as in teens with seizure disorders seeing that it does not interact with any of the antiepileptic agents (Kaunitz, 1994; Jones & Wild, 1994). Jones & Wild (1994) also showed that

DMPA has a favourable effect for patients with epilepsy, because it reduced seizure activities in these patients.

De Ceulaer *et al.* (1982) demonstrated that women who had sickle cell disease and used DMPA showed an increase in their hemoglobin erythrocyte levels and had a decrease in the frequency of their painful crisis, making DMPA the contraceptive of choice for teens suffering from sickle cell disease or coagulopathy. The mechanisms involved in this are not clear, but it is postulated that it could be due to the role of steroids in modifying the hematologic features of sickle cell disease by inhibition of sickling and by marrow stimulation (De Cealaer *et al.*, 1982). DMPA may possess other benefits like preventing iron deficiency anemia associated with heavy menses by decreasing or eliminating menstrual flow, and it has also been associated with a decreased risk of pelvic inflammatory disease (Khoiny, 1996).



1.3.3 Known side-effects of MPA

The Special Programme of Research, Development and Research Training in Human Reproduction (1993) organized a multicentre study of Cyclofem[®] (DMPA 25mg and estradiol cypionate 5mg) and Mesigyna[®] (Norethisterone enanthate 50mg and estradiol valerate 5mg) to determine the effects of these two injectable contraceptives on lipid metabolism and whether these effects, if any, persist after discontinuation. Although the lipid levels in Cyclofem[®] users were higher on almost all parameters than those in Mesigyna[®] users, both injectables reduced total cholesterol, LDL-cholesterol, HDL-cholesterol, apolipoproteins AI, AII and B. These studies concluded that the combination (estrogen + progestin) injectables have no significant or minor effects on

lipid metabolism, or the effects were mostly favourable. Small but significant changes in carbohydrate metabolism have been reported with contraceptive doses of DMPA. Among the different findings were an enhanced glucose and insulin response to a glucose tolerance test and a small increase in fasting glucose and insulin levels (Spellacy *et al.*, 1972; Vermuelen & Thiery, 1974).

Jaing *et al.* (1992) investigated the effects of Cyclofem (Cycloprovera) on carbohydrate metabolism in women and found that plasma glucose and area under the oral glucose tolerance curve were slightly increased at 9 months of treatment, whereafter it returned to pre-treatment levels within 3 months after treatment was discontinued. They found an exaggerated insulin response to the oral glucose tolerance test at 9 months when compared to pre-treatment levels (Jiang *et al.*, 1992). This insulin response appears to be due to a rise in biologically active insulin as well as proinsulin (Hausmann *et al.*, 1975). Plasma insulin levels were still elevated at three months in both groups after the treatment was stopped, although all values were in the normal range (Jiang *et al.*, 1992). Several studies indicated that changes in glucagon, growth hormone, cortisol, thyroxine or tryptophan metabolism or secretion are not responsible for any deterioration in glucose tolerance caused by DMPA (Vermuelen & Thiery, 1974; Tankeyoon *et al.* 1976), while one study (Amatayakul, 1979) postulated that it could be attributed to the corticosteroid-like effect of DMPA itself. Amatayakul *et al.* (1988) investigated the adrenal function and its reserve capacity in healthy non-lactating women receiving DMPA as injectable contraceptive. They found that DMPA did not impair the subjects' ability to release cortisol in response to a stressful situation, as well as in normal day-to-day activity. There are also discrepancies regarding the effect of

DMPA on carbohydrate metabolism (Fraser, 1981). The response to a glucose tolerance test became borderline or abnormal in 15% of the women tested in a study in the U.S.A. (Spellacy *et al.*, 1972), resulting in the questioning of possible adverse effects DMPA could have on carbohydrate tolerance in diabetics and prediabetics (Gershberg *et al.*, 1969).

A multicentre comparative study of the effects of Cyclofem® on coagulation and fibrinolysis showed that the combined injectable contraceptive decreased factor X and VII, plasminogen and fibrinogen (Special Programme of Research, Development and Research Training in Human Reproduction, April 1993). These results were seen as being of no clinical significance because most of the changes were within the normal range for the various parameters. A review on the results of liver function tests, collected by The Special Programme of Research, Development and Research Training in Human Reproduction multicentre lipid study, showed minor increases in bilirubin and a decrease in alkaline phosphatase (Special Programme of Research, Development and Research Training in Human Reproduction, May 1993). No significant changes in serum glutamic oxaloacetic acid (SGOT) and serum glutamic pyruvic transaminase (SGPT) were found, while similar results were obtained by Haiba *et al.* (1989). Two studies (Garza-Flores *et al.*, 1989; Wu *et al.*, 1991) on the effect of Cyclofem on prolactin levels found that prolactin increased after injection, gradually decreased to basal levels by the end of the injection interval, and that these changes were within the normal range.

Despite the effectiveness of MPA as a contraceptive, several side-effects associated with the use of DMPA exist. Some of these side-effects are adverse enough to cause discontinuation of use, while other side-effects are negligible and do not lead to discontinuation. One of the main disadvantages of DMPA contraceptive and the major reason for its discontinuation among adolescents and young adults is menstrual dysfunction. Depo-MPA is characteristically associated with a high incidence of amenorrhea, irregular (but frequent) bleeding, and a lower incidence of prolonged or heavy bleeding (Kaunitz, 2000; Benagiano *et al.*, 2000; Rome *et al.*, 2000; WHO Special programme of Research, Development and Research Training in Human Reproduction, 1983; Fraser, 1981). A cross-sectional study in Thailand on DMPA contraceptive use among Thai adolescents showed that with prolonged use the number of cases with irregular bleeding decreased and the duration was shortened, whereas the rate of amenorrhea increased (Chotnopparatpattara & Taneepanichskul, 2000). In a multicentre trial done by the World Health Organization (WHO) (1978), detailed analysis of menstrual patterns showed that over 70% of the women never experienced even one normal cycle throughout the treatment. Although bleeding irregularities are not life threatening, many users of DMPA stop the treatment due to discomfort.

Weight gain is the second most common reason for discontinuation of DMPA (Khoiny, 1996; Rome *et al.*, 2000; Fraser, 1981), while there are also studies that do not find a significant weight gain in adolescent DMPA users (Smith *et al.*, 1995; Moore *et al.*, 1995). A mean gain of 0.5kg to 2.0kg at the end of one year is reported by the majority of studies (Fraser, 1981). Individual tolerance to weight gain varies and although it is usually regarded as a problem, some cultures may welcome it as a sign of good health.

Other subjective effects related to DMPA use are breast tenderness, vaginal wetness, vaginal dryness, acne, hair loss, headaches, dizziness, mood changes, fatigue (asthenia), decreased libido, nervousness, abdominal bloating and discomfort, diarrhoea, nausea, asthma, chills, and insomnia (Khoiny, 1996; Kaunitz, 1994; Sharts-Hopko, 1993; Rome *et al.*, 2000; Smit *et al.*, 2002; Cummings & Brizendine, 2002; Fraser, 1981). All of these subjective side-effects are dependent on the individual whether they will lead to discontinuation or not. There have been many reports questioning the effects contraceptives could have on mood changes. Branham (1970) found that a disturbance in tryptophan metabolism occurred in up to 80% of oral contraceptive users. Izquierdo *et al.* (1978) and Ladisich (1977) both discovered that progesterone and progestogens may influence serotonin metabolism in women and in animals. Bjorn *et al.* (2000(78)) found more positive mood symptoms associated with MPA in HRT, while Cummings and Brizendine (2002) could not detect any effect on mood caused by MPA. These discrepancies arise out of the fact that the symptoms of mood changes are so subjective and that very careful evaluation is required.

An additional disadvantage associated with DMPA use is the effect it has on bone mass. Berenson *et al.* (2001) discovered that DMPA had an adverse effect on bone mineral density (BMD) when they compared the effect of DMPA on BMD among women 18 - 33 years of age with those not using hormonal contraception. Banks *et al.* (2001) did an overview of the published epidemiological literature on the relationship between the use of progestogen-only contraceptives and BMD. Sixty-eight percent of the data related to the effects of use of DMPA. They concluded that average BMD was reduced in current users of DMPA compared with non-users and that the reduction appeared to

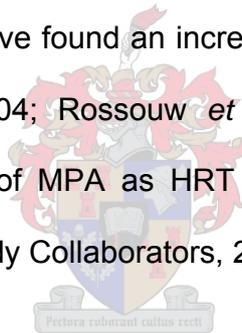
be greater at the lumbar spine, femoral neck and ultradistal forearm than at the midshaft of the ulna (Banks *et al.*, 2001). A greater reduction in BMD was also displayed in women with a longer average duration of DMPA use compared with studies of women with shorter duration of use (Banks *et al.*, 2001). In another study done by Kim *et al.* (2001), DMPA was associated with a greater risk of diabetes compared with combination oral contraceptive use only and this risk was associated with length of use and persisted after adjustment for body mass index.

Another area of theoretical concern is the association between MPA and cancer. Thuneke *et al.* (2000) investigated the effects of MPA on proliferation of T47D breast cancer cells. After 24 and 48 hours of MPA treatment they found an increase in proliferative activity followed by inhibition of proliferation after 72 hours, thus confirming the existence of a biphasic response of T47D cell proliferation in response to MPA treatment. Soderqvist (1998) showed that long-term continuous combined hormonal treatment with CEE and MPA induced a proliferative response in the breasts of surgically postmenopausal macaques. In this study the effect of combined treatment was more pronounced than that of oestrogen treatment alone. Both endogenous progesterone and exogenous progestogens down-regulate both oestrogen and progesterone receptors (Soderqvist, 1998). These findings suggest that progestogens and estrogens may have direct and indirect stimulating effects on proliferation.

World Health Organization studies and two other studies indicated that DMPA may increase the rate of breast cancer in women younger than 35 years of age (Paul *et al.*, 1998; Skegg *et al.*, 1995; WHO Collaborative Study of Neoplasia and Steroid

Contraceptives, 1991). It was suggested that these findings were the possible effect of progestin on advancing the pre-existing tumours and these speculations needed to be verified (Hatcher *et al.*, 1994). Although there is no evidence to suggest that DMPA may increase the risk of invasive carcinoma of the human cervix, two uncontrolled studies recorded abnormal cervical cytology, including carcinoma-in-situ, more frequently than expected in DMPA users (Powell & Seymour, 1971; Koetsawang *et al.*, 1972). Another study, which also had design limitations, found no abnormal cervical cytology in women using DMPA (Dabances *et al.*, 1974).

Only recently has the association between HRT and increased risk in breast cancer become clear. Various studies have found an increased risk in breast cancer amongst HRT-users (Stahlberg *et al.*, 2004; Rossouw *et al.*, 2002; Welnicka-Jaskiewicz & Jassem, 2003). Long-term use of MPA as HRT substantially increases the risk of breast cancer (Million Women Study Collaborators, 2003).



1.3.4 MPA and its use for other indications

Besides the contraceptive benefits MPA holds, it is also used in the treatment of various other diseases including hormone therapy. Medroxyprogesterone acetate is one of the most commonly prescribed progestins in the gynaecologic practice and for HRT after menopause (Ishida *et al.*, 2002). Medroxyprogesterone acetate is the most commonly used progestin in HRT in the USA (Stahlberg *et al.*, 2004). Medroxyprogesterone acetate is used in HRT either in conjunction with estrogens or when estrogens are contraindicated (Soderqvist, 1998; Bullock *et al.*, 1975). Doses used for HRT are about 10mg/day for about 11 days (Brunelli *et al.*, 1996). Medroxyprogesterone acetate is

used at higher doses in cancer therapy (Blossey *et al.*, 1984; Etienne *et al.*, 1992; Van Veelen *et al.*, 1986), including advanced or recurrent breast cancer (Kobayashi *et al.*, 2000; Gallagher *et al.*, 1987) and bone tumours (Sotaniemi *et al.*, 1978). Typical doses for cancer therapy ranges between 500 and 1500mg orally per day for about 12 weeks (Blossey *et al.*, 1984). In tumour regression, MPA may act partly via the PR (Blossey *et al.*, 1984; Braunsberg *et al.*, 1987), but predominantly via the GR (Bojar *et al.*, 1979) or the AR (Feghali & Wright, 1997) and an improved response is obtained when plasma MPA concentrations exceed 0.1 μ mol/L (Nishimura *et al.*, 1997) and when androstenedione secretion is suppressed (van Veelen *et al.*, 1984; van Veelen *et al.*, 1985).

Some other malignant conditions treated with MPA include metastatic disease of the kidney (Paine *et al.*, 1970; Bloom, 1971; Morales *et al.*, 1975) and various malignant conditions of the testes (Henry *et al.*, 1974). In women it has been used in the treatment of conditions requiring progestogen therapy, such as endometriosis (Irahara *et al.*, 2001; Telimaa *et al.*, 1989) and endometrial hyperplasia (Bonte, 1978). Medroxyprogesterone acetate is also employed in the treatment of auto-immune diseases (Gomez *et al.*, 1998; Bamberger *et al.*, 1999) due to its immunosuppressive properties and the fact that undesirable side-effects, such as the development of Cushingoid features (Leis *et al.*, 1980; Lang *et al.*, 1990), is less pronounced in patients treated with high dose MPA than with conventional GCs. Other conditions MPA is used in include acromegaly (Liuzzi *et al.*, 1972), hirsutism (Ettinger & Golditch, 1977), diabetic retinopathy (Hollows & Beaumont, 1973) and recurrent aphthous ulcers (Ferguson *et al.*, 1978).

1.3.5 Immunosuppression and GC properties

The immunosuppressive properties of MPA are well documented, but not fully understood due to the involvement of a variety of mechanisms and steroid receptors. In 1965, Hulka *et al.* published the first study on the immunosuppressive effects of progestins (Hulka *et al.*, 1965). Cosini and Puppo (1983) tested the inhibitory effect of MPA on the blastogenic response to mitogens of peripheral blood lymphocytes. They tested MPA *in vitro* at concentrations of 7ng/mL and 100ng/mL, corresponding respectively to plasma drug levels found during contraceptive treatment and during high dose oral therapy for cancer. At a concentration of 100ng/mL, MPA showed a significant inhibitory effect that can be attributed to a reduction in DNA synthesis and to a block of the uptake of ³H-thymidine (³H-TdR) into the cells. Their conclusions were based on four observations made. They found that 1.) cells preincubated with MPA showed a decreased blastogenic response, 2.) cells incubated throughout their entire 72hr culture period demonstrated a similar response, 3.) when MPA was added 1hr before ³H-TdR to activated cells, it inhibited the entry of ³H-TdR into the cells and, 4.) when MPA was added 1hr after ³H-TdR to activated cells only a slight and not significant decrease in ³H-TdR incorporation occurred.

The survivals of allografts done on animals have been used to demonstrate the immunosuppressive qualities of MPA (Pelner & Rhoades, 1965; Jeremiah *et al.*, 1968). Jeremiah and co-workers (1968) used the survival rate of renal allografts in dogs treated with MPA to evaluate the immunosuppressive properties of MPA. They obtained the most consistent evidence of immunosuppression in animals which received an intermediate dose of the drug (10mg/kg) and evidence for this suppression

consisted of prolonged survival, decreased serum creatinine, and the absence or modification of the allograft reaction on gross and microscopic examination. Skin allograft experiments also done by Jeremiah and co-workers (1968) on rabbits indicated that MPA can both suppress the primary antibody response and retard rejection of the allografts.

Gerretsen *et al.* (1979, 1980) tested the immune reactivity of women on hormonal contraceptives (including DMPA and oral contraceptives) in two separate experiments. Firstly, they evaluated the influence of sex steroids on immunity in women on hormonal contraceptives by performing sensitization tests with the contact allergen, 2,4-dinitrochlorobenzene (DNCB) (Gerretsen *et al.*, 1979). The DNCB sensitization test was used as a measure for cell-mediated immune reactivity. They found an increased DNCB reactivity in the group of women receiving DMPA, when compared to controls matched for age. Secondly, they investigated the influence of sex steroids on the lymphocyte response to the mitogens phytohemagglutinin (PHA) and concanavalin A (Con-A) in women on hormonal contraceptives (Gerretsen *et al.*, 1980). They found no significant changes between the different groups when compared to women not using hormonal contraceptives. They explained that these differences between their DNCB sensitization test and lymphocyte proliferation experiment were due to the fact that the *in vitro* lymphocyte proliferative response to mitogens is not a very sensitive measurement of cell-mediated immunity (CMI), compared to DNCB sensitization. Furthermore, DNCB sensitization test measures specific immune responsiveness involving the afferent, central and efferent phase of the immune response, while the *in vitro* lymphocyte to mitogens only reflects a non-specific proliferative response

(Gerretsen *et al.*, 1980). From these two experiments Gerretsen and co-workers (1980) suggested that sex steroids specifically influence the efferent phase of the CMI response and not the central proliferative response.

Since pregnancy can be regarded as a successful transplantation of an allograft *in utero*, studies have been done on the effects of sex steroids on CMI in pregnant women (Mori *et al.*, 1977; Tomoda *et al.*, 1976). Tomoda *et al.* (1976) demonstrated the suppressive action of progesterone *in vitro* by showing that pregnancy serum had a suppressive action on PHA-induced lymphocyte transformation. Progesterone was among the immune suppressive factors pointed out by them, concluding that CMI might be reduced in pregnant women and that this reduction might be one of the causes for the maintenance of pregnancy. Mori *et al.* (1977) examined the effects of different sex steroids, including progesterone, 17 α -hydroxyprogesterone and 20 α -dihydroxyprogesterone, on the response of human lymphocytes to PHA stimulation. Various concentrations of each steroid were used and incorporation of ³H-TdR into lymphocytes was measured. Of the nine sex steroids tested by them, progesterone and 20 α -dihydroxyprogesterone showed inhibition of the lymphocyte transformation induced by PHA and they considered progesterone to suppress maternal lymphocyte response at its site of production rather than at the systemic level.

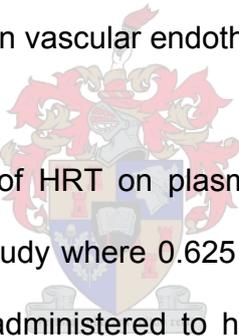
A prospective study was conducted by Yamashita and co-workers (1996) on the effects of MPA on serum IL-6 levels in patients treated with MPA for metastatic breast carcinoma, after it was discovered that IL-6 levels in these patients, receiving MPA treatment, were very low compared to those who had not. They found that serum IL-6

levels decreased in all the patients after four weeks since the initiation of MPA therapy and this regardless of whether or not the patients responded to the therapy. This effect on serum IL-6 levels did not correlate with the daily dose of MPA, but it correlated closely with the plasma MPA levels in these patients. A study done by Brunelli *et al.* (1996) indicated that HRT selectively affects various immune cell subsets. They treated 10 healthy menopausal women for 6 months by administering transdermal estradiol (100 micrograms/day for 21 consecutive days) and oral MPA (10 mg/day from day 10 to day 21). They found that on day 21, the only evaluation day during MPA administration, CD56⁺ cells and CD8⁺CD11b⁺ cells were decreased and that natural killer cell function was greatly reduced.

Vassiliadou *et al.* (1999) studied the effects of various physiological concentrations of progesterone on the expression of chemokines and chemokine receptors by T cells and macrophages. They found that at the maximum concentration progesterone significantly inhibited the secretion of all three chemokines by CD8⁺ cells, while it had no effect on the chemokine secretion by CD4⁺ cells. The proliferation of CD8⁺ cells were significantly suppressed by progesterone, which ultimately lead to the observed suppressive effect on chemokine secretion by CD8⁺ cell cultures.

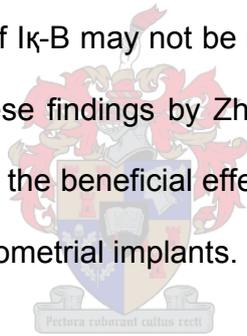
Several studies exist on the anti-inflammatory properties of MPA, although this capability of MPA is not completely clear (Wakatsuki *et al.*, 2002, Yildirim *et al.*, 2002). Because androgens have anti-inflammatory effects, Wakatsuki *et al.* (2002) investigated the effects of the androgenic progestin, MPA, on estrogen induced changes in acute inflammatory proteins and cell adhesion molecules in postmenopausal women. C-

reactive protein (CRP) stimulates the release of inflammatory cytokines (Galve-de Rochemonteix *et al.*, 1993), induces tissue factor expression from human monocytes (Nakagomi *et al.*, 2000), and induces the expression of cell adhesion molecules (Pasceri *et al.*, 2000). Conjugated equine estrogen (CEE) increases acute inflammatory proteins such as CRP and serum amyloid A protein (SAA) (Wakatsuki *et al.*, 2002). Wakatsuki *et al.* (2002) found that when MPA was used in combination with CEE, it tended to decrease the concentrations of the cell adhesion molecules ICAM-1 (intercellular adhesion molecule), VCAM-1 (vascular cell adhesion molecule) and E-selectin. They concluded that MPA's anti-inflammatory effect, but not its direct effect, may decrease cell adhesion molecule expression, since it was shown that MPA did not inhibit VCAM-1 expression in human vascular endothelial cells (Otsuki *et al.*, 2001).



In order to investigate the effect of HRT on plasma homocysteine and CRP levels, Yildirim *et al.* (2002) conducted a study where 0.625 mg/day CEE plus 2.5mg/day MPA or 0.625mg/day CEE alone was administered to healthy menopausal women. They discovered that estrogen alone significantly increased serum CRP concentrations, but that estrogen plus MPA therapy did not significantly alter serum CRP levels, indicating that the CRP raising effect of estrogen might be partially prevented by the addition of MPA. However, this reduction of CRP levels by MPA was not found by Koh *et al.* (2002). They administered 2.5mg of MPA with 0.625mg of CEE daily during 2 months to hypertensive and/or overweight postmenopausal women and found that CEE plus MPA significantly improved flow-mediated dilation and reduced plasma E-selectin, ICAM-1, MCP-1, and tumor necrosis factor-alpha, but not C-reactive protein. Zhao and co-workers (Zhao *et al.*, 2002) did an *in vitro* study on the possible inhibition of synthetic

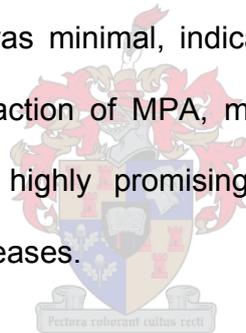
progestins, including MPA at a concentration of 10^{-7} M, on RANTES gene expression in isolated endometrial stromal cells. They transiently transfected endometrial stromal cells with wild-type RANTES promoter constructs cloned upstream of the firefly luciferase reporter gene in order to assess the roles of specific domains within the RANTES promoter. Their results showed that MPA exposure of 2 days did not influence RANTES transcriptional activity, but longer exposure (8 days) to MPA resulted in a $\pm 37\%$ decrease in RANTES promoter transcription. They hypothesized that the chronic inhibition of NF- κ B transcription activity by MPA was probably PR dependent, with a more potent repression mediated through PR-B than PR-A. Zhao and co-workers (Zhao *et al.*, 2002) found that MPA did not upregulate I κ -B α protein, which suggested that the accumulation of I κ -B may not be responsible for the down-regulation of NF- κ B by MPA treatment. These findings by Zhao *et al.* (2002) indicated the anti-inflammatory potential of MPA and the beneficial effects long-term treatment could have on pelvic pain associated with endometrial implants.



1.3.6 MPA and its interaction with cellular receptors

The ability of MPA to interact with progesterone, androgen and GC receptors is responsible for the multifaceted steroid hormone effects it possesses. Bamberger *et al.* (1999) used an electroporation protocol to show for the first time that MPA can suppress a human IL-2 promoter-luciferase construct to the same extent as the synthetic GC Dex in normal human lymphocytes. They found that MPA caused only marginal trans-activation, but strong trans-repression, in normal human lymphocytes and that this trans-repression of IL-2 promoter activity was at least equal to that produced by Dex. In their study MPA also markedly suppressed IL-2, IL-1 and IL-6 release. In a typical

experiment Bamberger and co-workers found that the trans-repressive capability of MPA was 1.91 times stronger than that of Dex and that MPA induced trans-activation was only 29% of that caused by Dex. Furthermore, their expression studies indicated that the observed effects of MPA were mediated by GR and not PR, since human lymphocytes only express GR (Bamberger *et al.*, 1999). Bamberger and Schulte (2000) went further to quantitate the immunosuppressive effects of MPA at the molecular level. Here too, they used the pGL3 luciferase reporter vector, but under the control of the human IL-2 promoter on a GRE, to transfect lymphocytes in order to measure luciferase activity in response to various steroid compounds (including MPA). They found that MPA profoundly suppressed IL-2 gene expression and that its stimulatory effects on the GRE-driven promoter construct was minimal, indicating the dissociative properties of MPA. The immunosuppressive action of MPA, mediated by the GR (Bamberger & Schulte, 2000), makes MPA a highly promising substance for the treatment of autoimmune and inflammatory diseases.



More recent data on the association of MPA with the GR include studies done by Koubovec *et al.* (2004) and Zhao *et al.* (2003). It was demonstrated by the research team of Koubovec that MPA behaved similar to Dex in that it significantly repressed TNF-stimulated IL-6 protein production in mouse fibroblast cells (Koubovec *et al.*, 2004). They found that MPA significantly repressed IL-6 and IL-8 promoter-reporter constructs at the transcriptional level and that MPA caused significant transactivation of a GRE-driven promoter-reporter construct in both mouse fibroblast and COS-1 cells. They concluded that the action of MPA in their system was at least in part mediated via the GR. Zhao *et al.* (2003) reported on the potency and efficacy of a panel of GR ligands

as a function of GR expression levels. They managed to show that the transrepression potency and efficacy of GR full agonist are affected by receptor density. Furthermore, Zhao and colleagues were able to demonstrate that MPA acted as a full agonist in transrepression in the presence of high levels of receptor density, while a decrease in receptor levels caused MPA to convert from a full agonist to a full antagonist.

A study conducted by Vermeulen *et al.* (2001), using a mouse model, suggested that MPA *in vivo* effectively interacts with PR to exert its enhancing effect on the humoral immune response. They found that low doses of MPA significantly increased the secretion of specific immunoglobulins (IgM and IgG) and that this enhancement of antibody production occurred through the direct interaction of MPA with immunocompetent leucocytes. The maximum release of immunoglobulins occurred at MPA concentrations of 10^{-9} and 10^{-10} M, which according to other studies (Briggs & Briggs, 1973; Kontula *et al.*, 1983), had no demonstrable GC effect. However, MPA had no effect at doses compatible with GC activity (10^{-8} and 10^{-7} M), suggesting that the stimulatory effects of MPA were counteracted by signals delivered through GC receptors.

Whether human lymphocytes expresses the PR has long been a matter of dispute. Some studies found that a marked proportion of T lymphocytes expressed the PR (Vermeulen *et al.*, 2001; Chiu *et al.*, 1996), while contradicting results were found by other studies (Schust *et al.*, 1996; Bamberger *et al.*, 1999). Females tend to generate increased pro-inflammatory stimuli, caused by sex hormones. It has been hypothesized that this pro-inflammatory effect of female sex hormones may be mainly mediated by a

sensitizing effect on the HPA axis toward inflammatory mediators, resulting in an increased inflammatory response mediated by GCs (Da Silva *et al.*, 1993). In one study MPA (100mg/day) was administered to five adrenalectomized humans who showed evidence of GC insufficiency after cortisone had been withdrawn (Camanni *et al.*, 1963). This caused an abrupt and complete disappearance of the symptoms attributable to GC insufficiency.

In 1973, while attempting to quantify the GC properties in humans of three widely used progestogens, Briggs and Briggs (1973) discovered that these GC properties were greater than those indicated by animal tests. In their study oral progesterone was devoid of GC activity in all three tests they used, but that the introduction of a 17 α -acetoxy group produced significant anti-corticotrophin activity and the further presence of a 6 α -methyl group gave significant eosinopenic and hyperglycemic properties.

Other indications of the possible detrimental immunosuppressive effects MPA can hold, exists in various infectivity rate studies that have been done in the past. Marx *et al.* (1996) used the SIVmac animal model to determine whether circulating progesterone had an effect on the vaginal transmission of simian immunodeficiency virus. They implanted 200mg progesterone pellets subcutaneously in treated macaques every 30 days for a total of 90 days, whereafter they inoculated the macaques vaginally with an intentional relative low dose (640 TCID₅₀, mean tissue culture infectious dose) of SIV virus stock. After inoculation SIV was isolated at multiple time points from PBMCs of only 1 of 10 placebo treated animals, compared to 14 of 18 progesterone-treated animals. Based on the high virus loads and the more rapid progression to AIDS, it is

safe to say that progesterone treatment also induced greater *in vivo* replication of SIV (Marx *et al.*, 1996).

Collectively, these findings suggest that women who are exposed to high levels of endogenous or exogenous progesterone may be at greater risk of HIV infection from vaginal intercourse. Thinning of the vaginal epithelium was pointed out be the major factor causing the enhanced vaginal transmission of SIV in this study. But noteworthy, the authors also mention that other progesterone-induced mechanisms, like other undetectable changes in the cervical or vaginal epithelium, changes in the immune system, and possibly even direct enhancement of viral replication, can play a role in this observed enhancement of vaginal transmission (Marx *et al.*, 1996).

The results of another study conducted by Marx showed that postmenopausal women and women using injectable, progestin-based contraceptives might be at an increased risk of HIV infection (Smith *et al.*, 2000). Ovariectomized female macaques were treated with either progesterone or estrogen prior to intra-vaginal inoculation of SIVmac. Of the progesterone-treated macaques they used, 83% became infected with SIVmac, compared to none of the estrogen-treated macaques, after intra-vaginal inoculation. These results raises concern considering that women who use long-acting, progestin-based contraceptives, such as DMPA, have low levels of estrogen.

Baker *et al.* (1980) investigated whether progesterone, by suppressing the CMI, enhanced the susceptibility of mice to herpes simplex virus type 2 (HSV-2) infection. They measured the proliferative phase of the CMI response by stimulating mouse

spleen lymphocytes with the non-specific T-cell mitogen ConA. The results obtained by them showed that progesterone depresses ConA stimulation of murine lymphocytes, but that it has to be continuously present to do so (Baker *et al.*, 1980). This suggests that either elevation of progesterone above a certain critical level is needed or that there is a critical period of time of progesterone exposure required for this effect to occur, and this is further supported by the observed increased mortality rates associated with increased progesterone concentrations. The conclusion drawn from this study of Baker *et al.* (1980) is that progesterone is capable of depressing the proliferative phase of CMI, thus increasing susceptibility to HSV-2 vaginal infection.

Opposing the findings of Marx *et al.* (1996) and Baker *et al.* (1980) is the findings of Vassiliadou and co-workers (1999). They found that progesterone dramatically and consistently inhibited infection of activated PBMCs with low titres of the HIV-1_{MN} T-tropic strain. In their study progesterone treatment resulted in significantly lower concentrations of RANTES, macrophage inflammatory protein-1 α (MIP-1 α) and MIP-1 β in supernatants from CD8⁺ T lymphocyte cultures caused by inhibition of cell proliferation (Vassiliadou *et al.*, 1999). The primary role of chemokines is to direct the movement of leukocytes in development, homeostasis, and inflammation via interactions with their receptors (Ward & Westwick, 1998). Progesterone had a suppressive effect on CCR5 and CXCR4 protein expression which occurred exclusively in activated T cells and not in resting T cells or macrophages, suggesting that the hormone interferes with early cellular activation events and that this suppressive effect could have implications for lymphocyte migration (Vassiliadou *et al.*, 1999). The data of Vassiliadou *et al.* (1999) suggest that progesterone may inhibit chemokine-mediated

recruitment and proliferation of activated CD8⁺ T lymphocytes and could thereby weaken mucosal antimicrobial defence functions. On the other hand, it could also be argued that the reduction in chemokine secretion by CD8⁺ T cells at sites of infection can result in a reduction of the number of CD4⁺ cells recruited to the mucosal epithelium, thereby decreasing the number of HIV-1 host cells at sites of transmission. Since progesterone reduced CXCR4 expression (but not CD4 expression), they concluded that this was the reason for their observed protection against HIV-1 infection, seeing that T cell-tropic viruses primarily use the CXCR4 co-receptor for entry (Vassiliadou *et al.*, 1999).

A number of studies exist on the association between contraceptives, sexually transmitted diseases and the incidence of HIV-1 infection. However, the result of studies examining this association has been somewhat inconsistent. One study found no association between injectable contraceptives and the prevalence of HIV infection (Mati *et al.*, 1995), while two cross-sectional studies (Bulterys *et al.*, 1994; Rehle *et al.*, 1992) and two prospective studies (Martin *et al.* 1998; Ungchusak *et al.* 1996) found a positive association between DMPA use and HIV-1 infection. However, the quality of these studies are not clear, although a trend towards a more positive association exists. The findings of two other studies indicated that the use of oral or injectable hormonal contraception altered susceptibility to STDs, which in turn may influence transmission of HIV-1 (Baeton *et al.*, 2001; Cottingham & Hunter, 1992), while Mostad *et al.* (1997) and Wang *et al.* (2004) found a significant association between cervical HIV-1 proviral shedding and the use of DMPA. Investigations on whether steroid hormones had a direct effect on the human immunodeficiency virus demonstrated that steroid hormones

can bind to the regulatory sequence of HIV-1 and up regulate expression of the virus (Kinter *et al.*, 2001; Ghosh, 1992; Furth *et al.*, 1990; Kolesnitchenko & Snart, 1977).

1.4 Norethisterone

1.4.1 NET-En in contraception

Different forms of the synthetic progestin, NET, are used for different applications. NET-A is most commonly used in HRT, while NET-En is widely used in contraception (Hapgood *et al.*, 2004). The first report on the contraceptive use of NET-En (17 beta-heptanoyl-17-alpha-ethinyl-4-oestren-3-one-7-3H) dates back to 1966 (Zanartu & Onetto, 1966) (Fig. 6). Although it was synthesized at about the same time as DPMA (Babock *et al.*, 1958), it has been much less extensively used which is also portrayed in the lack of available data compared to DPMA. Once NET-En is injected into the body it undergoes metabolism to form NET and its metabolites, which collectively is responsible for its progestogenic property (Stanczyk & Roy, 1990). Smit *et al.* (2001) compared the utilisation patterns of the injectable contraceptive products DPMA & NET-En issued from 4 South African provincial pharmaceutical depots (in rural KwaZulu-Natal) over 3 financial years. They found that while more DPMA than NET-En was issued, Net-En distribution from depots increased over the 3 year period and that younger woman were more likely to use Net-En than DMPA.

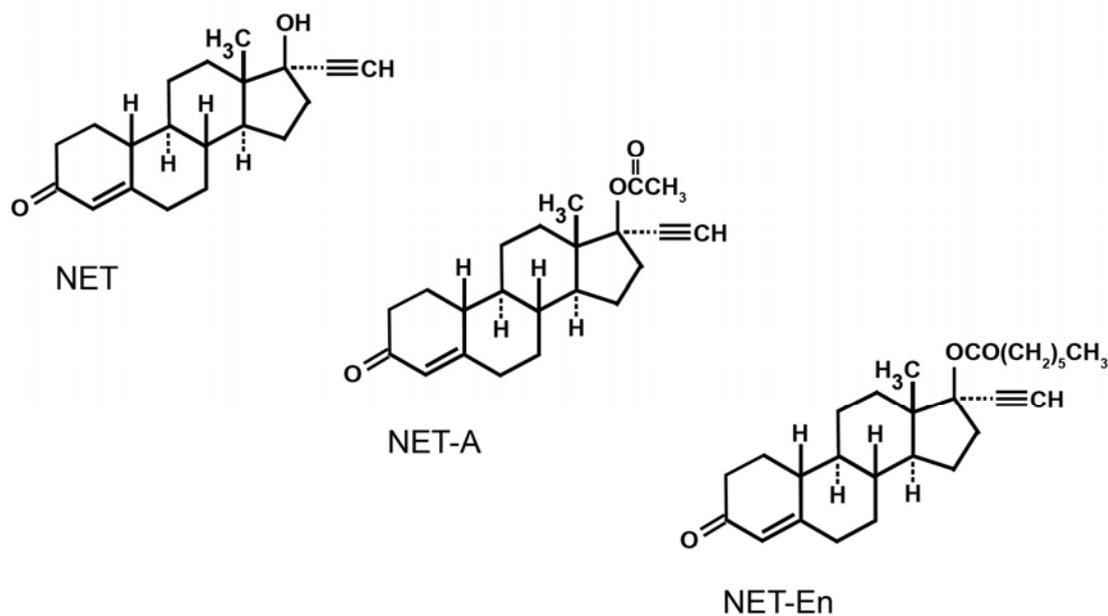


Figure 6. Structures of norethisterone, norethisterone acetate and norethisterone enanthate.

Norethisterone enanthate, a long chain ester of norethisterone prepared in oily solution, gives a high contraceptive efficacy when administered intramuscularly as a 60 to 90 day injection. The usual dosage contains 200mg of NET-En and peak plasma levels of 50nmol/L Norethisterone is reached one week after injection, whereafter a gradual decline occurs (Goebelsmann *et al.*, 1979; Fotherby *et al.*, 1978). Plasma levels of above 13nmol/L are usually maintained for one to four months depending on the demonstrated variation between individuals (Goebelsmann *et al.*, 1979; Howard *et al.*, 1975).

The mechanism of action of NET-En appears to be more complex than that of DPMA. An initial inhibition of ovulation coincide with very high initial plasma progesterone levels (Goebelsmann *et al.*, 1979; Fotherby *et al.*, 1978), which may wear off as early as 60 days (Howard *et al.*, 1975). As this ovulation-inhibiting effect wears off defective ovulation could occur with premature luteolysis together with a persisting peripheral fertility-inhibiting effect (Fraser, 1981). The median period for suppression of ovulation is 98 days (Achari, 1969). Distinct progesterone effects can be seen on the cervical mucus while different effects on endometrium occur at various times during the two to three months after administration (Achari, 1969), as well as probable effects on tubal function (Zanartu & Navarro, 1968).

The structural changes in the endometrial surface epithelium and subepithelial collagen III fibres were explored by Wonodirekso *et al.* (2000) by assessing endometrial biopsies from NET-En users and controls. They found that atrophic changes remained the dominant appearance for progesterone-exposed endometrium, with reduced cytokeratin staining, but apparently there is little change in subepithelial collagen III expression. The primal action is probably suppression of oestrogen-induced positive feedback mechanism and the sensitivity to this suppression is subjected to variability (Goebelsmann *et al.*, 1979).

The contraceptive efficiency appears to decline rapidly at around 80 to 100 days in the usual dosage of 200mg (WHO Expanded Programme of Research, Development and Research Training in Human Reproduction, 1977). Kesseru-Koos *et al.* (1973) conducted a large study where 1844 women were given 200mg every 84 days over

1810 woman-years of experience and recorded an over-all pregnancy rate of 1.49 per 100 woman-years. Some evidence of the WHO multicentre trial (WHO Programme of Research, Development and Research Training in Human Reproduction, 1977) show that a higher pregnancy rate occurs among thin woman who do not have adequate deposits of adipose tissue for storage of the NET-ester. NET-En has also been shown to be appropriate for use in lactating women without any detrimental side effects (Karim *et al.*, 1971). A study by Fine *et al.* (1962) indicated that NET-En should not be administered during known pregnancy as masculinization of the female fetus may occur with the use of NET late in the first and early in the second trimesters.

Another NET-En containing contraceptive is the once-a-month injectable, Mesigyna[®]. Mesigyna[®] consists of a progestogen (50mg NET-En) and an estrogen (5mg estradiol valerate) and is known to disrupt vaginal bleeding patterns less than which is observed by the widely used progestogen-only preparations. In a WHO study done by Hall (1998) the annual pregnancy rate for Mesigyna[®] users were below 0.4% and in a phase III clinical study done by Bassol *et al.* (2000) the pregnancy rate at 1 year was 0 per 100 woman-years for a total experience of 4688 woman-months.

Another contraceptive application of NET-En is the use of it in hormonal contraceptive for males in combination with testosterone. Several studies have been done by Kamischke *et al.* on this subject (Kamischke *et al.*, 2000; Kamischke *et al.*, 2001; Kamischke *et al.*, 2002). In the first study Kamischke *et al.* (2000) assessed the potential of NET-En for male contraception by evaluating the suppressive effect of a single injection of 200mg NET-En on serum gonadotropins, serum testosterone, lipids,

spermatogenesis, well-being and sexual function in seven healthy men. Based on its strong, rapid and sustained suppression of serum FSH and testosterone, they concluded that NET-En offers great potential for hormonal male contraception if combined with testosterone esters. In their second study they determined the efficacy of the long-acting testosterone undecanoate ester (TU) alone or in combination with NET-En in a phase II clinical trial (Kamischke *et al.*, 2001). Their results showed that the combination of TU and NET-En can be considered as the most promising in further studies of hormonal contraception. Seeing that they obtained a high efficacy with the combination of TU and NET-En, they next attempted to achieve high rates of suppression of spermatogenesis as reflected by sperm counts and monitoring of gonadotropins as well as other hormones (Kamischke *et al.*, 2002). They found in all groups that marked suppression of gonadotropins resulted in a significant decrease of spermatogenesis and azoospermia and that the dose and mode of action they used (1000mg TU i.m. every 6 wk plus 400mg NET-En i.m. every 6 wk) was as effective as the previously reported regimen containing 1000mg TU and 200mg NET-En i.m. every 6 weeks.

1.4.2 Pharmacokinetics and influence on lipid metabolism

Gerhards *et al.* (1976) described the kinetics and metabolism norethisterone enanthate after intra-muscular injection in two female subjects. Respectively 177.4mg and 174.5mg NET-En were injected. They found that maximum ³H-activity in plasma was reached 8 to 14 days after the injection which, in terms of NET-En, amounted to 70-100µg/100mL. Maximum NET-En concentration of about 1µg/100mL was reached on the 4th to 8th day. Furthermore, the concentration of NET-En was still about 0.05 to

0.1µg/100mL after 4 weeks and NET-En was still detectable in plasma even after 6 weeks.

Amatayakul *et al.* (1988) studied adrenal function and its reserve capacity in 9 healthy non-lactating women who received NET-En as long-acting injectable contraception. They found that NET-En did not impair the ability to release cortisol in response to a stressful situation as well as to normal day-to-day activity. Primiero and Benagiano (Primiero & Benagiano, 1993, as cited by Giwa-Osagie, 1994) determined the effect of Mesigyna® and the triphasic oral contraceptive Trinovum® (norethisterone 0.5 / 0.75 / 1.0 mg plus ethinyl estradiol 35µg) on carbohydrate metabolism. Each treatment group consisted out of 30 women and they used the euglycaemic glucose clamp technique to quantify glucose and insulin response before contraception and after six cycles of contraception. They found no significant change in glucose tolerance or insulin sensitivity, therefore concluding that the once-a-month injectable contraceptive has no significant effect on carbohydrate metabolism.

Another study on the effect of Mesigyna® on carbohydrate metabolism was done by Jaing *et al.* (1992) in 33 Chinese women. They discovered that plasma glucose and area under the oral glucose tolerance curve were slightly increased at 9 months of treatment and that it returned to pre-treatment levels within 3 months of treatment discontinuation. Furthermore, they found that plasma insulin levels were significantly increased at 9 months when compared to pre-treatment levels and they were still elevated at 3 months, although all values were in the normal range, once the treatment was stopped.

Herkert *et al.* (2001) examined whether sex steroids promote hemostasis indirectly by increasing the procoagulant activity of blood vessels. They were surprised to find that upregulation of proteolytically activatable thrombin receptor (PAR-1) and surface procoagulant activity occurred selectively with certain progestins, such as progesterone, gestodene, KDG, and MPA, whereas norgestimate, levonorgestrel, and norethisterone, despite their similarly strong progestin potency, did not.

A cross-sectional survey done by Meng *et al.* (1990) investigated coagulation in 64 Chinese women using Mesigyna, compared to 59 controls who were not using steroidal contraceptives. They found no significant differences between the two groups in values of prothrombin time, fibrinogen, factor X activity, anti-thrombin III antigen, anti-thrombin activity and fibrinolytic activity expressed by euglobulin lysis time. A longitudinal study (Meng *et al.*, 1990) was done by the same group of investigators on the effects Mesigyna could have on haematological parameters in 42 Chinese women. They discovered a progressive and significant decrease in factor X and anti-thrombin III functional activity, decreasing by about 14% and 20% respectively, after 12 months of treatment. These reduced levels were significantly lower than in the pre-treatment or the control groups. An increase in haemoglobin levels occurred in the treatment group after the third injection and remained at the higher level during the study period. It is doubtful whether these changes in factor X and anti-thrombin III will affect coagulation as they are antagonistic to each other.

Mueck *et al.* (2002) used endothelial cell cultures from human female coronary arteries to evaluate the effect of progestin addition to Estradiol ($E_{(2)}$) on the production of the

following endothelial markers: prostacyclin, endothelin, plasminogen activator inhibitor-1, E-selectin, ICAM-1, MCP-1, and the precursor of matrix metalloproteinase-1 (pro-MMP-1). They demonstrated for the first time in human coronary cell cultures that the addition of MPA or NET can elicit different effects. Medroxyprogesterone acetate antagonized the $E_{(2)}$ -induced reduction of MCP-1 synthesis, while an enhancement of the positive $E_{(2)}$ -effect on pro-MMP-1 production occurred after the addition of both MPA and NET-En. Their results showed that neither the addition of MPA or NET negatively interfered with the $E_{(2)}$ -induced benefits and that no impact was found on markers representing primarily vasotonus and thrombogenicity. After 6 months of Mesigyna use, Haiba *et al.* (1989) found no significant changes in albumin/globulin ratio, SGOT and SGPT. These findings are in agreement with a review on the data on liver function tests from the HRP multicentre lipid study (Special Programme of Research, Development and Research Training in Human Reproduction, May 1993), which showed no significant changes in SGPT, SGOT and gamma-glutanyl transpeptidase and showed minor increases in bilirubin and a decrease in alkaline phosphatase associated with the use of Mesigyna[®].

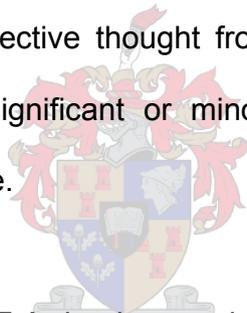
The association of adverse lipid patterns with atherogenesis and cardiovascular complications emphasises the importance of the effects contraceptives have on lipid metabolism (Fotherby, 1985; Deslypere *et al.*, 1985). A study done in Chinese women showed that the use of Mesigyna[®] was associated with reduced HDL-cholesterol and Triglycerides (TGs) from the first to the twelfth treatment cycle and that recovery to pre-treatment levels occurred after discontinuation. LDL-cholesterol remained unchanged during the 12 months of treatment, resulting in a decrease in HDL-cholesterol / LDL-

cholesterol ratio which also returned to pre-treatment level after discontinuation of treatment (Yang *et al.*, 1993, as cited by Giwa-Osagie, 1994). Kesserü *et al.* (1991) did a prospective study on serum lipid parameters during 12 months on controls, women on triphasic oral pill containing gestodene, and the injectable Mesigyna®. They found that total serum lipid values in Mesigyna users decreased significantly, up to six months, then increased to levels comparable to those in controls at 12 months.

Unlike the results of Yang *et al.* (1993), Kesserü *et al.* (1991) found that the HDL-cholesterol levels did not show any significant differences between the groups and that LDL-cholesterol levels of injectable users decreased progressively and significantly up to 12 months, thereby causing an increase in HDL/LDL ratio which is in the direction of reduced cardiovascular risk. The TG levels in Mesigyna® users also decreased significantly in their study up until 6 months then became similar to those of controls by 12 months. The study by Primiero and Benagiano (1993) on Mesigyna demonstrates that Mesigyna causes minor changes in cholesterol, HDL, LDL and very low density lipoprotein (VDL) and that these changes are likely to be of no clinical significance.

Perry and Wiseman (2002) determined the effect of continuous estradiol valerate 2mg and NET 0.7mg daily as hormone replacement on lipid profiles, coagulation factors, haematology and biochemistry over 3 years in an open label trial with 107-133 postmenopausal women. They found that lipoprotein A decreased significantly and that this decrease might be an indicator of long-term decreases in atherosclerotic events. McEwan *et al.* (1992) did a metabolic study on the blood levels of lipoprotein fractions of 56 long-term users of NET-En as injectable contraceptive. There were no differences

between their study group and a group of non-users in TGs, total cholesterol, LDLs and very low density lipoproteins (VDLs). There was however a significant reduction in mean HDL levels between the controls and the user group. The multicentre study on Cyclofem[®] and Mesigyna[®] organized by the HRP (Special Programme of Research, Development and Research Training in Human Reproduction, May 1993) investigated the effects these contraceptives had on lipid metabolism and if these changes persisted after discontinuation. Both injectables were shown to reduce total cholesterol, LDL-cholesterol, HDL-cholesterol, apolipoproteins AI, AII and B, and slightly reduce the HDL/LDL cholesterol ratio. The lipid levels in Mesigyna[®] users were virtually on all parameters lower than those in Cyclofem[®] users and TG levels were significantly reduced by Mesigyna[®]. The collective thought from all these studies suggests that Mesigyna[®] produces either no significant or minor changes on lipids, or produce changes that are mostly favourable.



Norethisterone, in the form of NET-A, is also used in HRT and is the most commonly used progestin for HRT in Europe (Stahlberg *et al.*, 2004). Lippert *et al.* (2001) compared the effects of MPA and NET on the estradiol stimulated proliferation in MCF-7 cells with respect to the different regimens used in combined HRT and found that NET did not show any significant effect on proliferation in the sequential combined model. In 1965, Hulka *et al.* (1965) experimented with skin allografts on castrated adult rabbits and found that norethindrone and norethynodrel significantly and consistently prolonged allograft survival to an average of 13 days, but had a variable effect on circulating antibody production. Numerous recent studies have revealed that the risk of breast cancer is higher in HRT-users (Welnicka-Jaskiewicz & Jassem, 2003; Stahlberg *et al.*,

2004; Beral *et al.*, 2002). The recent 'Million Women Study' found that NET significantly increased the risk of breast cancer in long-term HRT users (Million Women Study Collaborators, 2003).

1.4.3 Known side effects

There are a number of documented side effects associated with NET-En, although very much less information is available on the metabolic effects of NET-En than on those of DMPA. When given alone at contraceptive doses all progestogens (irrespective of their mechanism of action) cause some disruption of menstrual bleeding patterns and this is maximal with injectable, long-acting progestogens, including NET-En (Benagiano *et al.*, 2000). Swenson *et al.* (1980) conducted a randomized, single blind comparative trial between norethindrone enanthate and DMPA in the Model Clinic, Decca, Bangladesh, to determine possible differences in reported side effects, reasons for discontinuation and discontinuation rates of these two injectable contraceptives. Their data shows that on all follow-up visits the proportion of women reporting irregular bleeding was consistently higher for the NET-En clients compared to those receiving DMPA.

In a phase III clinical study by Bassol *et al.* (2000) among Latin American women the discontinuation rates for bleeding problems and amenorrhea were 5.1% and 1.1% respectively. They also found that Columbian women had a marked increase in bleeding problems compared to other countries. From the available data it is estimated that between 10% and 25% of women will experience amenorrhoea of greater than 3 months duration at the end of one year of treatment (Fraser, 1981; WHO Expanded

Programme of Research, Development and Research Training in Human Reproduction, 1978; Kessler-Koos *et al.*, 1973).

A study by Smit *et al.* (2002) reported on the common experience of vaginal wetness amongst South African users of progestogen-only injectable contraceptives. They interviewed 848 women aged 15-49 years of which 22% were currently using injectable contraception in the form of either DMPA or NET-En (Nur-Isterate®). Amenorrhea was the most common side effect (62.5%), followed by vaginal wetness (18.4%) which was also what 17.5% of the women liked least about using this method. In a WHO study (WHO Expanded Programme of Research, Development and Research Training in Human Reproduction, 1977) headaches were reported by 6% of women at some time during the first year, while Giwa-Osagie *et al.* (1978) noted an increase in body weight in less than 4%.



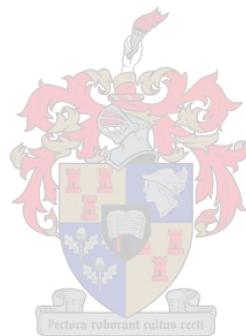
Rugarn *et al.* (2001) investigated the effects of progesterone and NET on regional immune-like reactivity of neuropeptide Y (NPY), substance P (SP), neurokinin A (NKA) and neurotensin (NT) in brains of female ovariectomized estradiol-substituted rats. They concluded that the effects of NET on SP-and NKA-like immunoreactivity in the frontal cortex contributed to the mood effects associated with this progestogen in clinical use. There appears to be no association between NET-En and change in blood pressure (Giwa-Osagie *et al.*, 1978). As mentioned previously, NET-En has no substantial effect on blood clotting factors, carbohydrate or lipid metabolism or hepatic function. Several side effects were also noted in the hormonal male contraceptive study of Kamischke *et al.* (2000). These reversible side effects included an increase in body

weight, erythrocytes, hemoglobin, and hematocrit, and a decrease in HDL-cholesterol and alkaline phosphatase.

Wonodirekso *et al.* (2000) investigated the structural changes caused by NET-En in the endometrial surface epithelium and subepithelial collagen III fibres. They assessed endometrial biopsies from NET-En users and controls during routine haematoxylin- and eosin staining, immunohistochemical staining for cytokeratins 8, 18 and 19, and collagen III and found most of the NET-En tissues to be of reduced surface epithelial cell height compared to controls. NET-En has been involved in the controversy of whether it may induce tumors. Neumann *et al.* (1974) demonstrated that it caused breast and pituitary nodules in rats. However, NET-En has strong oestrogenic effects in rats which are not seen in most species, including humans, therefore explaining the findings of Neumann and co-workers. An interesting study was done by Mildvan *et al.* (2002) on possible interaction between nevirapine, a non-nucleoside reverse transcriptase inhibitor of HIV-1 and P450 inducer, and ethinyl estradiol (EE) / norethindrone (NET), a widely used oral contraceptive. They found that concomitant administration of nevirapine at steady state with the oral contraceptive resulted in a significant (29%) median reduction in the area under the plasma concentration time curve and a significant reduction in mean residence time and half-life of EE. There was also a significant (18%) median reduction in the area under the plasma concentration time curve for NET that was not associated with a detectable change in NET C(max), mean residence time, or half-life. They concluded that oral contraceptives should not be the primary method of birth control in women of child-bearing potential who are

treated with nevirapine. From their results and conclusion the question can also be raised about the possible influence of the injectable contraceptives.

With this comprehensive literature review as background, the aim of our study became to investigate the effects the synthetic progestins, MPA and NET, had on various immunological parameters in an *in vitro* model. What follows in the next chapters is the relevant research conducted by us.



CHAPTER 2

AN INVESTIGATION OF THE EFFECTS OF MPA AND NET-A ON THE INFLAMMATORY MARKERS, IL-6 AND TNF- α .

Abstract

Aim: This study was conducted to determine whether MPA and NET-A displayed any pro-inflammatory and/or anti-inflammatory properties compared to that of the known anti-inflammatory agent, Dex.

Methods: The effects these three synthetic hormones had on monocytes *in vitro* were determined by measuring the release of two of the major pro-inflammatory cytokines, IL-6 and TNF- α , by means of ELISA.

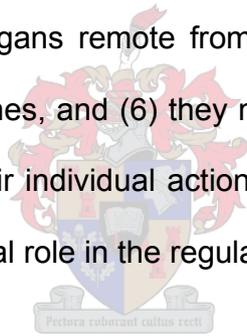
Results: Dex significantly inhibited IL-6 release from sub-optimally as well as optimally activated monocytes in a dose dependent manner. Neither MPA nor NET-A had a significant effect on sub-optimally activated monocytes. MPA and NET-A were able to significantly reduce IL-6 release from optimally activated monocytes in a combined gender group. The inhibitory effect of MPA on optimally activated monocytes was more pronounced in male donors than in female donors. While NET-A had no significant effect on optimally activated monocytes in the female group, it significantly reduced IL-6 release in the male group.

Conclusion: Dex significantly reduced TNF- α release from moderately as well as optimally activated monocytes. MPA had a more pronounced effect on optimally activated monocytes than on moderately activated monocytes. NET-A displayed a much weaker effect on TNF- α release than the other two compounds. Collectively, we were able to demonstrate various differences amongst the compounds, their effectiveness as well as differences amongst genders.

[KEY WORDS: MPA, NET-A, Dex, IL-6, TNF- α , contraceptives]

2.1 Introduction

Cytokines are small-molecular-weight soluble factors that are released by cells (cyto-) to communicate with and influence the functions (-kines) of other cells through the interaction with specific surface receptors. Cytokines typically have a molecular mass of 15-20 kDa. There are several main characteristics of cytokines, namely, (1) they possess a pleiotropic property where each cytokine has many different effects on different cells, (2) they possess an autocrine function (that serves as a feedback mechanism) thereby acting directly on the cell that released it, (3) they have a paracrine effect on cells immediately around them, (4) they may act like hormones and have endocrine effects on cells and organs remote from the site of release, (5) they may induce the release of other cytokines, and (6) they may act synergistically to achieve a greater effect than the sum of their individual actions. The expression of cytokines by immune system cells plays a crucial role in the regulation of the immune response.

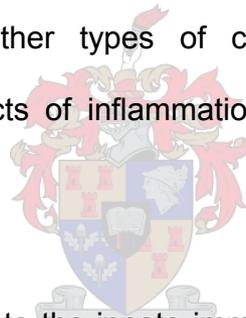


Interleukins can be described as factors that are released by white blood cells and which subsequently act on white blood cells. IL-6, a glycoprotein of around 21-28 kDa, is a member of the cytokine family and functions in both innate and adaptive immunity. It is secreted mainly by mononuclear-phagocyte system cells, vascular endothelial cells, fibroblasts and T- and B lymphocytes (Akira *et al.*, 1990). IL-6 is not normally produced constitutively in normal cells, but its synthesis and secretion is rapidly induced by bacteria (Blanchard *et al.*, 1991; Van Damme *et al.*, 1989; Zhang *et al.*, 1994), viruses (Gessani *et al.*, 1997; Van Damme *et al.*, 1989; 30), bacterial products (Van Damme *et al.*, 1989; Zhang *et al.*, 1994), cytokines (May *et al.*, 1988; Fujisawa *et al.*, 1997),

cytokine receptors (Hess *et al.*, 1995) and chemicals/biologicals that induce inflammatory reactions (Dentener *et al.*, 1993). Binding of specific nuclear factors to the 5' flanking promoter region of the IL-6 gene mediates the induction of IL-6. Among these nuclear factors are NF- κ B (Okada *et al.*, 1997) and nuclear factor IL-6 (Zhu *et al.*, 1996). The human IL-6 cDNA transcribes a 212 amino acid precursor protein containing two potential N-glycosylation sites (Hirano *et al.*, 1986). A hydrophobic 28 amino acid signal peptide is cleaved off from the precursor protein (Hirano *et al.*, 1986) to produce the functional form of IL-6 which is a homodimer with subunit forming a four- α -helical globular domain.

IL-6 exerts its activity by binding to specific receptors on cells. The receptor for IL-6 consists of a cytokine-binding protein, called IL-6R α , and a signal-transducing subunit, called gp130, both of which belongs to the type 1 cytokine receptor family (Hibi *et al.*, 1996). The association of IL-6R α and gp130 is induced by the binding of IL-6 to IL-6R α (Taga *et al.*, 1989). The signal-transducing subunit (gp130) activates a JAK/STAT signalling pathway and is also the signalling component of other cytokine receptors. JAK/STAT pathways are activated by several cytokine receptors that do not possess intrinsic kinase activities. Binding of a cytokine causes its receptor to dimerize, thereby providing the signal to associate with and activate a JAK kinase which in turn phosphorylates transcription factors called STATs. The JAK/STAT pathway is much simpler than other signal transduction pathways which consist of a large number of components.

IL-6 is a multi-functional cytokine that is involved in a number of activities. Early in infection, metabolism is switched to fighting the microorganism. Inflammation is basically a protective response aimed to eradicate the initial cause of injury to cells by e.g. microbes, toxins and the necrotic cells and tissues arising as a result of such cellular injury. The purpose of the inflammatory response is to dilute, neutralize or remove the threatening agent and initiate the process of recovery or repair. Pyrogens and inflammatory agents induce monocytes to secrete IL-6, which in turn initiates the APR by inducing hepatocytes to synthesize acute-phase proteins like CRP, clotting as well as complement factors. IL-6 knockout mice have been used to confirm the central role of IL-6 in mediating the APR (Kopf *et al.*, 1994). The APR is often used to distinguish inflammation from other types of clinical problems. Therefore, by contributing to the systemic effects of inflammation, IL-6 plays a key role in innate immunity.



The effect of IL-6 is not exclusive to the innate immune system, but it also plays a big role in adaptive immunity. IL-6 has a potent effect on the differentiation of B cells and induces mitogen-activated B cells to produce IgM, IgG and IgA (Keller *et al.*, 1996). IL-6 causes the proliferation of T cells and thymocytes by inducing IL-2 production and IL-2 receptor expression in these cells (Feghali & Wright, 1997). In addition to stimulating natural killer (NK) cell activity (Akira *et al.*, 1990), IL-6 also induces the production of pro-inflammatory cytokines, by macrophages and endothelial cells, and ICAMs, by endothelial cells (Mantovani, 1997). It has also been shown that IL-6 levels are a good indication of disease outcome and bacterial infection (Helfgott *et al.*, 1989; Yoon *et al.*, 1995; Romero *et al.*, 1993).

TNF- α is a 25kDa cytokine secreted mainly by activated mononuclear phagocytes, antigen-stimulated T cells, NK cells and mast cells. It serves as a principal mediator of the acute inflammatory response to bacteria and other infectious microorganisms and is responsible for many of the systemic complications associated with severe infection. Furthermore, it may also play a role in other aspects of immune pathology. The lipopolysaccharide (LPS) of bacterial cell walls is the most potent inducer of TNF- α production by macrophages, therefore large amounts of this cytokine may be produced in infections by gram-negative bacteria. The release of TNF- α is also augmented by IFN- γ released by T and NK cells during an immune response.

TNF is synthesized in mononuclear phagocytes as a non-glycosylated type II membrane protein consisting of an intracellular amino terminus and a large extracellular carboxyl terminus. There are two types of TNF molecules. Membrane TNF is expressed as a homotrimer, while a membrane-associated metalloproteinase cleaves this protein to release a 17kDa polypeptide of which three polymerase to form the 51kDa circulating TNF protein. The TNF receptors form part of a large family of proteins of which many are involved in inflammatory and immune responses. There are two distinctly different TNF receptors based on their molecular size. The 55kDa receptor is known as type I TNF receptor (TNF-RI) and is also called p55, while the 75kDa receptor is known as TNF-RII or p75. Binding of membrane-associated TNF is restricted to the type II receptor (TNF-RII). Cytokine binding to TNF-RII leads to the recruitment of proteins (TNF receptor-associated factors) to the cytoplasmic domains of the receptors, thereby activating transcription factors. These transcription factors

include NF- κ B and activation protein-1 (AP-1). TNF-RI signalling is also associated with activation of transcription factors.

Besides being one of the foremost mediators of inflammatory responses, TNF also plays a key role in bridging the innate and adaptive immune systems. Local release of TNF causes a range of activities that are important in the immune responses to bacteria and viruses. These activities include: (1) stimulation of the recruitment of neutrophils and monocytes to sites of infection, (2) activation of neutrophils and macrophages to eradicate microbes, (3) enhancement of cytokine release by mononuclear-phagocyte system cells, (4) amplification of the expression of class I major histocompatibility (MHC) molecules to enhance presentation of viral peptides in intracellular infections, and (5) with the presence of IFN- γ induce the expression of class II MHC molecules. TNF enhances cell migration through several actions on vascular endothelial cells and leukocytes. First, TNF causes up-regulation of adhesion molecules on vascular endothelial cells thereby making them more adhesive for neutrophils, monocytes and lymphocytes. Secondly, TNF induces leukocyte chemotaxis and recruitment by stimulating endothelial cells to secrete chemokines. Furthermore, mononuclear-phagocytes are stimulated by TNF to secrete IL-1, which almost has the same functions as TNF. The actions of TNF are noticeably crucial for local inflammatory responses and if there are inadequate quantities of TNF present, ineffective containment of infection may occur.

TNF is also a major factor of the APR and plays a major role in the shock associated with gram-negative bacterial sepsis. Local inflammatory reactions that are injurious to

the host, like autoimmune diseases, can also be attributed to the involvement of TNF. Systemic clinical and pathologic abnormalities are caused in severe infections where TNF is produced in large amounts. This occurs when the amount of cytokine produced is so large that it enters the blood stream and acts as an endocrine hormone at distant sites. Some of the systemic effects of TNF include stimulation of the hypothalamus to induce fever, working synergistically with IL-1 and IL-6 to stimulate the release of proteins from hepatocytes which amounts to the APR, decrease in blood pressure due to myocardial contractility and inhibition of vascular smooth muscle tone, and intravascular thrombosis.

It is well documented that GCs suppress cytokine production (Munck & Náray Fejes Tóth, 1994; Chrousos, 1995; De Rijk & Berkenbosch, 1994). Not only do GCs inhibit cytokine production, but they have also been shown to promote the production and release of IL-6 and TNF- α (Liao *et al.*, 1995). It has been shown that Dex inhibited LPS-induced, as well as enterotoxin-induced IL-6 and TNF- α production in peripheral leukocytes (Ebrecht *et al.*, 2000; Lanza *et al.*, 1999; Krakauer, 1995). TNF-induced IL-6 gene expression is also repressed by GCs (Vanden Berghe *et al.*, 1999; De Bosscher *et al.*, 1997), while this repression might be the result of both transcriptional and post-transcriptional events (Vanden Berghe *et al.*, 1999; Chen *et al.*, 1998; Swantek *et al.*, 1997). A marked decrease in serum IL-6 levels was found in patients with metastatic breast carcinoma treated with MPA (Yamashita *et al.*, 1996). This decrease correlated closely with the plasma levels of MPA. Studies on hormone replacement therapy demonstrated that MPA significantly reduced TNF- α levels (Koh *et al.*, 2002), as well as CRP and serum amyloid A protein (Wakatsuki *et al.*, 2002), thereby indicating that MPA

might have an anti-inflammatory effect. Taking the various effects of GCs into consideration, the aim of this study was to evaluate the effects of the synthetic steroids, MPA and NET-A, on IL-6 and TNF- α production *in vitro*.

2.2 Materials and Methods

2.2.1 Influence on IL-6 production by monocytes

2.2.1.1 Study design

Blood samples were collected from 10 healthy individuals, 5 females (between 20-34 yr of age) and 5 males (between 20-40 yr of age), who were not on any hormonal treatment, including any form of contraception. Medroxyprogesterone acetate (Sigma, Sigma Aldrich), Norethisterone acetate (Sigma, Sigma Aldrich) and Dexamethasone (Sigma, Sigma Aldrich) were used at 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} and 10^{-10} M final concentrations. A 70% ethanol solution was used to dissolve as well as dilute all three steroids. These concentrations were chosen to represent different physiologic states these steroids are found in, where higher concentrations e.g. 10^{-7} M to 10^{-9} M are found in hormone replacement therapy (Adams *et al.*, 1997; Stanczyk, 2003) and lower concentrations e.g. 10^{-8} M and 10^{-9} M are reached in hormonal contraception (Mishell, 1996; Zalanyi *et al.*, 1984; Smit *et al.*, 2004).

2.2.1.2 Preparation and incubation of samples

The following were added into 96 well flat bottom cell culture plates (Costar, Corning Incorporated):

1. 50µL whole heparinized blood per well.
2. Three sets of each steroid (MPA, NET-A or Dex) at six different final concentrations (10^{-5} to 10^{-10} M).
3. Each well was filled up to a final volume of 250µL with Roswell Park Memorial Institute medium 1640 (RPMI 1640) (Bio Whittaker, Sterilab).

Two positive (stimulated) and two negative (non-stimulated) control wells were also set up in the same way previously mentioned with an equal volume (2.5 µL) of a 70% ethanol solution to compensate for the 70% ethanol solution used to dilute the steroids. The cell culture plate was then incubated at 37°C and 98% humidity in a CO₂ incubator (NuAir Water-Jacketed Incubator, Centrotec) for 40 minutes. After this incubation, one set of cells containing the different concentrations of each steroid was stimulated with 10µg/mL LPS (Sigma, Sigma Aldrich) and another set of cells, also containing different concentrations of each steroid, was stimulated with 100µg/mL LPS. The two positive controls wells were also stimulated with 10µg/mL and 100µg/mL LPS respectively. The remaining sets of each steroid represented the effect the steroids would have on IL-6 production without any stimuli. The plate was then incubated for 4 hours at 37°C and 98% humidity in a CO₂ incubator. The plate was then centrifuged for 5 minutes at 2000 rpm and enough supernatant was drawn off from each well to use 50µL in duplicate in an IL-6 ELISA. The samples were kept overnight at -20°C.

2.2.1.3 IL-6 ELISA

An in-house IL-6 ELISA kit was used and the assays were carried out in 96 well Maxisorp plates (Nunc, AEC Amersham). The wells were coated with 100 μ L a 1:500 dilution (in PBS) of rabbit anti-IL6 (Sigma Aldrich) and incubated sealed overnight at ambient temperature. The coating antibody was aspirated and the wells were blocked with 200 μ L of 0.1% human serum albumin (HSA) (Albumin Bovine, Sigma) in phosphate-buffered saline (PBS) and incubated for 30 minutes at ambient temperature, after which the plates were washed once with PBS using an autowasher (Denley Wellwash 4, Alltech Medical Services).

The standards were set up with a final volume of 50 μ L/well in duplicate as follow: A five point 2-fold serial dilution (diluted with 10% HSA in PBS) of human recombinant IL-6 (Sigma Aldrich) with a high standard of 1000 pg/mL was used. The blanks were filled with 50 μ L of 10% HSA in PBS. Fifty (50) μ L of each sample was loaded in duplicate on the plates. Each plate had its own standards in duplicate. 50 μ L of biotinylated monoclonal IL-6 (Mouse anti human interleukin 6: Biotin, Serotec, Separations) was added to all the wells. The concentration of the biotinylated monoclonal anti-IL6 used was 0.5 μ g/mL diluted with 0.1 % HSA-PBS solution.

The plates were then incubated at ambient temperature for 2 hours on a plate shaker (New Brunswick Scientific CO.) at 200 rpm. After the incubation period the plates were washed 3 times with an autowasher using PBS, whereafter a 100 μ L of Streptavidin-horseradish peroxidase (HRP) (Sterilab) was added to all the wells. After 20 minutes of incubation at ambient temperature, the plates were washed 4 times with an autowasher

and PBS. BM Blue POD Substrate (Roche Diagnostics) was added at 100 μ L/well and the plates were incubated at ambient temperature in the dark for approximately 10 to 15 minutes or until a light blue colourification occurred. The chromogenic reaction was stopped by adding 50 μ L 2M H₂SO₄ to each well. The plates were read on a microplatereader (Organon Teknika Microwell system, Alltech Medical Services) at an optical density of 450nm and a reference wavelength of 690nm using Organon Teknika MIMS software. Sterile techniques and materials were used throughout the whole experiment and the experiments were performed on the blood of 5 female donors and 5 male donors.

2.2.2 Influence on TNF- α production by monocytes

2.2.2.1 Study design

Blood samples were collected from 10 healthy individuals, 5 females (between 20-34 yr of age) and 5 males (between 20-40 yr of age), who were not on any hormonal treatment, including any form of contraception. Medroxyprogesterone acetate (Sigma, Sigma Aldrich), Norethisterone acetate (Sigma, Sigma Aldrich) and Dexamethasone (Sigma, Sigma Aldrich) were used at 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹ and 10⁻¹⁰ M final concentrations. A 70% ethanol solution was used to dissolve as well as dilute all three steroids. These concentrations were chosen to represent different physiologic states these steroids are found in, where higher concentrations e.g. 10⁻⁷ M to 10⁻⁹ M are found in hormone replacement therapy (Adams *et al.*, 1997; Stanczyk, 2003) and lower

concentrations e.g. 10^{-8} M and 10^{-9} M are reached in hormonal contraception (Mishell, 1996; Zalanyi *et al.*, 1984; Smit *et al.*, 2004).

2.2.2.2 Preparation and incubation of samples

The following were added into a 96 well flat bottom cell culture plate (Costar, Corning Incorporated):

1. 100 μ L whole heparinized blood per well.
2. Three sets of each steroid (MPA, NET-A or Dex) at six different concentrations (10^{-5} to 10^{-10} M).
3. Each well was filled up to a final volume of 250 μ L with RPMI 1640 (Bio Whittaker, Sterilab)

Two positive (stimulated) and two negative (non-stimulated) control wells were also set up with an equal volume (2.5 μ L), as the volume of steroids added, of a 70% ethanol solution added to compensate for the 70% ethanol solution used to dilute the steroids. The cell culture plate was then incubated at 37°C and 98% humidity in a CO₂ incubator (NuAir CO₂ Water-Jacketed incubator, Centrotec) for 40 minutes. After this incubation, one set of cells containing the different concentrations of each steroid was stimulated with 50 μ g/mL LPS (Sigma) and another set of cells, also containing different concentrations of each steroid, was stimulated with 100 μ g/mL LPS. The two positive control wells were also stimulated with 50 μ g/mL and 100 μ g/mL LPS respectively. The remaining set of concentrations of each steroid represented the effect the steroids would have on TNF- α production without any stimuli. The plate was then incubated for

6 hours at 37°C and 98% humidity in a CO₂ incubator. The plate was then centrifuged for 5 minutes at 2000 rpm and 120µL supernatant of each well was drawn off and double diluted with RPMI 1640. The samples were kept overnight in eppendorf tubes at -20°C.

2.2.2.3 TNF-α ELISA

The human TNF-α DuoSet[®] ELISA Development kit by R&D Systems (Whitehead Scientific, SA) was used to assay the samples.

Ninety-six (96) well plates (Nunc-Immunoplate, Maxisorp, AEC Amersham) were coated with 100µL of a 4µg/mL in PBS working concentration of the capture antibody and incubated sealed overnight at ambient temperature. The plates were then washed three times with wash buffer (0.05% Tween[®] 20 in PBS, pH 7.2-7.4) using an autowasher (Denley Wellwash 4, Alltech Medical Services). Any remaining wash buffer was removed after the wash by inverting the plate and blotting it against clean paper towels. The plates were blocked by adding 200µL of block buffer (1% HSA, 5% Sucrose, and 0.05% NaN₃ in PBS) and incubating for a minimum of 1 hour at room temperature. After the blocking procedure the plates were washed again as described previously with wash buffer.

The standards were set up with a final volume of 100µL/well in duplicate as follow: A seven point 2-fold serial dilution [diluted with reagent diluent – 0.1% HSA, 0.05% Tween 20 in Tris-buffered saline (20mM Trizma base, 150mM NaCl), pH 7.2 – 7.4, 0.2µm filtered] of the standard with a high standard of 1000pg/mL was used. The blanks were filled with 100µL of reagent diluent per well. One-hundred (100) µL of each sample was

loaded in duplicate on the plates and the plates were incubated sealed for 2 hours at room temperature. Thereafter the plates were washed as previously described and 100µL of detection antibody, with a working concentration of 300ng/mL diluted with reagent diluent, was added to each well. The plates were sealed and incubated for another 2 hours at room temperature, where after the plates were washed again as previously described.

One hundred (100) µL of the working dilution of Streptavidin-HRP (1:200 dilution in reagent diluent) was added to each well and the plates were incubated in the dark for 20 minutes at room temperature. The plates were washed again as described previously and 100µL of substrate solution (BM Blue Pod substrate, Roche Diagnostics) was added to each well. The plates were incubated in the dark at room temperature for 10-15 minutes or until a slight light-blue colourification occurred. The reaction was stopped by adding 50µL of stop solution (2M H₂SO₄) to each well and gently tapping the plates to ensure thorough mixing. The plates were read directly after the reaction was stopped on a microplate reader (Organon Teknika Microwell system, Alltech Medical Services) at an optical density of 450nm and a reference wavelength of 690nm using Organon Teknika MIMS software. Sterile techniques and materials were used throughout the whole experiment and the experiments were performed on the blood of 5 female donors and 5 male donors.

2.2.3 Data analysis

Analysis of datasets were achieved by repeated measures ANOVA followed by post testing using Dunnett's test. Post testing was only done when there was significant

pairing ($p < 0.05$) among the groups. Analysis was done on the combined male and female groups ($n=10$), as well as on the male and female groups separately ($n=5$) in case the biological effect was more pronounced on a particular gender. All results were considered statistically significant when $p < 0.05$.

2.3 Results

Dex is well known as an anti-inflammatory agent. Medroxyprogesterone acetate has been shown to possess certain GC-like properties as previously discussed in the literature review. Although NET has been shown to display certain immunosuppressive properties, it is still uncertain whether it has any GC-like properties. The hypothesis that MPA and NET-A could have anti-inflammatory capabilities was tested by determining the effects MPA and NET-A had on inflammatory cytokines and by using the effects Dex had on these cytokines as a guideline. The effects that MPA, NET-A and Dex had on the cytokines, IL-6 and TNF- α , were measured by ELISA. These cytokines play a vital role in the acute-phase response to inflammatory episodes by inducing the liver to synthesize plasma proteins that are involved in acute-phase reactions, such as clotting factors, complement factors and CRP. Initial experiments indicated that there might be significant discrepancies between genders; therefore statistical analysis was done on the group as a whole as well as separately on the male donor group and female donor group.

We are aware that our assays have been done on whole blood which contains a variety of different white blood cells. For this reason we used the monocyte-specific stimulus, LPS. Monocytes are the only cells which express CD14, the ligand for LPS. Binding of LPS leads to the activation of monocytes, allowing us to conclude that the release of both IL-6 and TNF- α is monocyte-derived. The possibility that any other cell can influence the monocytes can also be excluded. For any other blood cells to influence the monocytes in the *in vitro* assay would require that the cells be activated. Due to the fact that the cells are resting, the release of any inhibitory factor from the non-monocyte fraction of the whole blood is not possible (even though they may have bound steroids, e.g. Dex).

2.3.1 Influence on Interleukin-6

The production of IL-6 *in vitro* was achieved by using two different concentrations of the stimulus, LPS. The concentrations we used were 10 μ g/mL and 100 μ g/mL LPS. These two concentrations of LPS were chosen because they represent a mild infection state (10 μ g/mL) and a state of severe infection (100 μ g/mL) e.g. septicaemia. Since our assays were done on whole blood samples, we specifically used LPS for the reason that it only targets monocytes. The following percentages of inhibition were found in each group when the different samples were stimulated with a 100 μ g/mL LPS. All percentages were calculated using average values.

The presence of MPA, NET-A as well as Dex significantly influenced the production of IL-6 *in vitro* in the combined gender groups. MPA showed a very significant ($p < 0.01$)

inhibition of IL-6 production at all concentrations tested (Fig. 7). The highest inhibition of 59.4% occurred at 10^{-6} M MPA and the lowest of 35.9% at 10^{-5} M MPA.

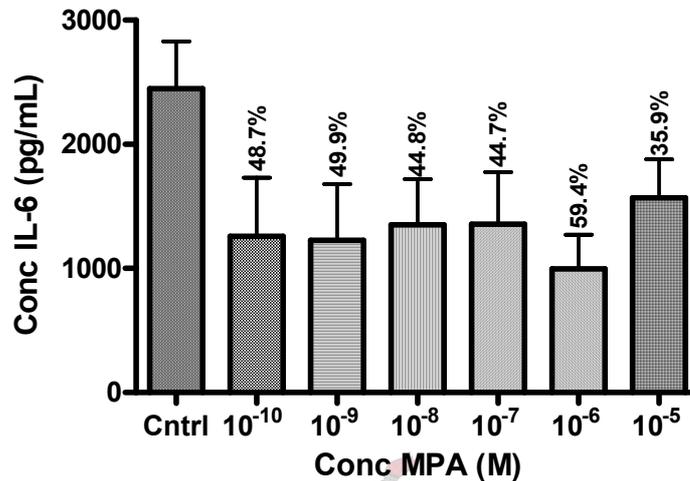


Figure 7. Inhibition of IL-6 production by MPA at different concentrations found in whole blood samples stimulated with LPS (100 μ g/mL), compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

Although a general decrease occurred at all the concentrations tested, NET-A only exerted a significant effect on IL-6 production at concentration ranging from 10^{-5} to 10^{-8} M (Fig. 8). A significant average reduction of 45.8% ($p < 0.01$) was found at a concentration of 10^{-6} M, while the lowest average reduction of 32.2% ($p < 0.05$) occurred at 10^{-7} M NET-A.

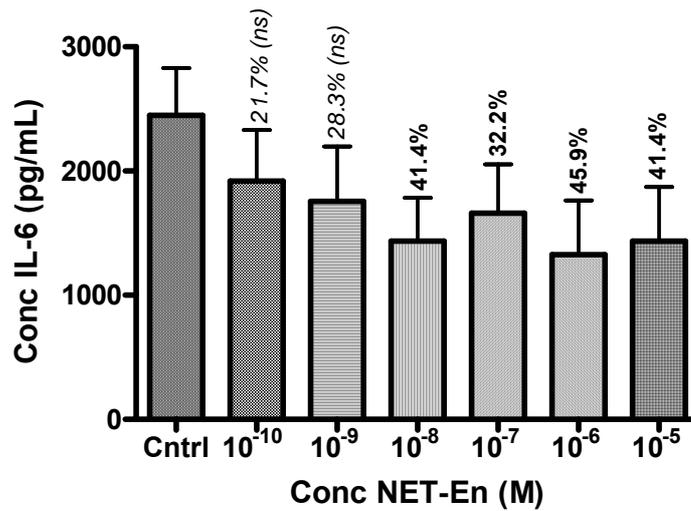


Figure 8. Inhibition of IL-6 production by NET-A at different concentrations found in whole blood samples stimulated with LPS (100µg/mL), compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.



Dex had a very significant ($p < 0.01$) inhibitory effect on IL-6 production in a dose dependent fashion at all concentrations tested, with an average inhibition ranging from 71.6% to 47.9% (Fig. 9).

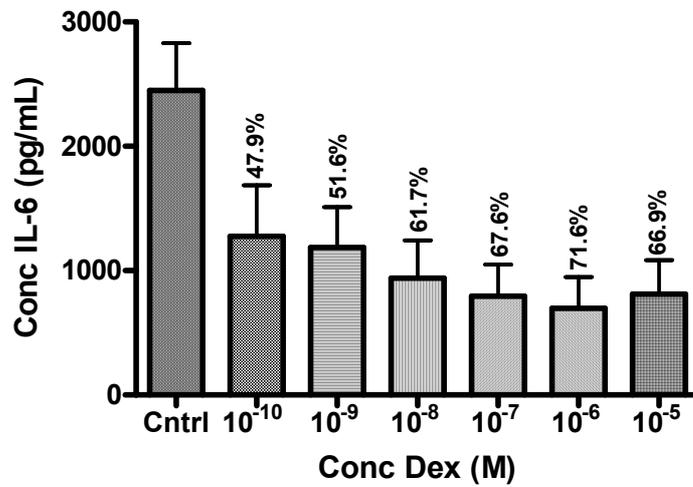


Figure 9. Inhibition of IL-6 levels by Dex at different concentrations found in whole blood samples stimulated with LPS (100µg/mL), compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

MPA caused a general decrease of IL-6 production in females throughout the concentration range. This observed reduction was however only statistically significant at concentrations of 10⁻⁶ M (61.6%), 10⁻⁹ M (52.6%) and 10⁻¹⁰ M (50.9%) (Fig. 10).

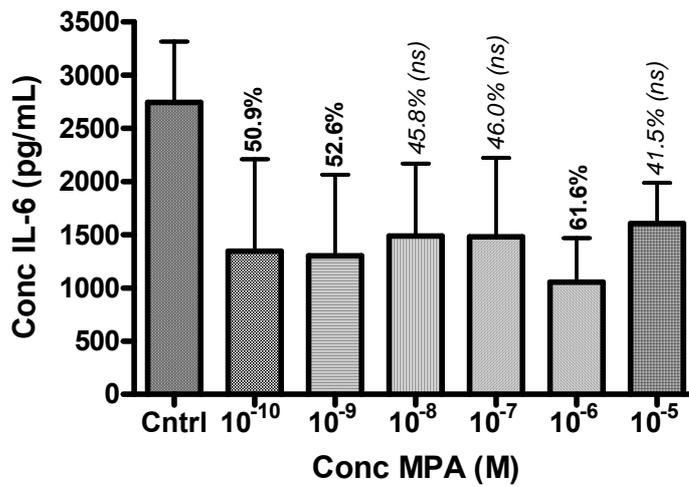


Figure 10. Inhibition of IL-6 production by MPA at different concentrations found in female donors when whole blood samples were stimulated with LPS (100µg/mL), compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.



NET-A did not have any significant influence on females when samples were stimulated with 100µg/mL LPS *in vitro* (Data not shown). Dex, on the other hand, caused a very significant ($p < 0.01$) inhibition of IL-6 production in females (Fig. 11). This effect was dose dependent and inhibition ranged from 77.1% at 10^{-6} M to 57.8% at 10^{-10} M.

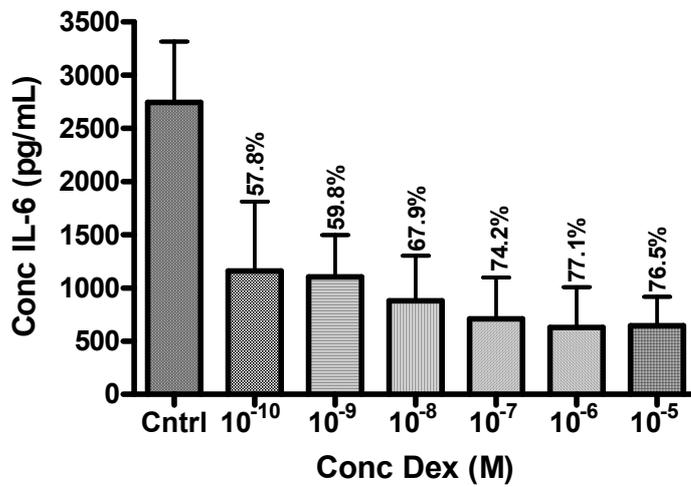


Figure 11. Inhibition of IL-6 production by Dex at different concentrations found in female donors when whole blood samples were stimulated with LPS (100µg/mL), compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant values are represented in italics.

The influence that MPA and NET-A had on males differed from the influence it had on females. This can be seen by the much more prominent inhibition MPA had on IL-6 production in males (Fig. 12). At concentrations ranging from 10⁻⁶ M to 10⁻¹⁰ M, MPA had an extremely significant (p<0.01) effect on IL-6 levels with average inhibition ranging from 56.6% at 10⁻⁶ M to 43.0% at 10⁻⁷ M.

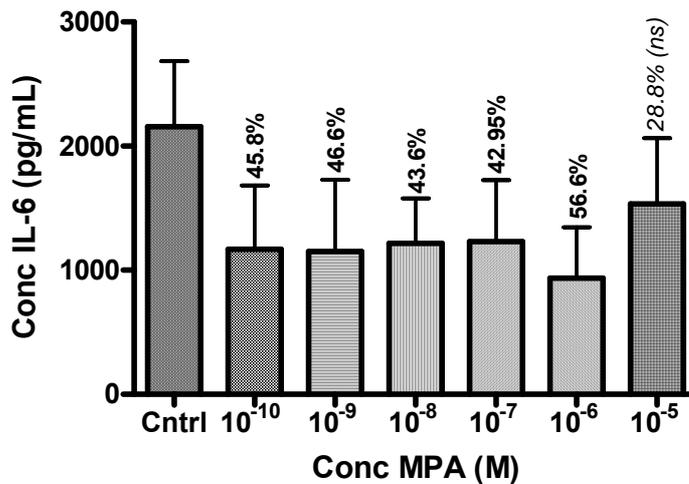
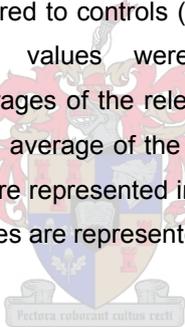


Figure 12. Inhibition of IL-6 production by MPA at different concentrations found in male donors when whole blood samples were stimulated with LPS (100µg/mL), compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.



Unlike the effect it had in females, NET-A significantly reduced IL-6 production in males at all concentrations tested with the exception of 10⁻¹⁰ M (Fig. 13). This reduction ranged from 44.8% at 10⁻⁸ M (p<0.01) to 30.0% at 10⁻⁹ M (p<0.05). Considering that NET-A had no significant effect on females, it can be concluded that the effect NET-A had on the group as a whole was exclusively carried by the effect it had on the males.

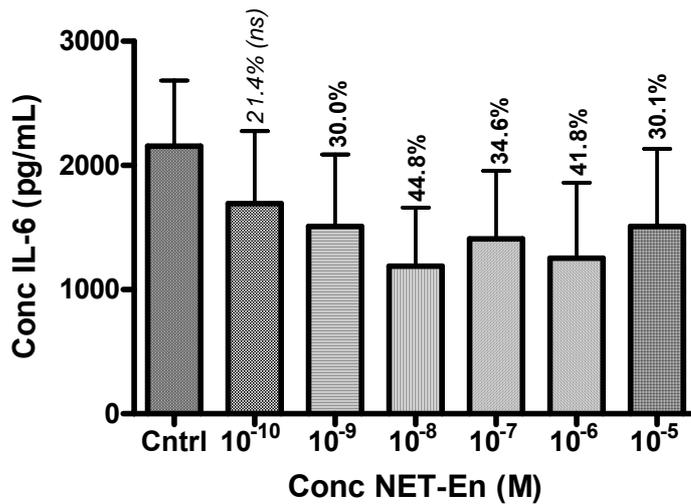
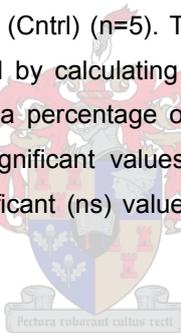


Figure 13. Inhibition of IL-6 production by NET-A at different concentrations found in male donors when whole blood samples were stimulated with LPS (100µg/mL), compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.



Dex caused a very significant ($p < 0.01$) decrease in IL-6 production amongst males in a dose dependent manner (Fig. 14). The highest reduction of 64.6% transpired at 10^{-6} M where after the effect gradually decreased to 35.3% at 10^{-10} M.

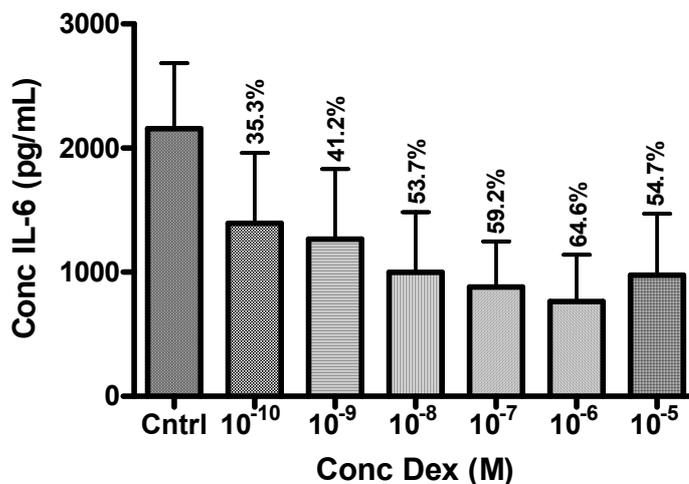
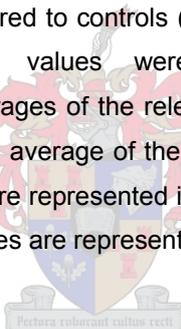


Figure 14. Inhibition of IL-6 production by Dex at different concentrations found in male donors when whole blood samples were stimulated with LPS (100µg/mL), compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.



Dex was the only compound to have a significant effect on samples when they were stimulated with 10µg/mL LPS. This was the same for the different gender groups. In the combined gender group Dex significantly inhibited the production of IL-6 in more or less a dose dependent fashion at all concentration except 10⁻¹⁰ M (Fig. 15). The observed reduction of IL-6 by Dex at 10⁻¹⁰ M was not statistically significant, although Dex clearly reduced IL-6 production at this concentration. The highest inhibition of 76.8% occurred at a concentration of 10⁻⁶ M (p<0.01) while 39.4% was the lowest at 10⁻⁹ M (p<0.01).

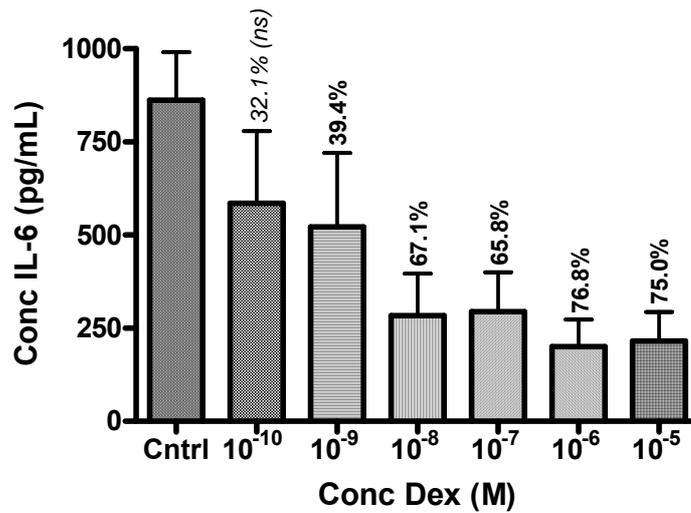


Figure 15. Inhibition of IL-6 production by Dex at different concentrations found in whole blood samples stimulated with LPS (10µg/mL), compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.



The presence of Dex had a more significant effect in females than in males when cells were stimulated with 10µg/mL LPS. In females Dex significantly reduced the amount of IL-6 in a dose dependent manner at all concentrations tested except at 10⁻¹⁰ M, which did not demonstrate statistical significance (Fig. 16). This reduction ranged from 83.4% (p<0.01) at 10⁻⁵ M to 55.1% (p<0.05) at 10⁻⁹ M.

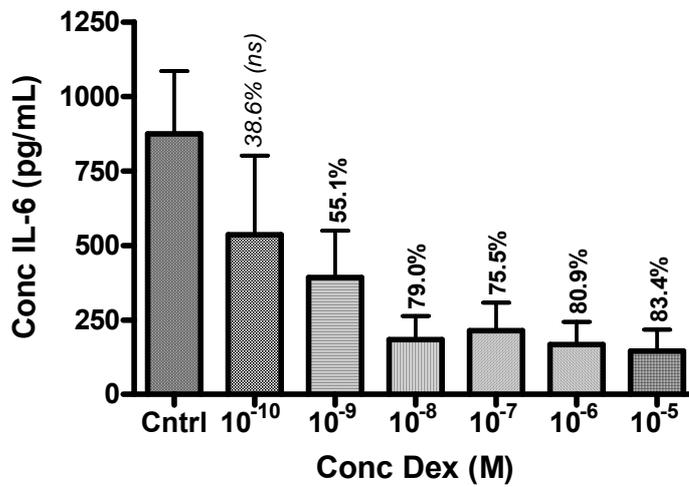
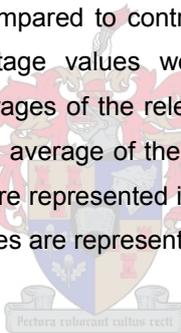


Figure 16. Inhibition of IL-6 production by Dex at different concentrations found in female donors when whole blood samples were stimulated with LPS (10µg/mL), compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.



The production of IL-6 was much less affected by Dex in males, with Dex only having a significant ($p < 0.05$) influence at 10^{-5} M (66.4%) and 10^{-6} M (72.6%) (Fig. 17). The values obtained from Dex at the lower concentrations were not statistically significant, although these concentrations displayed a visible inhibiting effect on IL-6 production.

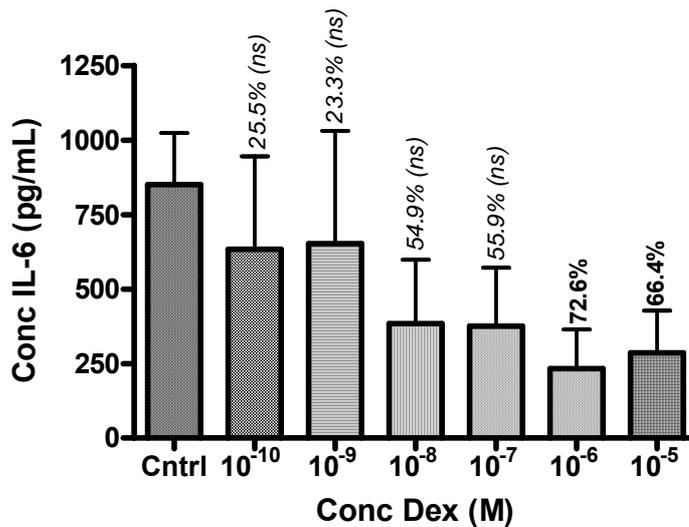


Figure 17. Inhibition of IL-6 production by Dex at different concentrations found in male donors when whole blood samples were stimulated with LPS (10µg/mL), compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

With one exception, none of the three steroids significantly affected the basal levels of IL-6 in any of the non-activated cultures of the combined gender groups as well as the gender groups. The only significant effect occurred at a concentration of 10⁻⁷ M NET-A in the combined gender group, where the presence of NET-A caused a 102.5% (p<0.05) increase in the amount of IL-6 in the supernatants of cells (Fig. 18). Although none of the other concentrations of NET-A produced any significant results, it can clearly be seen that NET-A exhibits a tendency to act as a pro-inflammatory factor by enhancing the basal IL-6 levels in donors.

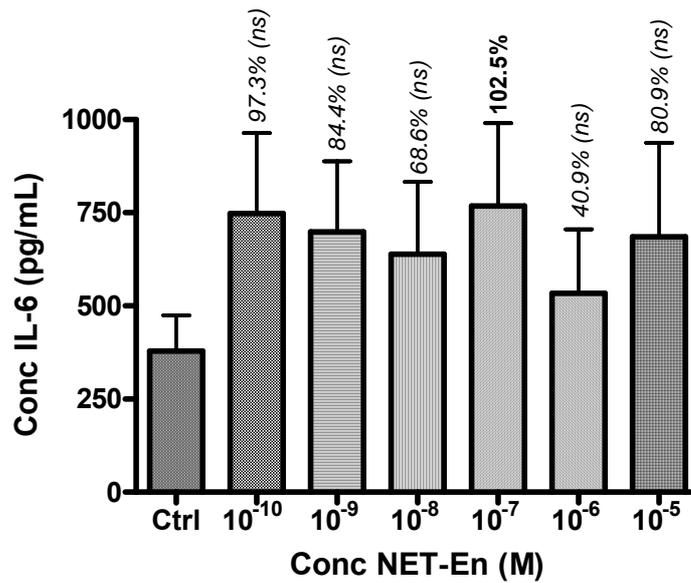


Figure 18. Effects on the basal levels of IL-6 levels in whole blood samples treated with different concentrations of NET-A, compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

2.3.2 Influence on TNF- α

The production of TNF- α *in vitro* was achieved by stimulating whole blood samples with two different concentrations of the stimulus, LPS. Whole blood samples were stimulated *in vitro* with 50 μ g/mL and 100 μ g/mL LPS, as mentioned previously in the materials and methods section. These different concentrations of stimuli were used to represent a moderate state of infection and a severe state of infection. Since our assays were done on whole blood samples, we specifically used LPS for the reason that it only targets monocytes. All percentages were calculated using average values.

MPA and Dex affected the combined gender group stimulated with 100µg/mL LPS more prominently than NET-A. TNF-α production was significantly inhibited by MPA at concentrations of 10⁻⁶ M through to 10⁻¹⁰ M (Fig. 19), although the values do not follow a true dose-response tendency. The highest inhibition of 65.76% (p<0.01) occurred at 10⁻¹⁰ M MPA, while the least inhibition of 51.3% (p<0.01) was caused by 10⁻⁶ M MPA.

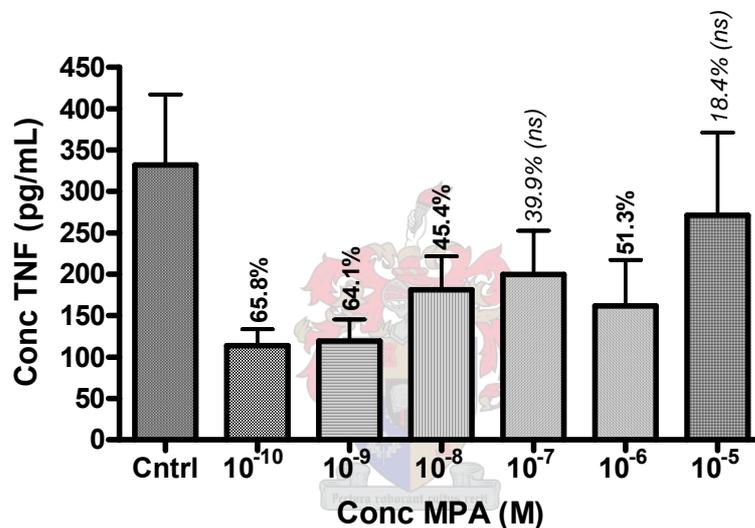


Figure 19. Inhibition of TNF-α production by MPA at different concentrations found in whole blood samples stimulated with LPS (100µg/mL), compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

The only significant reduction of TNF-α production, in the presence of NET-A, was a 60.2% (p<0.05) decrease occurring at a concentration of 10⁻⁵ M NET-A (Fig. 20).

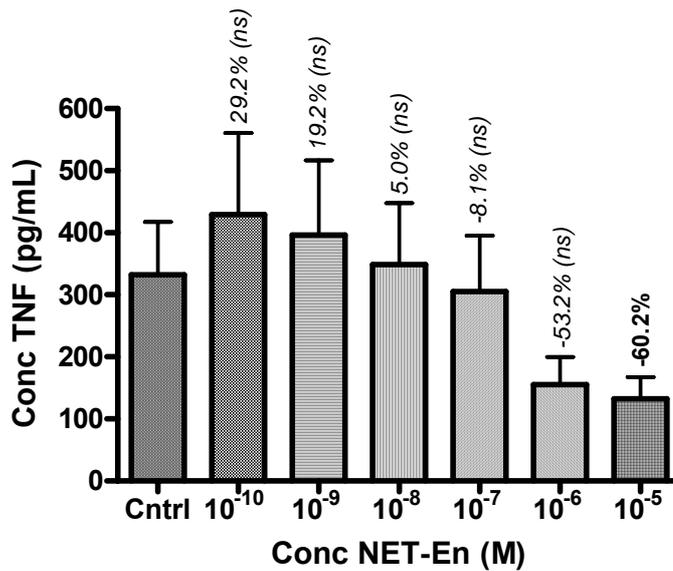


Figure 20. Inhibition of TNF- α production by NET-A at different concentrations found in whole blood samples stimulated with LPS (100 μ g/mL), compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

Dex caused a very significant ($p < 0.01$) decrease in TNF- α production in the combined gender group when stimulated with 100 μ g/mL LPS (Fig. 21). This dose dependent inhibition by Dex reached a high of 96.3% at 10^{-7} M Dex and gradually declined to 63.6% at 10^{-10} M. This clearly confirms the potency of Dex as an anti-inflammatory agent.

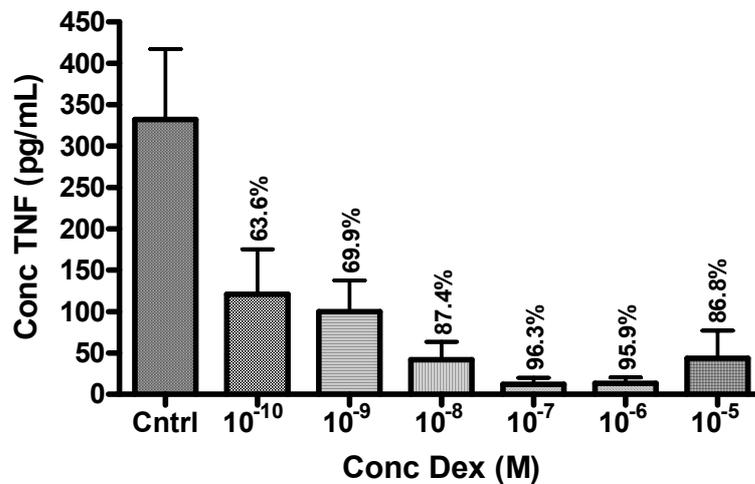


Figure 21. Inhibition of TNF- α production by Dex at different concentrations found in whole blood samples stimulated with LPS (100 μ g/mL), compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

MPA and NET-A had a more varied effect, when the data was separated according to gender, than Dex in samples stimulated with a 100 μ g/mL LPS. MPA showed a significant inhibition of TNF- α production in females at 10⁻⁶ M, 10⁻⁹ M and 10⁻¹⁰ M (Fig. 22). This inhibition ranged from 53.0% (p<0.05) at 10⁻⁶ M to 47.4% at 10⁻¹⁰ M. Although not statistically significant, MPA still had an inhibitory effect at 10⁻⁵ M, 10⁻⁷ M and 10⁻⁸ M.

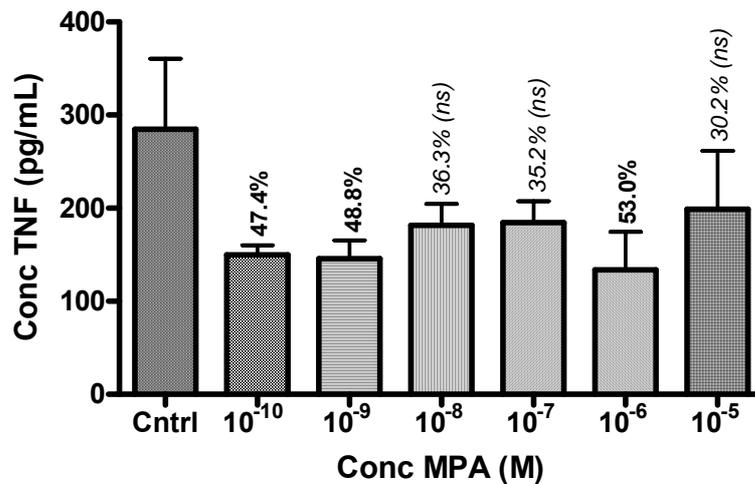


Figure 22. Inhibition of TNF- α production by MPA in female donors at different concentrations found in whole blood samples stimulated with LPS (100 μ g/mL), compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

NET-A showed no significant effect in female blood samples stimulated with 100 μ g/mL LPS (Data not shown). Dex, on the other hand, significantly inhibited TNF- α production in females in a dose dependent manner at all concentrations tested (Fig. 23). This dose response reached a maximum at a concentration of 10⁻⁷ M with a 97.2% (p<0.01) decrease in TNF- α levels, where after the effects of Dex gradually declines reaching a minimum of 63.9% (p<0.05) at the lowest concentration of 10⁻¹⁰ M.

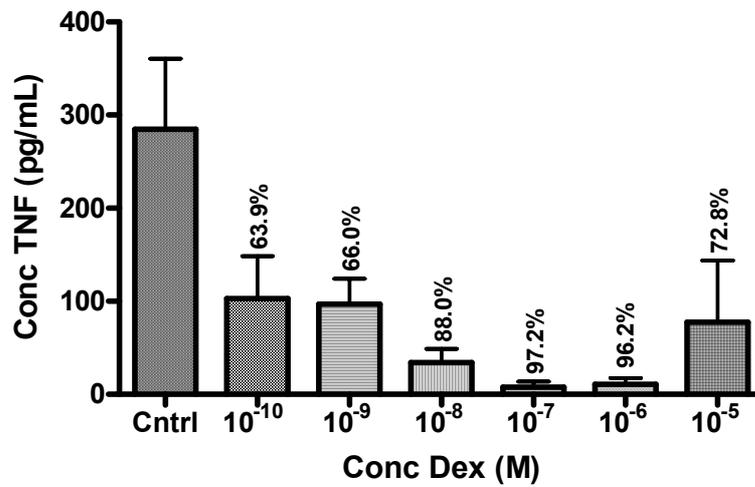


Figure 23. Inhibition of TNF- α production by Dex in female donors at different concentrations found in whole blood samples stimulated with LPS (100 μ g/mL), compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

Although a general decrease can be observed, MPA only had a statistical significant affect on TNF- α production in males at the two lowest concentrations tested, causing a 79.6% ($p < 0.05$) decrease at 10⁻¹⁰ M and a 75.5% ($p < 0.05$) decrease at 10⁻⁹ M (Fig. 24). Again, the dose-response observed does not fit into a logical/true dose-response curve.

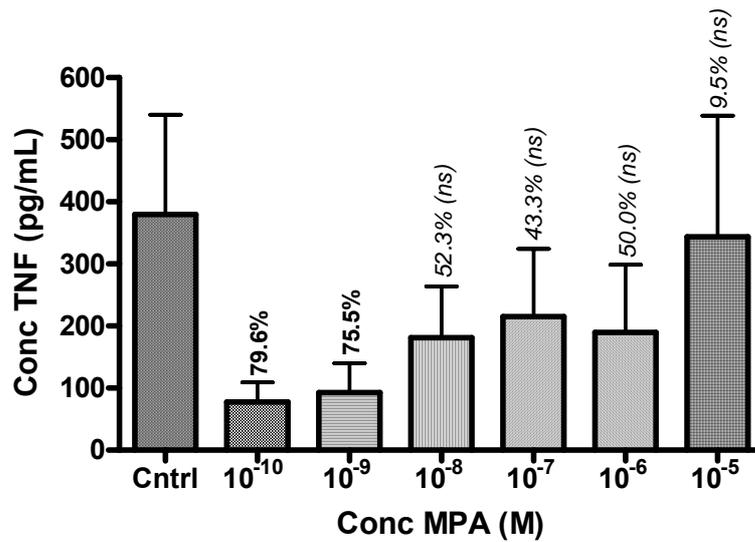


Figure 24. Inhibition of TNF- α production by MPA in male donors at different concentrations found in whole blood samples stimulated with LPS (100 μ g/mL), compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.



The influence of NET-A on males was similar to that on females, showing no significant change in TNF- α levels (Data not shown). The effects of Dex in males corresponded to that in females, considering that it significantly inhibited TNF- α production in a strict dose dependent fashion (Fig. 25). Dex caused a maximum inhibition of 97.3% ($p < 0.01$) at 10^{-5} M, where after the potency of Dex declined to reach a minimum inhibition of 63.3% ($p < 0.01$) at 10^{-10} M.

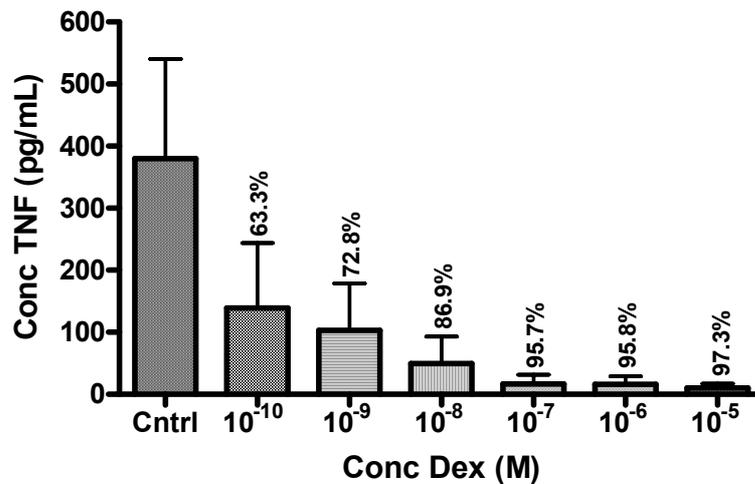


Figure 25. Inhibition of TNF- α production by Dex in male donors at different concentrations found in whole blood samples stimulated with LPS (100 μ g/mL), compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

The production of TNF- α was also influenced by MPA and Dex, but not by NET-A, when blood samples were stimulated with 50 μ g/mL LPS *in vitro*. MPA inhibited the production of TNF- α in a sort of reverse dose dependent manner in the combined gender group, but only had a significant effect at the two lowest concentrations tested (Fig. 26). MPA at 10⁻¹⁰ M caused an 81.3% (p<0.01) decrease of TNF- α levels and a 76.0% (p<0.01) decrease at 10⁻⁹ M.

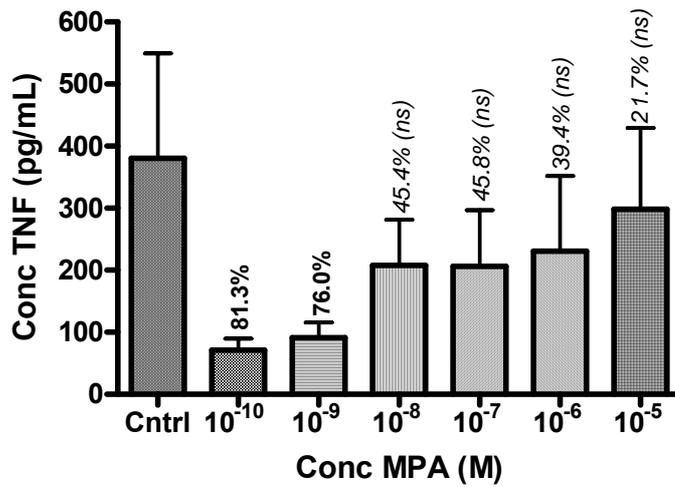
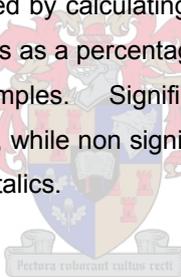


Figure 26. Inhibition of TNF- α production by MPA at different concentrations found in whole blood samples stimulated with LPS (50 μ g/mL), compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.



NET-A had no significant effect on the combined gender group (Data not shown). TNF- α levels were very significantly ($p < 0.01$) reduced by Dex in a dose dependent manner at all concentrations tested (Fig. 27). A maximum inhibition of 94.2% transpired at a concentration of 10^{-6} M Dex thereafter declining to 75.6% at 10^{-10} M.

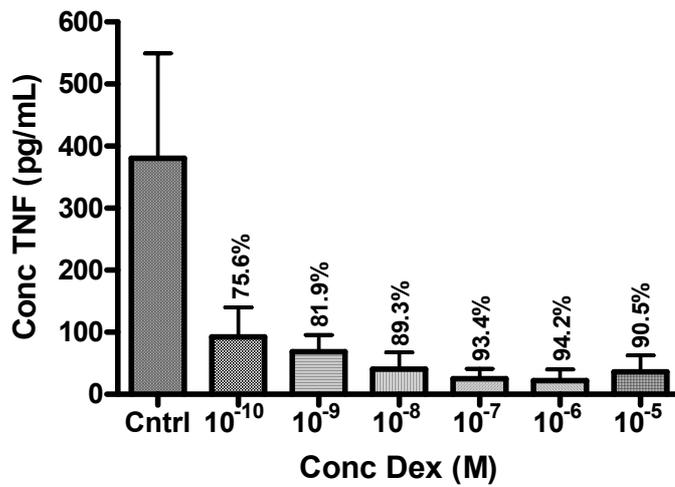


Figure 27. Inhibition of TNF- α production by Dex at different concentrations found in whole blood samples stimulated with LPS (50 μ g/mL), compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

Neither MPA nor NET-A showed any significant inhibition of TNF- α production in the female group stimulated with 50 μ g/mL LPS. Dex was the only steroid that caused a significant reduction in TNF- α levels in the female group stimulated with 50 μ g/mL LPS (Fig. 28). This reduction was dose dependent in nature with the amount of inhibition ranging from 97.1% ($p < 0.05$) at 10⁻⁶ M to 92.6% ($p < 0.05$) at 10⁻⁸ M, while concentrations that had a significant effect were from 10⁻⁵ M Dex down to 10⁻⁸ M Dex.

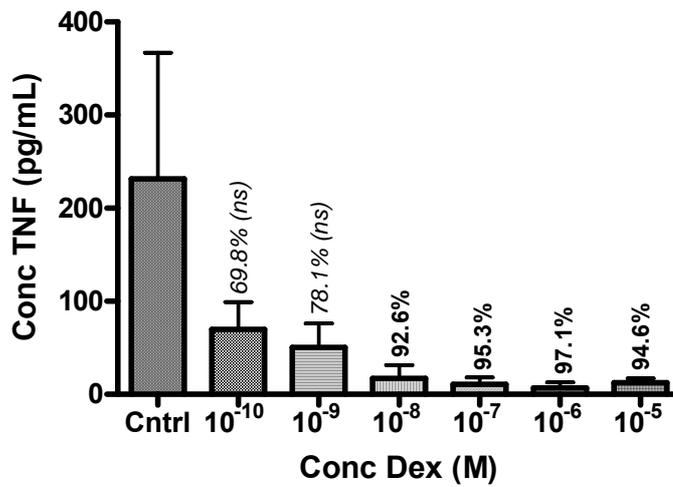
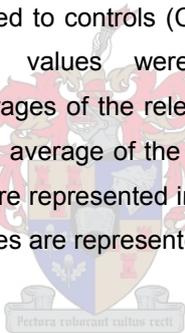


Figure 28. Inhibition of TNF- α production by Dex in female donors at different concentrations found in whole blood samples stimulated with LPS (50 μ g/mL), compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.



The effects on TNF- α levels in male donors stimulated with 50 μ g/mL LPS were slightly different from that in female donors. Although MPA caused a general reduction of TNF- α levels in males, it was only statistically significant at the two lowest concentrations tested. MPA significantly reduced TNF- α levels by 75.5% and 79.6% respectively in males at concentrations of 10⁻⁹ M and 10⁻¹⁰ M, thereby showing that the weight of the influence MPA had on the combined gender group was mainly carried by the influence it had on males (Fig. 29).

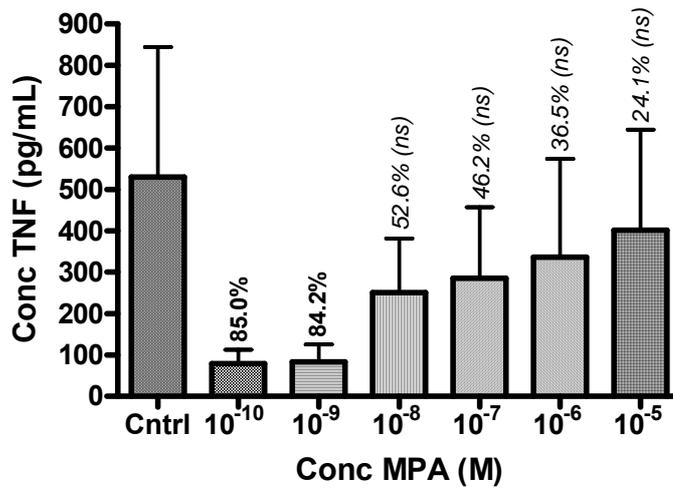


Figure 29. Inhibition of TNF- α production by MPA in male donors at different concentrations found in whole blood samples stimulated with LPS (50 μ g/mL), compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

No significant effects by NET-A on TNF- α levels in males were found (Data not shown). A more prominent dose dependent reduction in TNF- α levels occurred in males under the influence of Dex at all concentrations tested (Fig 30). This significant inhibition by Dex reached a high of 92.9% ($p < 0.05$) at 10⁻⁶ M and declined to 78.1% ($p < 0.05$) at 10⁻¹⁰ M.

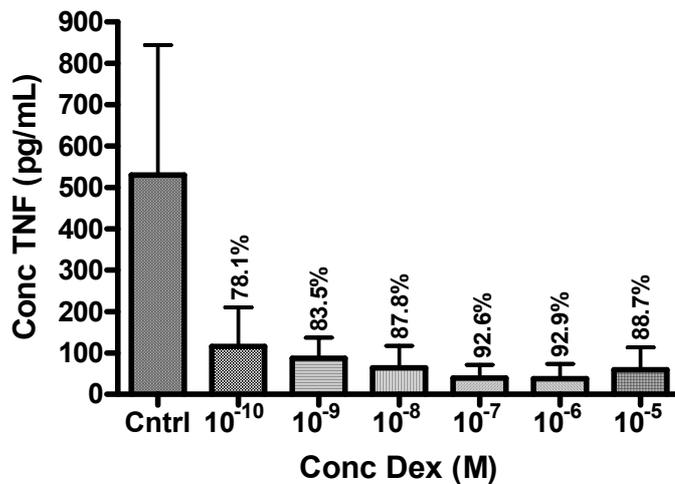


Figure 30. Inhibition of TNF- α production by Dex in male donors at different concentrations found in whole blood samples stimulated with LPS (50 μ g/mL), compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

In the combined gender groups, NET-A was the only compound to have a significant effect on the basal levels of TNF- α in non-stimulated samples (Fig 31). This effect, however, was only statistically significant at a concentration of 10⁻⁹ M, where NET-A caused a 353.8% (p<0.05) increase in TNF- α levels. Once again, this could imply the pro-inflammatory effects of NET-A *in vitro*.

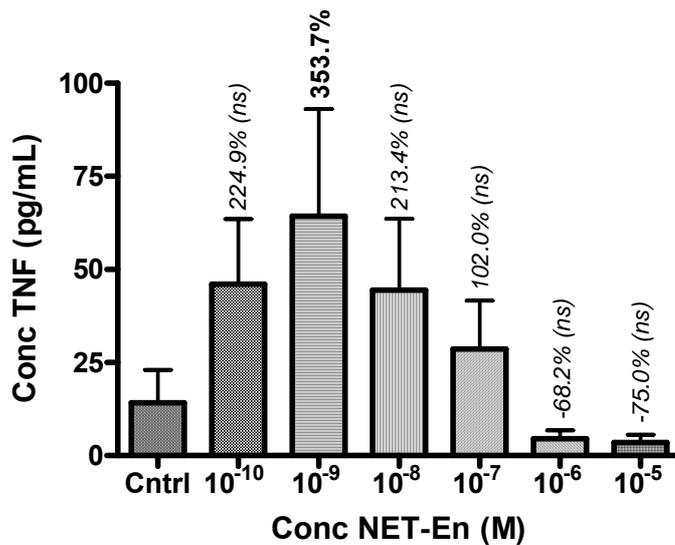


Figure 31. Effects on the basal levels of TNF- α in whole blood samples treated with different concentrations of NET-A, compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics. A negative value indicates a decrease.

In the separate gender groups, NET-A and Dex were the only compounds that significantly affected TNF- α production and this occurred only in the male donor groups for both the compounds. As was the case in the combined gender group, NET-A caused a statistically significant increase in TNF- α levels of 285.8% ($p < 0.05$) in male donors at a concentration of 10^{-9} M, while the observed increases by the surrounding concentrations were not statistically significant (Fig. 32).

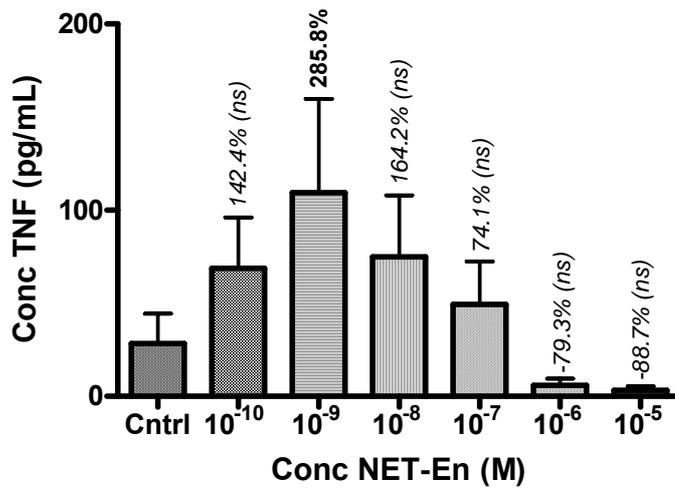
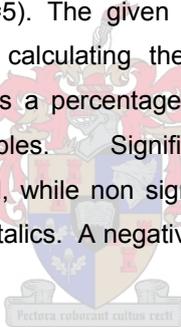


Figure 32. Effects on the basal levels of TNF- α in whole blood samples of male donors treated with different concentrations of NET-A, compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics. A negative value indicates a decrease.



In males, Dex significantly reduced the basal levels of TNF- α in a dose dependent manner (Fig. 33). An immense reduction of 100% was achieved at a concentration of 10⁻⁵ M Dex, whereafter it declined to a still substantial reduction of 88.4% (p<0.05) at 10⁻¹⁰ M.

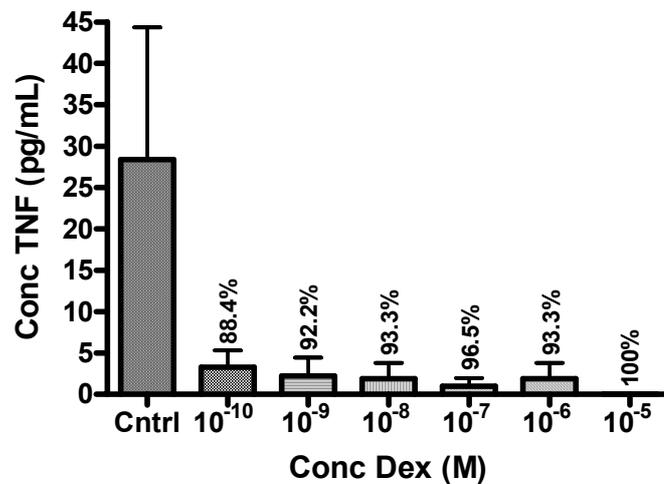


Figure 33. Effects on the basal levels of TNF- α in whole blood samples of males treated with different concentrations of Dex, compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.



2.4 Discussion

The anti-inflammatory properties of GCs, and specifically Dex, are well documented (Munck & Náray Fejes Tóth, 1994; Chrousos, 1995; De Rijk & Berkenbosch, 1994). Given the known GC-like activity of MPA, this study determined the effects of this progestin at a number of concentrations, including contraceptive doses, on the production of the two major pro-inflammatory cytokines *in vitro*. The effect of NET-A on inflammation was also determined, although its GC-like capability is not known. The first part of the results of this study demonstrated the effects MPA and NET-A had on

the production of the pro-inflammatory cytokine, IL-6, while the second part demonstrated the effects on the production of another pro-inflammatory cytokine, TNF- α . The production of these two cytokines was induced by two different concentrations of LPS. LPS is a component of bacterial cell walls and is the main stimulus for inflammatory events. LPS specifically stimulates monocytes to secrete pro-inflammatory cytokines, therefore any observed changes in the levels of these cytokines would occur if the function of these monocytes was influenced.

Dex is a very potent inhibitor of IL-6 release from monocytes. This was demonstrated by the occurrence of Dex being the only compound to affect cells stimulated with 10 μ g/mL LPS in the gender groups as well as in the combined group. This concentration of LPS represented a small stimulus which translated into sub-optimal activation of monocytes. Analysis of the combined group showed that Dex significantly inhibited the release of IL-6 from sub-optimally activated monocytes in a dose dependent manner. This inhibitory effect of Dex once again substantiates why it is registered as an anti-inflammatory agent and used extensively clinically. The effect of Dex on sub-optimally activated monocytes was more pronounced in females than in males. IL-6 release was very significantly reduced in females at all concentrations tested except 10⁻¹⁰ M, while only the two highest concentrations of Dex had a significant effect in males. This observed difference in the potency of Dex between genders clearly indicates that Dex has a varied influence on different genders and this is most likely caused by a difference in receptor levels amongst males and females.

MPA significantly inhibited IL-6 release from optimally activated monocytes (100µg/mL LPS) at all concentrations tested in the combined group. This anti-inflammatory effect of MPA correlates with the findings of previous studies (Yamashita *et al.*, 1996; Wakatsuki *et al.*, 2002). The inhibiting effect of NET-A on IL-6 release from optimally activated monocytes in the combined group was less potent, but still strong enough to have a significant effect at the four highest concentrations tested. Not surprisingly, Dex very significantly inhibited IL-6 release from optimally activated monocytes of the combined group in a dose-dependent fashion at all concentrations tested, confirming its strong anti-inflammatory capabilities.

Analysis conducted, based on gender criteria, on the influence of the three synthetic hormones on IL-6 release from optimally activated monocytes revealed variability amongst genders. In females the effect of MPA was less pronounced than in males, where the compound was only able to significantly inhibit IL-6 release at 10^{-6} M, 10^{-9} M and 10^{-10} M. Although inhibition of IL-6 production also occurred at the other concentrations of MPA, it was not statistically significant. NET-A had no significant effect at all on optimally activated monocytes from female donors. As was expected, Dex significantly reduced IL-6 concentrations in female donors in a dose-dependent manner. Although less potent in females, MPA was still able to significantly inhibit IL-6 release at low concentrations (10^{-9} M) corresponding with levels reached during contraception. Compared to the potent anti-inflammatory agent Dex at the same concentration, MPA yielded an average inhibition of 52.6% whereas Dex had an average inhibition of 59.8%. The inhibition of IL-6 release by Dex occurred in a proper dose-dependent manner, while MPA-inhibition showed no dose-dependent tendency.

The difference in inhibition curves between MPA and Dex could possibly indicate that MPA utilizes the same receptors as Dex as well as other receptors which will be discussed later.

The release of IL-6 from optimally activated monocytes obtained from male donors in the presence of MPA and NET-A differed from that in female donors. MPA had a very significant ($p < 0.01$ for all concentrations) inhibitory effect on IL-6 release from male donor cells at all concentrations except 10^{-5} M (inhibition occurred, but it was not statistically significant). This effect was more potent in male donors and was again not dose-dependent, indicating the involvement of different receptor levels and possibly a difference in the metabolism of MPA between genders. While NET-A had no significant effect in females, it significantly reduced IL-6 release in a non-dose-dependent manner from male donor monocytes at all concentrations tested except 10^{-10} M. This observed effect of NET-A indicates the possible existence of complete different mechanisms of action amongst male and female donors. Although the influence of Dex was less potent in males, it still significantly reduced IL-6 release from male donor monocytes dose-dependently at all concentrations. Compared to the dose-dependent inhibition (ranging from 77.1% to 57.8%) in female donors, Dex-mediated inhibition in male donors ranged from 64.6% to 35.3%.

NET-A was the only compound exhibiting pro-inflammatory properties by inducing the release of IL-6 from non-activated monocytes. This occurred only in the combined group and the only statistically significant increase occurred at 10^{-7} M NET-A. It is worth mentioning that by looking at the effect the other concentrations of NET-A (although not

statistically significant) had on basal IL-6 levels (Fig. 14), the pro-inflammatory nature of NET-A becomes apparent.

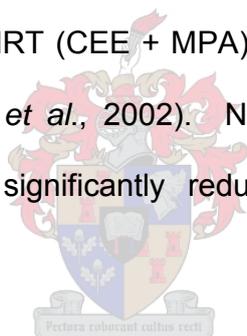
The results of the second part of this study are discussed henceforth. This entails the influence of MPA, NET-A and Dex (a known anti-inflammatory agent) on TNF- α release from moderately (stimulated with 50 μ g/mL LPS) as well as optimally (stimulated with 100 μ g/mL LPS) activated monocytes.

Analysis of TNF- α release from moderately activated monocytes in the combined group revealed that MPA acted in a reverse dose-response manner and while a general inhibition of TNF- α levels occurred, only the two lowest concentrations of MPA had a statistically significant effect on TNF- α levels by reducing it with as much as 81.3%. In its proper anti-inflammatory manner, Dex reduced TNF- α release from moderately activated monocytes of the combined group at all concentrations tested. This immunosuppressive effect of Dex on cytokine production concurs with findings from other studies (Ebrecht *et al.*, 2000; Lanza *et al.*, 1999; Krakauer, 1995).

Results from the separate genders demonstrated the existence of a difference in the activity of MPA found between male and female donors. MPA had no significant effect on moderately activated monocytes of female donors. This was however not the case with male donors. The two lowest concentrations of MPA significantly reduced TNF- α levels with as much as 85.0% in this group, while a non-significant decrease in TNF- α levels occurred at the other concentrations. The possibility of Dex having a slightly more potent effect on moderately activated monocytes in males than in females was

demonstrated by its ability to have a significant effect in male donors at all concentrations, while it only had a significant potency up to a concentration of 10^{-8} M in female donors. NET-A showed no significant activity on TNF- α release from monocytes of either gender group.

The overall effect on TNF- α release from monocytes upon optimal activation (100 μ g/mL LPS) was more prominent than in the moderately stimulated groups. The strongest inhibition of TNF- α release by MPA in the combined group occurred at the two lowest concentrations and the average inhibition at these two concentrations were similar to that found in samples treated with Dex. This coincides with findings from a study which found that continuous combined HRT (CEE + MPA) significantly reduced TNF- α levels in postmenopausal women (Koh *et al.*, 2002). NET-A showed a lesser amount of activity by only being able to significantly reduce TNF- α levels at the highest concentrations tested.



As was the case with moderately activated monocytes, NET-A had no effect in either gender group on TNF- α release from optimally activated monocytes. The gender differentiation of MPA was further elucidated by the results of its influence on TNF- α release from optimally activated monocytes. Although MPA had a significant effect at more concentrations in females than in males (10^{-6} M, 10^{-9} M and 10^{-10} M vs. 10^{-9} M and 10^{-10} M), it was more potent in males than in females (75.5% vs. 48.8% at 10^{-9} M). Female donors were slightly more sensitive as male donors to the potent dose-dependent anti-inflammatory effects of Dex. Compared to the inhibition of IL-6 at contraceptive doses in female donors, the effects of MPA was less potent on TNF- α

release. In comparison, Dex was able to inhibit TNF- α levels in female donors with as much as 66% at 10^{-9} M, while MPA managed a 48.8% reduction at the same concentration.

The possible pro-inflammatory nature of NET-A was further strengthened by its effect on TNF- α release from non-activated monocytes. The only significant increase of 285.8% in the male donor group occurred at a concentration of 10^{-9} M and this effect was carried through to the combined group (353.7% at 10^{-9} M). By looking at the two relevant graphs (Fig. 31 and Fig. 33) it can be seen that the pro-inflammatory effect of NET-A increases up to 10^{-9} M, where after it decreases. We cannot explain this behaviour, but only speculate that optimal NET-A-induced receptor activation occurs at a much lower concentration of the hormone. The collective effect NET-A had on IL-6 and TNF- α release indicates the possible pro-inflammatory tendency of this hormone. Dex significantly reduced the basal levels of TNF- α in male donors with as much as 100% at a concentration of 10^{-5} M. This effect, together with the effect it had on activated monocytes, suggests that Dex may influence transcriptional as well as post-transcriptional events (Chen *et al.*, 1998; Swantek *et al.*, 1997).

Although the suppressive effect of MPA on IL-6 and TNF- α production was more pronounced in male donors than female donors during a severe inflammatory episode, it still affected the release of these two cytokines to a great extent in female donors. NET-A had a greater effect on male donors regarding IL-6 release during a severe inflammatory episode, while it had no effect on TNF- α release in either gender. Dex on

the other hand had a greater effect on IL-6 production in female donors, while this potency affected TNF- α release more in male donors.

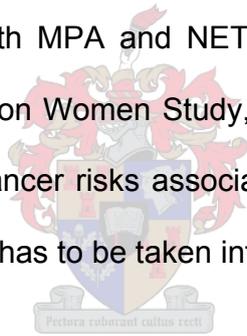
The obscure dose-response displayed by MPA and NET-A throughout the study, together with the differences in sensitivity between genders (also seen with Dex), is a clear indication of the magnitude of complex receptor-interactions involved. The role of steroid receptors in mediating the effects of these steroids is a vast field and, although this study did not include receptor interactions, certain explanations can be given for the differences observed between the steroids and the different effects they had on the genders. Firstly, the occurrence where NET-A is hydrolysed into NET and its metabolites could play a role (Stanczyk & Roy, 1990). It is not known to what extent NET-A is metabolised in whole blood samples, but if this occurs and these metabolites have different effects in genders it can partially explain the variation observed by us. Secondly, the concentration of the receptors involved plays an important role. Therefore, the differences these steroids displayed amongst genders could have been caused by a variation in the receptor levels of males and females. Receptor levels directly influences the EC₅₀ value of a dose-response curve in that the curve would shift to the left in the presence of more receptors and to the right in the presence of less receptors. Thirdly, the percentages of steroids occupying different receptors they have an affinity for, would greatly influence the behaviour of these steroids in a particular experimental model. Fourthly, the differences in affinities MPA and NET-A has for their different receptors as well the different receptors they are able to interact with, could be responsible for the observed effects of these two steroids. MPA has been shown to bind with high affinity to the PR, GR, AR, and with a low affinity

to the mineralocorticoid receptor (MR) (Bamberger *et al.*, 1999; Teulings *et al.*, 1980; Bentel *et al.*, 1999; Bojar *et al.*, 1979; Bergink *et al.*, 1983; Kemppainen *et al.*, 1999; Wambach *et al.*, 1979; Hapgood *et al.*, 2004), while it has been demonstrated that MPA does not bind to the estrogen receptor (ER) (Teulings *et al.*, 1980; Bergink *et al.*, 1983). On the other hand, NET has been shown to bind to the PR and AR (Bergink *et al.*, 1983), while it had a low affinity for the GR (Kontula *et al.*, 1983; Schoonen *et al.*, 2000) and MR (Wambach *et al.*, 1979). It has been demonstrated that NET could display estrogenic activity by binding to the ER (Markiewicz & Gurpide, 1994; Mendoza-Rodriguez *et al.*, 1999), however, conflicting findings also exist (Bergink *et al.*, 1983; Schoonen *et al.*, 2000).

The strange effects of MPA and NET-A observed by us, where for instance a certain steroid had a more pronounced effect at a lower concentration, are difficult to explain properly. We can, however, conclude that the above mentioned receptor interactions could be, if not entirely, at least partially responsible for the observed effects displayed by these two synthetic progestins. Experimental error is ruled out by the fact that the influence of Dex on IL-6 and TNF- α in our experiments occurred in a proper dose-dependent manner and that the assays on the effects of Dex, MPA and NET-A were all run in parallel on the same blood sample of a particular individual.

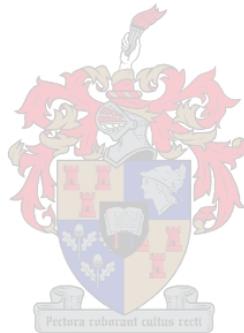
Considering the role IL-6 plays in the induction of the APR and the maturation of B cells, as well as the role TNF- α has in host inflammatory responses, it is possible that MPA could exert some side-effects by compromising the release of these two essential cytokines. If our *in vitro* data is a reflection of an *in vivo* environment, than this

observed action of MPA suggests that the functions of monocytes from females undergoing MPA treatment are suppressed during a severe inflammatory episode and this is possibly mediated via binding to various receptors as well as the GR, where it interferes with NF- κ B and AP-1 transcription factors (Koubovec *et al.*, 2004; Vanden Berghe *et al.*, 1999). Our findings raises concern when it is taken into consideration that MPA and NET-A are the most commonly used progestins for HRT in the USA and Europe (Stahlberg *et al.*, 2004). Recent studies have shown that long-term HRT, consisting of MPA and estrogen, was associated with an increased risk in cardiovascular complications and breast cancer (Rossouw *et al.*, 2002). NET has also been implicated in the findings indicating an increased risk of breast cancer. A recent study of long-term HRT users demonstrated that both MPA and NET significantly increased the risk of breast cancer in these users (Million Women Study, 2003). Our findings suggest that, besides the cardiovascular and cancer risks associated with these progestins, there is also an immunological aspect that has to be taken into consideration.



These negative effects of MPA could also be applicable to women who are on contraception containing MPA, seeing that it exerted the above mentioned side-effects even at concentrations corresponding to levels reached during contraception. Furthermore, the findings of this study suggest that NET-A has a less harmful effect on the inflammatory response of individuals. Although we used NET-A in our experiments and that NET-En is the synthetic progestin used in contraception, we can safely speculate that this progestin would have more or less the same effect in HRT and contraceptive users. Our grounds for this conclusion are that NET-En and NET-A both undergo metabolism *in vivo* to form NET and its respective metabolites. Both NET and

its metabolites have been shown to be the active compounds (Stanczyk & Roy, 1990). We are however not sure to what extent NET-A or NET-En is metabolized in whole blood cell cultures (*in vitro*). The difference in sensitivity of Dex on IL-6 and TNF- α amongst genders suggests that the gender of a patient being treated with this anti-inflammatory agent should be taken into consideration when dealing with the appropriate clinical dose.



CHAPTER 3

AN INVESTIGATION OF THE EFFECTS OF MPA AND NET-A ON THE PROCESS OF CD4⁺ AND CD8⁺ T CELL ACTIVATION

Abstract

Aim: The effects of MPA and NET-A on the activation of human lymphocytes were determined *in vitro*.

Methods: Measurement of the expression of the activation membrane marker CD69 by CD4 and CD8 T lymphocytes was used for this purpose. These effects were compared to that of the known glucocorticoid, Dex.

Results: The inhibitory effect of MPA on the activation of CD4⁺ T lymphocytes in female donors was comparable, and in some cases more potent, than the effect of Dex. NET-A had no significant effect on the activation of CD4⁺ T lymphocytes. MPA, NET-A and Dex significantly inhibited the activation of CD8⁺ T lymphocytes.

Conclusions: Dex, MPA and NET-A displayed a marked inhibitory effect on the activation of CD8⁺ T cells in both female and male donors. These effects of MPA and NET-A raise concern since they are used extensively in HRT and contraception.

[KEY WORDS: MPA, NET-A, Dexamethasone, CD4 T cells, CD8 T cells, T cell activation, contraceptives, HRT]

3.1 Introduction

T lymphocytes play an integral part in the immune response and are primarily involved in CMI, such as guarding against virally infected cells, fungi, parasites and foreign tissue. T lymphocytes are generated in the thymus. The recirculation of lymphocytes occurs throughout the body as they move out of the blood and into sites of infection or move to particular lymphoid organs. They respond to cytokines and other molecules which are either released from or expressed on the cell surface of activated lymphoid and other cells, in particular antigen presenting cells (APCs). Examples of APCs are macrophages, B cells and various dendritic cells. APCs need to process protein antigens in order for them to produce the antigenic peptides recognized by T cells. Antigen is presented by APCs in association with major MHC molecules (In: Cellular and Molecular Immunology, Ed. Abbas & Lichtman, 2003)

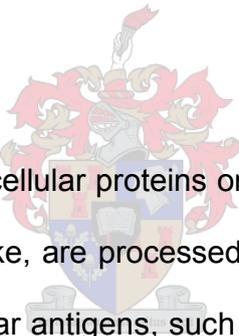
The surfaces of immune cells are covered with molecules that are crucial for the function of the cells. Monoclonal antibodies are used to detect these molecules which are given "CD" numbers. CD4 and CD8 facilitates signal transduction and cell activation through stabilization of the TCR-peptide-MHC complex by binding to different MHC molecules and bringing a tyrosine kinase into the proximity of the cytoplasmic tails of the CD3 and zeta proteins. T cells consist of two subsets, namely CD4⁺ helper cells and CD8⁺ cytotoxic cells. CD4⁺ T cells promote the responsiveness of other cells while CD8⁺ T cells, also known as cytotoxic T lymphocytes (CTLs), have a killing function, e.g., lysis of virally infected cells. The mechanisms of CMI are carried out by these different subsets of mature T cells. This includes killing virally infected cells and tumor

cells, secretion of various cytokines, aiding B cells and CD8⁺ T cells to mature into effector cells, and activating the bactericidal functions of macrophages. T cells also express an antigen-specific TCR, making them the foremost source of antigen specific protection against viral infection and other intracellular infections (In: Immunology for Medical Students, Ed. Nairn & Helbert, 2002; Cellular and Molecular Immunology, Ed. Abbas & Lichtman, 2003).

T cell activation is subjected to several requirements. Binding of the TCR to major MHC-peptide antigen complexes on the APC generates the primary signal. This is also known as clustering of the TCRs. Optimal signalling through the TCR only occurs when co-receptor molecules are involved. CD4 and CD8 act as co-receptor molecules on T cells and contribute to the activation of T cells. A second co-stimulatory signal required for T cell activation is provided by the APC. Finally, contact between the T cell and the APC, as well as TCR-MHC peptide binding, is facilitated by accessory molecules (mostly adhesion molecules or leukocyte function associated antigen found on T cells). Cell adhesion molecules play an important role in the trafficking of lymphocytes. The link between receptor activation and activation of biochemical pathways (that amplify and transmit the signal) is created by intracellular molecules, mainly protein tyrosine kinases and protein tyrosine phosphatases (In: Basic and Clinical Immunology, Ed. Peakman & Vergani, 1997; Cellular and Molecular Immunology, Ed. Abbas & Lichtman, 2003).

Several biochemical pathways are utilized to propagate the signal from the membrane to the nucleus and amplify it. Three main signalling pathways are used. The first

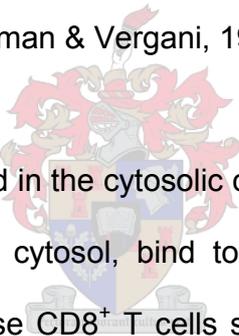
involves phosphorylation and activation of a phospholipase enzyme (PLC- γ) which in turn stimulates two pathways involving (i) diacylglycerol and protein kinase C and (ii) inositol 1,4,5-triphosphate (IP₃) and calcineurin. The third main pathway consists of activation of single-chain guanosine triphosphate (GTP)-binding proteins (e.g. Ras) by activated adaptor proteins. Activation of transcription factors occurs when these Ras proteins activate signalling pathways which lead to the mitogen-activated protein kinases (MAP kinases). A variety of these activated transcription factors, including NF-AT (nuclear factor of activated T cells), NF- κ B and AP-1, exert their effects on several lymphocyte genes. This increases transcription of these genes, leading to an enhancement in cytokine expression in T cells to ultimately form an effective T cell response.



Extracellular antigens, either extracellular proteins or a protein derived from a pathogen in a cytoplasmic vesicle after uptake, are processed for eventual presentation on MHC class II molecules, while intracellular antigens, such as viral proteins, are processed by target cells for eventual presentation on class I MHC molecules. The primary factor in determining if an antigen will be presented on class I or class II MHC molecules is not particular about the antigen itself, but rather by the intracellular pathway followed by an antigen.

CD4⁺ T cells recognize peptide antigens that are generated in intracellular vesicles from the endocytic uptake of extracellular antigens, such as toxins or from microbes growing in intracellular vesicles, which are bound to class II MHC molecules. CD4⁺ T cells are able to differentiate into one of two helper cell subsets (T_H1 or T_H2). This differentiation

is determined by the cytokines present in the environment of the cell. If APCs are responding to infection by microbes they release cytokines that stimulate primed CD4⁺ T cells to differentiate into T_H1 cells. The major role of this subset is to activate the bactericidal functions of macrophages and this is largely accomplished by the release of IFN-γ by T_H1 cells. The destruction of bacteria and viruses present in the macrophages is increased by IFN-γ which acts as a potent activator of macrophage phagocytic activity. On encountering of a parasitic worm or allergen, mast cells secrete IL-4 which favours the differentiation of CD4⁺ T cells into the T_H2 cells. T_H2 cells release cytokines (IL-4 and IL-5) that stimulate B cells, enhance antibody class switching and promote the secretion of IgE (In: Immunology for Medical Students, Ed. Nairn & Helbert, 2002; Basic and Clinical Immunology, Ed. Peakman & Vergani, 1997).



Peptide antigens that are generated in the cytosolic compartment of the cell, like viruses and bacteria that replicate in the cytosol, bind to MHC class I molecules and are recognized by CD8⁺ T cells. These CD8⁺ T cells start off as naive CD8⁺ T cells that emerge from the thymus. Further activation and differentiation is required in order for them to reach the stage where they can lyse virally infected target cells and tumor cells. Activation of CD8⁺ T cells to become effector T cells can occur by means of either one of two events. Firstly, activation can occur if CD8⁺ T cells encounter an antigen displayed on a professional APC and receive activation signals from both MHC class I and co-stimulatory molecules. Secondly, activation can also occur when an antigen is encountered on a non-APC target cell and second signals are received from cytokines released by CD4⁺ T helper cells. MHC class I molecules are found on essentially all nucleated cells, thereby enabling CD8⁺ T cells to monitor all cells for signs of infection.

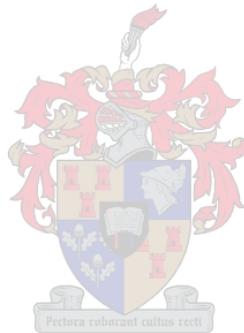
During early phases of infection with viruses an extensive proliferation of CD8⁺ T cells can be observed (In: Immunology for Medical Students, Ed. Nairn & Helbert, 2002; Cellular and Molecular Immunology, Ed. Abbas & Lichtman, 2003).

Because of their different MHC restrictions, the different functions of CD4⁺ T cells and CD8⁺ T are harmonized in that CD4⁺ T cells monitor the extracellular environment while CD8⁺ T cells monitor the intracellular environment for pathogens.

Measurements of the cellular proliferation or cytokine expression in bulk cultures of PBMC cultures in long term incubations have traditionally been used to analyze T cell responses to specific antigen (Clerici *et al.*, 1993; Croft *et al.*, 1994; Cua *et al.*, 1996). There is however several drawbacks associated with these techniques. This includes the inability to detect responses in very low frequency populations and prohibiting analysis of single cell responses in the context of unselected cellular backgrounds (Suni *et al.*, 1998). CD69 is one of the earliest markers expressed on T, B, and natural killer (NK) lymphocytes after stimulation. Rapid and transient expression of the CD69 molecule is uniquely suited for timely analysis of discrete subsets of responding cells. Fluorescent monoclonal antibodies directed against CD69 are used in quantitative flowcytometric analysis to determine CD69 expression. There are several advantages to using CD69 expression as a marker for activation. A four hour culture period is needed, opposed to the 3 days incubation period associated with the measurement of ³H-thymidine incorporation, thereby giving relatively rapid results. CD69 expression is an early event in cell activation, therefore its measurement gives sensitive and unbiased results.

Medroxyprogesterone acetate and norethisterone are the major progestins used in injectable contraceptives and HRT. The immunosuppressive properties of MPA are well documented (Bamberger *et al.*, 1999; Jeremiah *et al.*, 1968; Bunelli *et al.*, 1996; Corsini & Puppo, 1983) compared to that of NET-A. Besides the progestogenic activity of MPA and NET-A, they also display certain GC-like properties (Bamberger *et al.*, 1999). In a study by Vassiliado *et al.* (1999) they found that progesterone significantly inhibited the proliferation of CD8⁺ cells, while it did not significantly affect the proliferation of CD4⁺ cells. What follows is an investigation of the possible effects MPA and NET-A can have on the activation of human lymphocytes, in particular, by measuring the expression of the activation membrane marker CD69 by CD4 and CD8 T cells.

3.2 Materials and methods



3.2.1 Study design

Blood samples were collected from 10 healthy individuals, 5 females (between 20-34 yr of age) and 5 males (between 20-40 yr of age), who were not on any hormonal treatment, including any form of contraception. Medroxyprogesterone acetate (Sigma, Sigma Aldrich), Norethisterone acetate (Sigma, Sigma Aldrich) and Dexamethasone (Sigma, Sigma Aldrich) were used at 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} and 10^{-10} M final concentrations. A 70% ethanol solution was used to dissolve as well as dilute all three steroids. These concentrations were chosen to represent different physiologic states these steroids are found in, where higher concentrations e.g. 10^{-7} M to 10^{-9} M are found in hormone replacement therapy (Adams *et al.*, 1997; Stanczyk, 2003) and lower

concentrations e.g. 10^{-8} M and 10^{-9} M are reached in hormonal contraception (Mishell, 1996; Zalanyi *et al.*, 1984; Smit *et al.*, 2004).

3.2.2 Preparation of samples and flow cytometer analysis

The following sterile FACScan (Falcon[®], Becton Dickinson, Scientific Group) tubes were set up:

One hundred (100) μ L of whole EDTA blood was added to 38 tubes respectively. Eighty-nine (89) μ L of PBS was added respectively to 19 of the 38 tubes, which would later become the stimulated samples, and 98 μ L of PBS was added to the remaining 19 tubes as the non-stimulated samples. Eighteen (18) tubes of each set were divided into 3 groups of 6 each. One group of tubes out of each set was made up with six different concentrations of each steroid, MPA, NET-A or Dex, in order to reach final concentrations of 10^{-5} to 10^{-10} M of the steroids. Two (2) μ L of a 70% ethanol solution was added respectively to the remaining two tubes to compensate for the 70% ethanol solution that was used to dilute the steroids.

All the tubes were then incubated in a CO₂ incubator (NuAir CO₂ Water-Jacketed Incubator, Centrotec) at 37°C and 98% humidity for 30 minutes, where after the 19 stimulated samples were stimulated with 0.25 μ g/mL Phorbol Myristate Acetate (TPA) and 2.0 μ g/mL Ionomycin. All the samples were then incubated again in a CO₂ incubator for 4 hours according to the previously mentioned methods. The rapid,

transient expression of the CD69 antigen is inimitably suited to timely analysis of distinct subsets of responding cells, whereas the CD3 antigen is exclusive to T lymphocytes.

Monoclonal antibodies (mAbs) directed against CD3 and CD69 were used to identify activated lymphocytes, while mAbs directed against CD4 or CD8 were used to differentiate between the subsets of CD4⁺ and CD8⁺ T cells. After the incubation 10µL of FastImmune CD4/CD69/CD3 (CD4 FITC, CD69 PE, CD3 PerCP) or CD8/CD69/CD3 (CD8 FITC, CD69 PE, CD3 PerCP) monoclonal antibody mixture (Becton Dickinson, Scientific Group) was added to each tube and the tubes were further incubated for 20 minutes in the dark at room temperature. Thereafter 500µL of lysing solution (FACS lysing solution, Becton Dickinson, Scientific Group) was added to each tube and the tubes were incubated in the dark at room temperature for 10 minutes, after which another 500µL of lysing solution was added to each tube and incubated for another 10 minutes in the dark in order to insure complete lysing. The tubes were then centrifuged at 1800 rpm for 5 minutes. The supernatants were discarded and 2mL of PBS was added to each tube and centrifuged again at 1800 rpm for 5 minutes. After the supernatant was discarded, 500µL of fixative (5% Formaldehyde in PBS) was added to each tube and vortexed.

The samples were analysed on a flow cytometer (FACSCalibur, Becton Dickinson, Scientific Group) using CellQuest software (Becton Dickinson, Scientific Group). Figure 34 and 35 are examples of our flow cytometric analysis on the activation of CD4⁺ T cells (Fig. 34) and CD8⁺ T cells (Fig. 35) without and in the presence of the three synthetic

hormones. Experiments were performed on the blood of 5 female donors and 5 male donors, thereby assuring reproducibility.

3.2.3 Data analysis

Analysis of datasets were achieved by repeated measures ANOVA followed by post testing using Dunnett's test. Post testing was only done when there was significant pairing ($p < 0.05$) among the groups. Analysis was done on the combined male and female groups ($n=10$), as well as on the male and female groups separately ($n=5$) in case the biological effect was more pronounced on a particular gender. All results were considered statistically significant when $p < 0.05$.



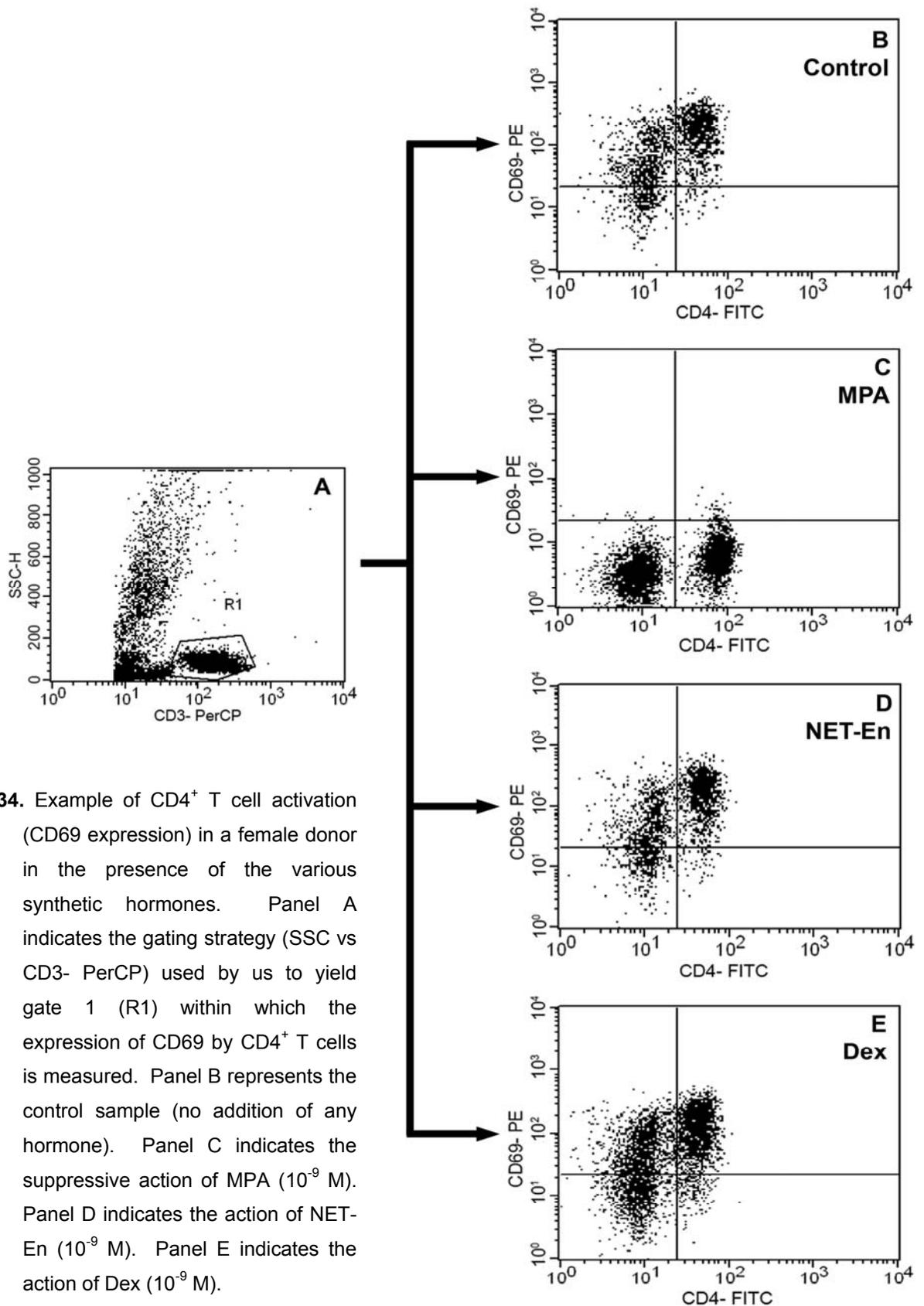


Fig. 34. Example of CD4⁺ T cell activation (CD69 expression) in a female donor in the presence of the various synthetic hormones. Panel A indicates the gating strategy (SSC vs CD3- PerCP) used by us to yield gate 1 (R1) within which the expression of CD69 by CD4⁺ T cells is measured. Panel B represents the control sample (no addition of any hormone). Panel C indicates the suppressive action of MPA (10⁻⁹ M). Panel D indicates the action of NET-En (10⁻⁹ M). Panel E indicates the action of Dex (10⁻⁹ M).

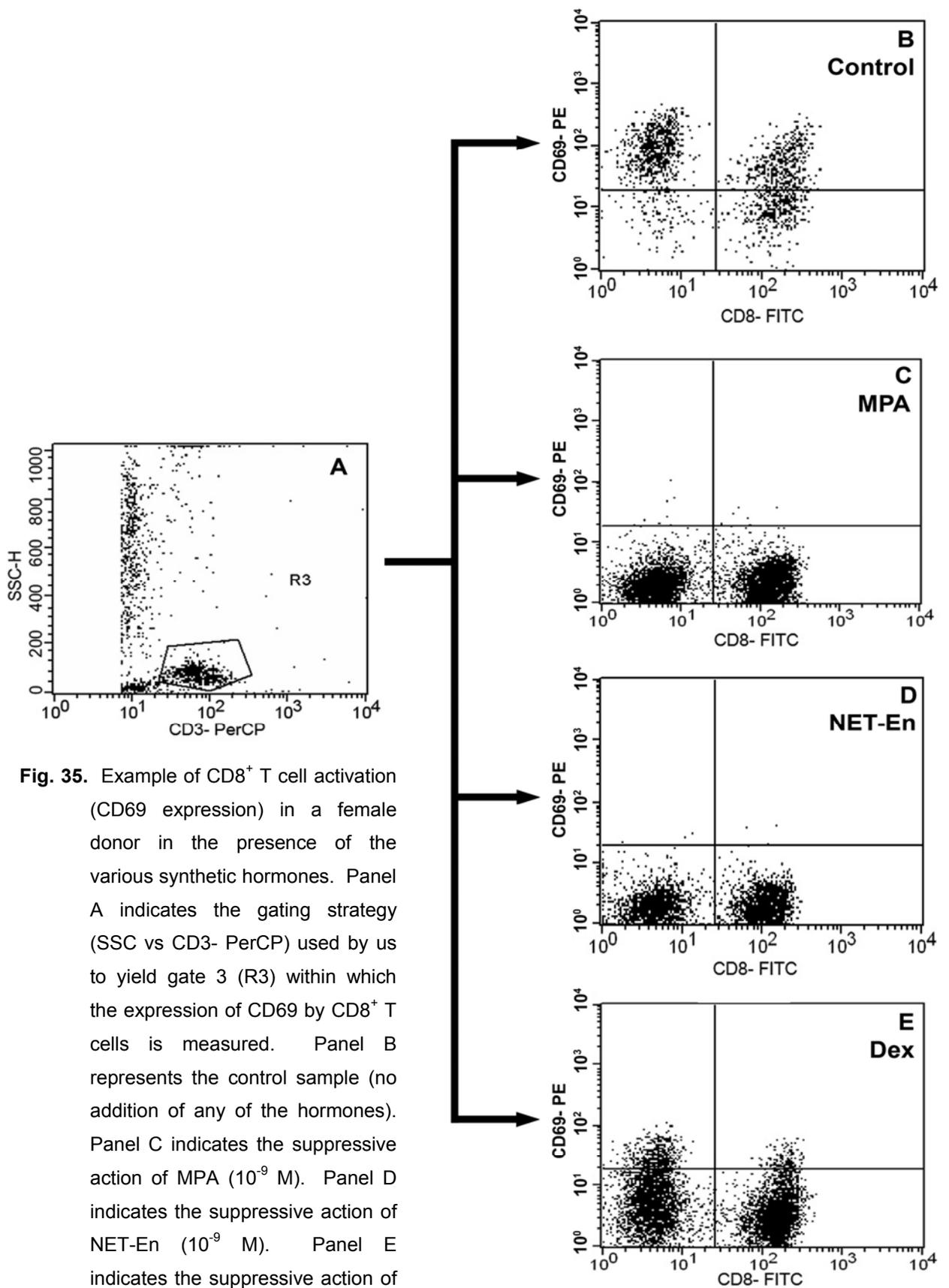


Fig. 35. Example of CD8⁺ T cell activation (CD69 expression) in a female donor in the presence of the various synthetic hormones. Panel A indicates the gating strategy (SSC vs CD3- PerCP) used by us to yield gate 3 (R3) within which the expression of CD69 by CD8⁺ T cells is measured. Panel B represents the control sample (no addition of any of the hormones). Panel C indicates the suppressive action of MPA (10⁻⁹ M). Panel D indicates the suppressive action of NET-En (10⁻⁹ M). Panel E indicates the suppressive action of Dex (10⁻⁹ M).

3.3 Results

3.3.1 Effects on CD4 T cells

The effects that MPA and Dex had on stimulated and non stimulated CD4⁺ T cells are discussed in the following paragraphs. NET-A had no significant effect on any of the different samples and the data is therefore not shown.

MPA had a general suppressive effect on the activation of CD4⁺ T cells in the combined gender group. However, the suppressive effect of 28.2% was only statistically significant at a concentration of 10⁻⁷ M (p<0.05) (Fig. 36).

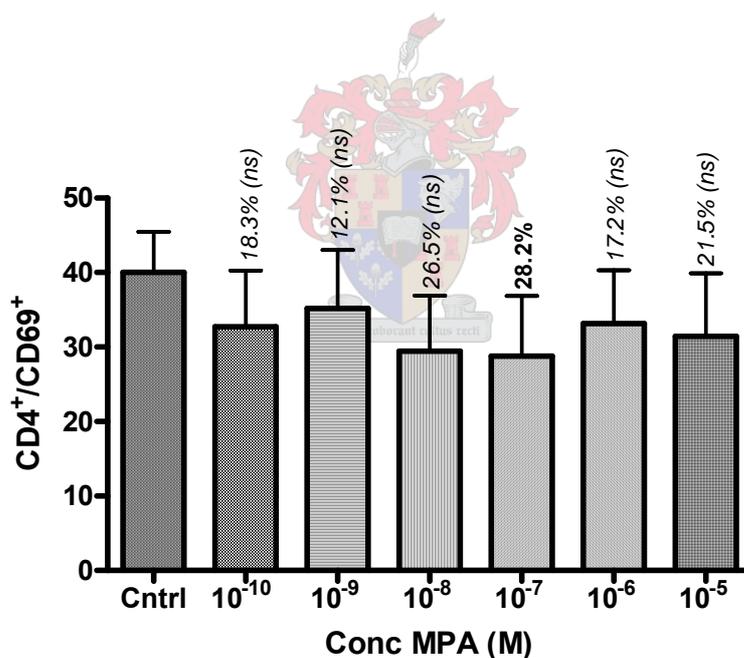


Figure 36. Suppressive effects of MPA at different concentrations on the activation of CD4⁺ T cells, compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

Similarly, Dex had a general suppressive effect at all concentrations, although only two were statistically significant. Dex significantly suppressed the activation of CD4⁺ T cells by 22.6% at 10⁻⁷ M (p<0.01) and 18.6% at 10⁻¹⁰ M (p<0.05) (Fig. 37).

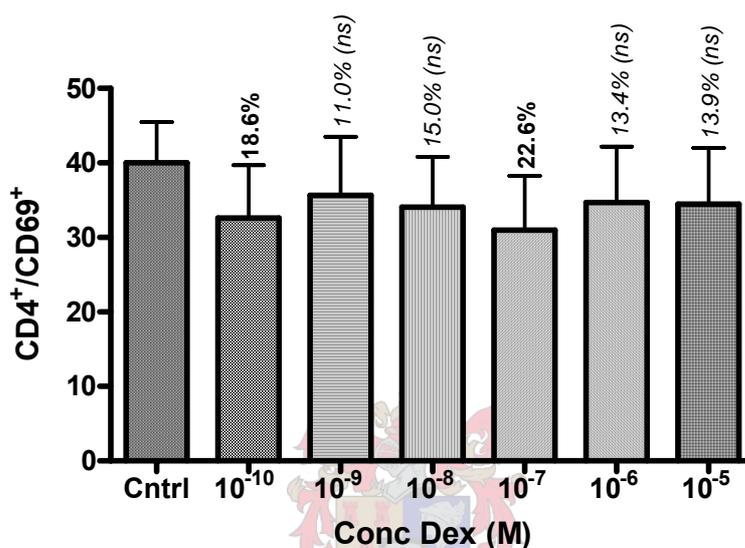


Figure 37. Suppressive effects of Dex at different concentrations on the activation of CD4⁺ T cells, compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

However, when data analysis was done on the different gender groups a surprisingly different story unfolded. MPA had a very significant inhibitory effect on the activation of CD4⁺ T cells obtained from female donors (p<0.01 for all concentrations) (Fig. 38). A maximum suppressive effect of 43.4% was obtained at a concentration of 10⁻⁸ M, while

the least effect of 36.3% occurred at 10^{-6} M. It is worth mentioning that a striking 43.1% inhibition occurred at the lowest MPA concentration of 10^{-10} M.

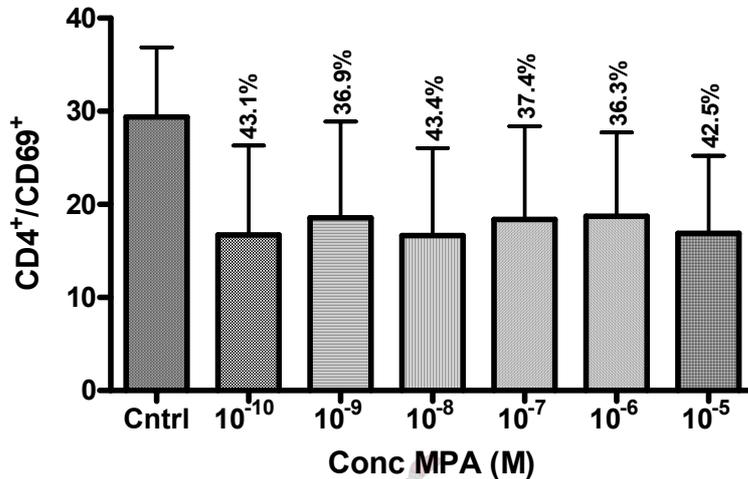


Figure 38. Suppressive effects in females of MPA at different concentrations on the activation of CD4⁺ T cells, compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

Similarly, Dex had an alternating effect on the activation of CD4⁺ T cells from female donors with the greatest inhibition of 42.83% ($p < 0.01$) occurring at a concentration of 10^{-7} M and the lowest being 29.8% ($p < 0.01$) at 10^{-8} M (Fig. 39).

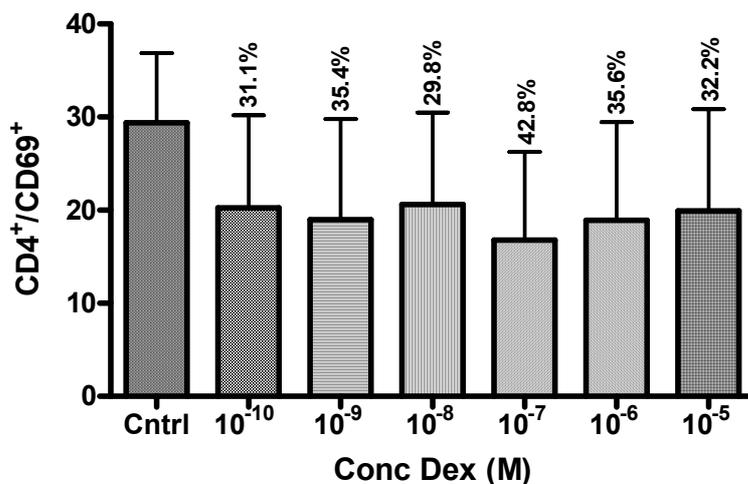


Figure 39. Suppressive effects in females of Dex at different concentrations on the activation of CD4⁺ T cells, compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

Noteworthy, MPA (10⁻⁹ M) induced the activation of CD4⁺ T cells in non-activated samples from male donors with 27.2% (p<0.05). Before writing this off as experimental error it is worth mentioning that MPA also had an enhancing tendency in males, although not statistically significant, at 10⁻⁵, 10⁻⁶, 10⁻⁷, and 10⁻⁸ M (Fig. 40).

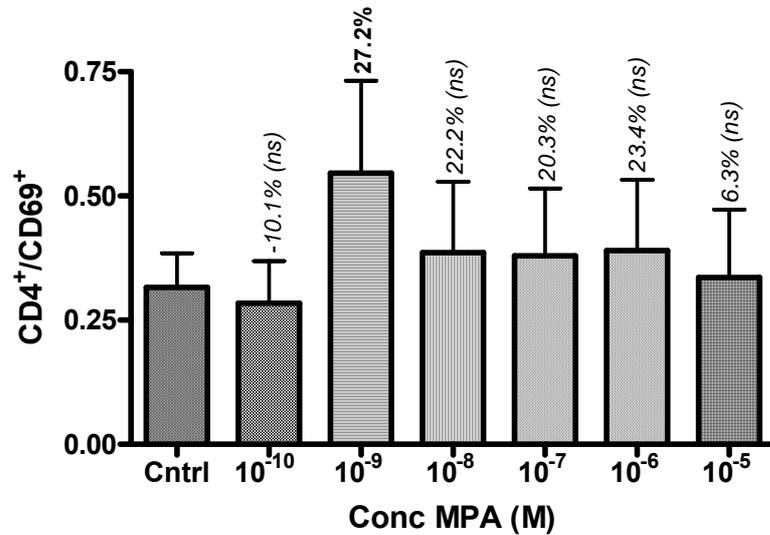
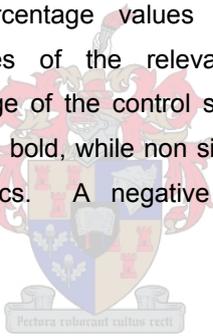


Figure 40. Activation of CD4⁺ T cells by MPA at different concentrations, compared to non stimulated controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics. A negative value indicates a decrease.



3.3.2 Effects on CD8 T cells

The effects of MPA, NET-A and Dex were much more pronounced on the activation of CD8⁺ T cells. The following was found when analysis was done on the different combined gender groups. MPA significantly ($p < 0.01$) inhibited the activation CD8⁺ T cells at all concentrations, with the highest inhibition of 47.4% ($p < 0.01$) occurring at 10⁻⁸ M and the lowest of 31.6% ($p < 0.01$) at 10⁻⁵ M (Fig. 41).

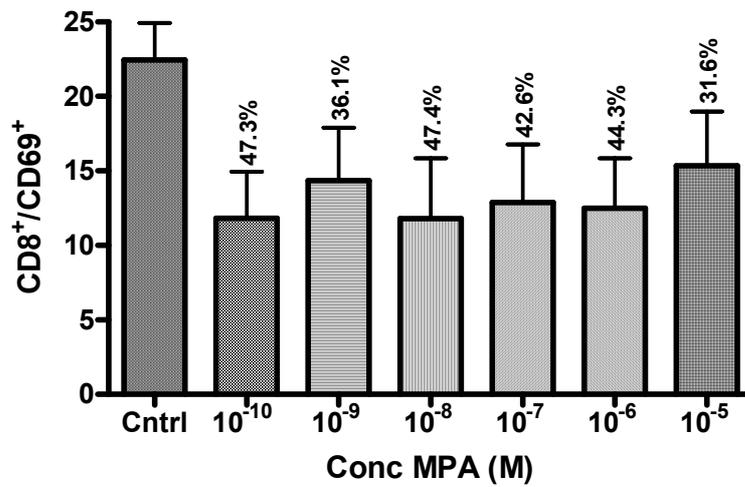


Figure 41. Suppressive effects of MPA at different concentrations on the activation of CD8⁺ T cells, compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

Similar to MPA, NET-A also significantly ($p < 0.01$) reduced the activation of CD8⁺ T cells, with a peak of 52.7% reduction at 10⁻⁷ M and a low of 42.4% reduction at 10⁻⁵ M (Fig. 42).

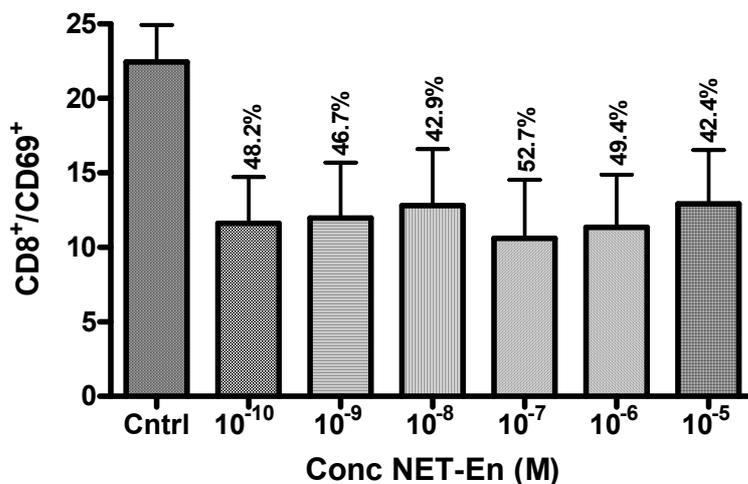


Figure 42. Suppressive effects of NET-A at different concentrations on the activation of CD8⁺ T cells, compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

As was expected, Dex also significantly ($p < 0.01$) reduced the number of activated CD8⁺ T cells with as much as 61.6% at 10⁻⁷ M and the lowest amount of inhibition of 35.0% occurring at 10⁻⁸ M (Fig. 43).

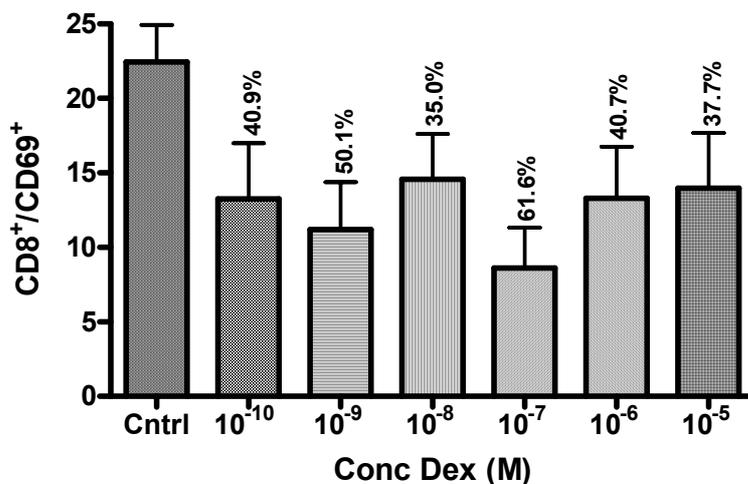


Figure 43. Suppressive effects of Dex at different concentrations on the activation of CD8⁺ T cells, compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

A much more pronounced effect by MPA, NET-A, as well as Dex, was witnessed in females when the data was analysed separately according to gender. MPA at all concentrations had a very significant ($p < 0.01$) inhibitory effect on the activation of CD8⁺ T cells in females (Fig. 44). The highest percentage of inhibition was seen at 10⁻⁶ M (52.5%), while 40.4% at 10⁻¹⁰ M was the least inhibition.

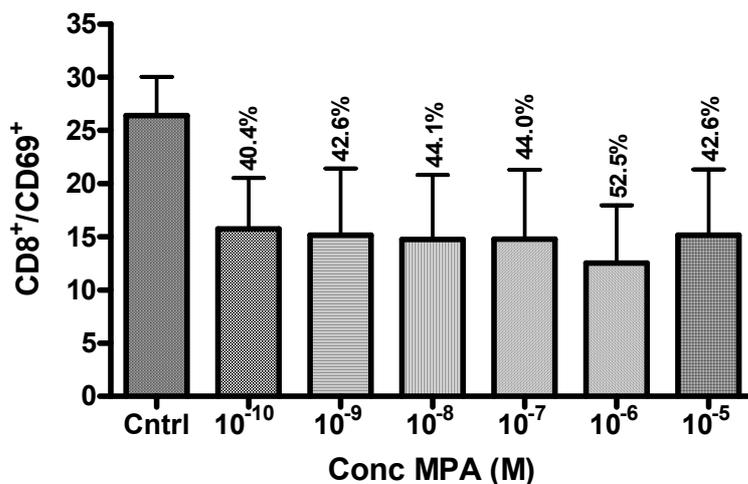


Figure 44. Suppressive effects of MPA at different concentrations on the activation of CD8⁺ T cells in females, compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

NET-A significantly ($p < 0.01$) decreased the activation of CD8⁺ T cells in a similar fashion as MPA in females, but with the highest inhibition of 73.7% occurring at 10⁻¹⁰ M and the least inhibition of 41.7% at 10⁻⁵ M (Fig. 45).

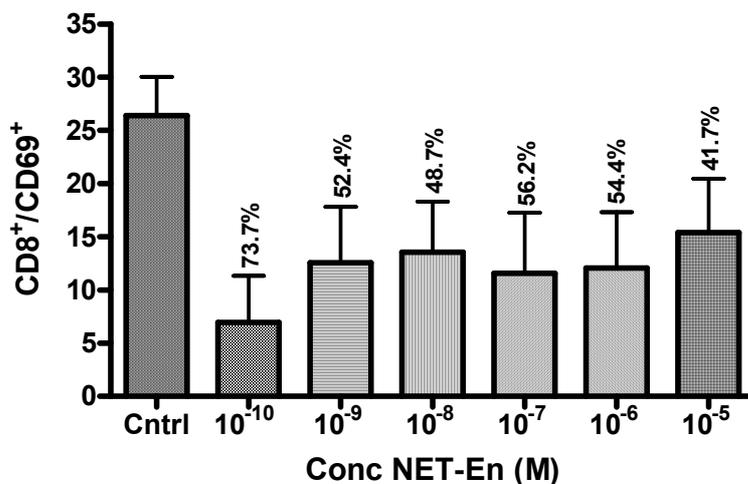


Figure 45. Suppressive effects of NET-A at different concentrations on the activation of CD8⁺ T cells in females, compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

The activation of CD8⁺ T cells was also significantly ($p < 0.01$) reduced by Dex at all concentrations tested in females, with a high of 74.2% at 10⁻⁷ M and a low of 39.3% at 10⁻⁸ M (Fig. 46).

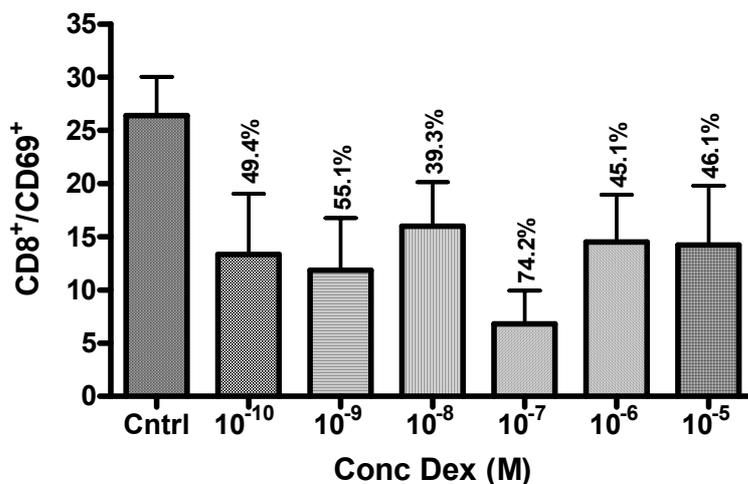


Figure 46. Suppressive effects of Dex at different concentrations on the activation of CD8⁺ T cells in females, compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

While a general decrease in CD8⁺ T cell activation occurred in males, MPA only produced a significant result at 10⁻⁷, 10⁻⁸ and 10⁻¹⁰ M with the amount of inhibition ranging from 40.5% (10⁻⁷ M) to 57.2% (10⁻¹⁰ M) (Fig. 47). This indicates that the incredibly significant (p<0.01 for all concentrations) effect MPA had on the combined gender group was manifested by the effect MPA had on the females rather than what it had on the males.

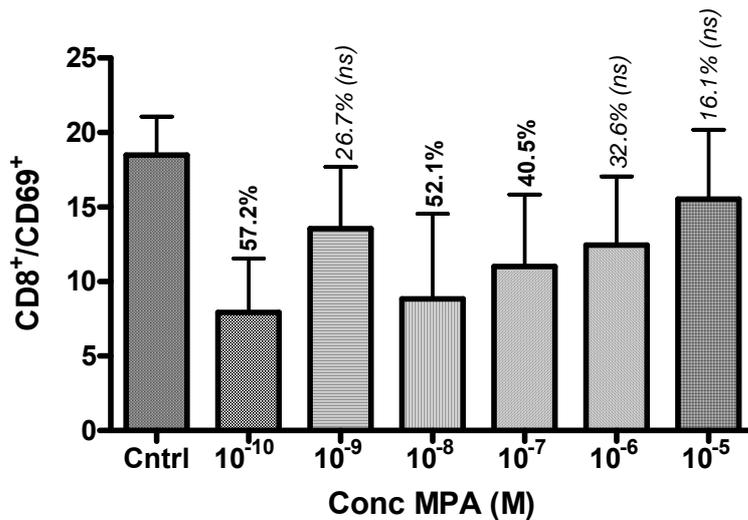


Figure 47. Suppressive effects of MPA at different concentrations on the activation of CD8⁺ T cells in males, compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

NET-A also demonstrated a general inhibiting effect on CD8⁺ T cell activation, but not as clear and prominent as with MPA. NET-A inhibited the activation of CD8⁺ T cells in males with varying significant p values at concentrations of 10⁻⁵, 10⁻⁶, 10⁻⁷ and 10⁻⁹ M (Fig. 48). A highest inhibition of 47.6% was reached at 10⁻⁷ M, while the lowest was 38.4% at 10⁻⁹ M NET-A. Surprisingly, Dex had no significant effect on the activation of CD8⁺ T cells in males at any concentration tested (data not shown), thereby indicating that the inhibitory effect of Dex at all concentrations on the group as a whole is solely carried by the effect it had on the females.

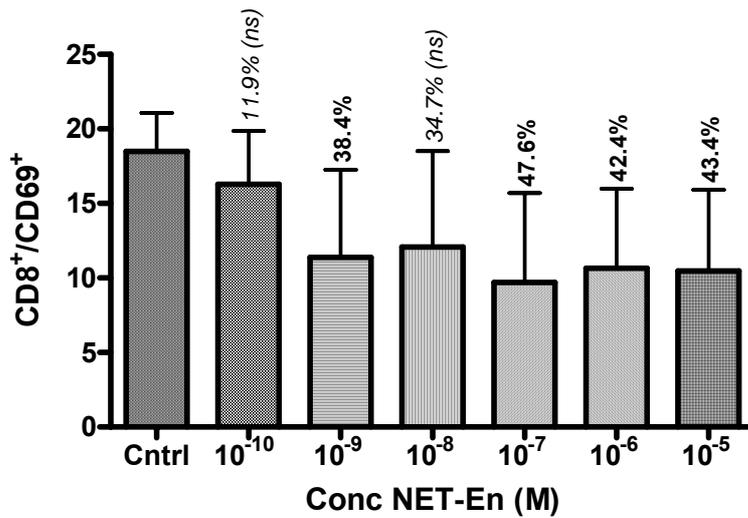


Figure 48. Suppressive effects of NET-A at different concentrations on the activation of CD8⁺ T cells in males, compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

A marked influence on the basal level of CD8⁺ T cells was observed in the non stimulated groups treated with MPA and NET-A, but not in the group treated with Dex. In the combined group MPA caused a general decrease in the amount of activated CD8⁺ T cells, although not all statistically significant. MPA at concentrations of 10⁻⁶ M – 10⁻⁸ M, and 10⁻¹⁰ M caused a statistically significant decrease in CD8⁺ T cells of up to 76.2% (p<0.05) at 10⁻¹⁰ M and as low as 67.5% (p<0.05) at 10⁻⁶ M (Fig. 49).

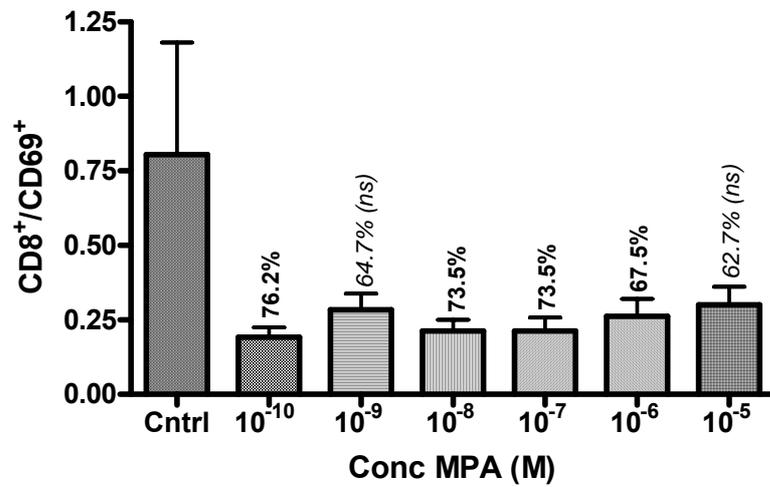


Figure 49. Reduction of activated CD8⁺ T cells by MPA at different concentrations, compared to non stimulated controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

NET-A reduced the basal levels of activated CD8⁺ T cells significantly at all concentrations tested in the group as a whole, with the highest amount of inhibition (78.3%; p<0.05) occurring at 10⁻¹⁰ M and the least amount of inhibition (67.3%; p<0.05) occurring at 10⁻⁹ M (Fig. 50).

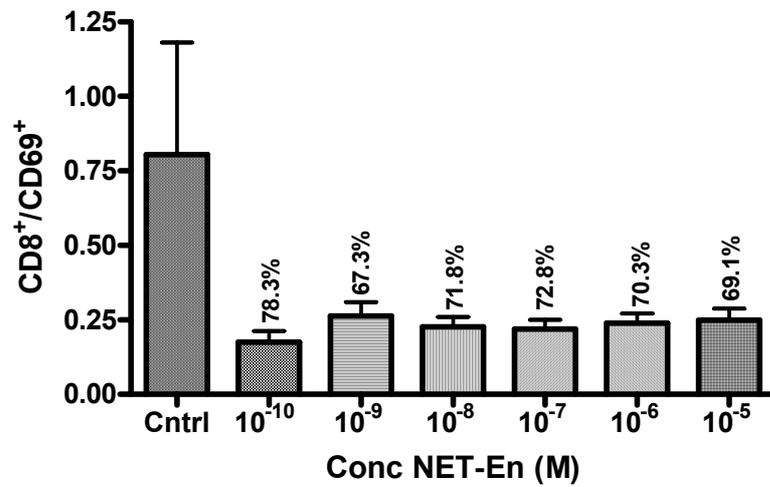


Fig. 50. Reduction of activated CD8⁺ T cells by NET-A at different concentrations, compared to non stimulated controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

Analysis of the different gender groups revealed that neither MPA, NET-A nor Dex had any significant effect on the basal levels of activated CD8⁺ T cells in the female group. This was however not the case in males. MPA significantly affected the basal levels of activated CD8⁺ T cells in males the least, with only a statistically significant decrease of 60% occurring at a concentration of 10⁻⁷ M (p<0.05) (Fig. 51).

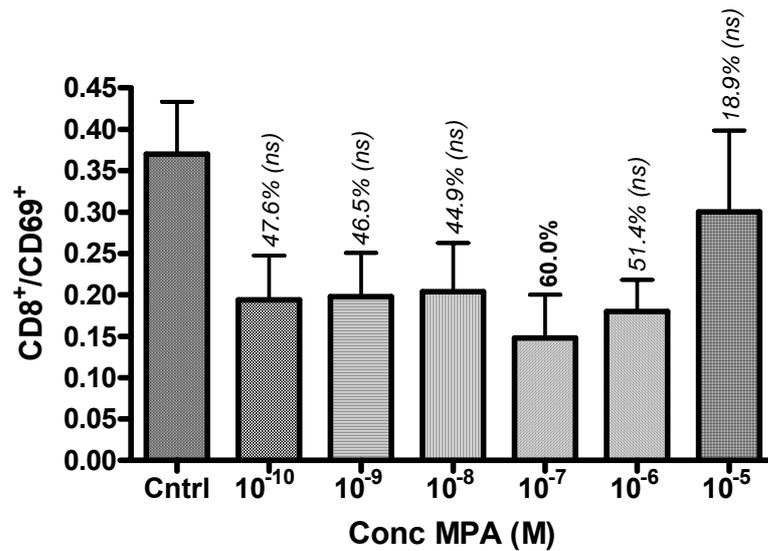


Figure 51. Reduction of activated CD8⁺ T cells in males by MPA at different concentrations, compared to non stimulated controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

NET-A on the other hand had a more marked effect on CD8⁺ T cell levels in non stimulated samples from males by causing a general decrease (Fig. 52). All concentrations tested, except 10⁻⁹ M, displayed a statistically significant reduction in the basal levels. NET-A had the greatest effect of 60% reduction at 10⁻¹⁰ M (p<0.01) and the smallest amount of 39.5% reduction at 10⁻⁸ M (p<0.05).

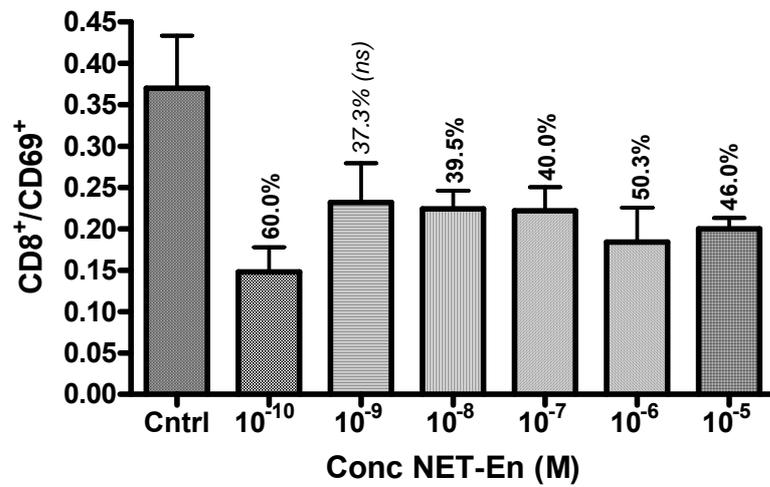


Figure 52. Reduction of activated CD8⁺ T cells in males by NET-A at different concentrations, compared to non stimulated controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

Dex had an even greater effect on activated CD8⁺ T cell basal levels in males by causing a significant reduction at all concentrations tested (Fig. 53). A concentration of 10⁻⁹ M Dex caused a 57.8% (p<0.01) reduction in basal CD8⁺ T cell levels and the least reduction (43.8%; p<0.05) occurred at 10⁻⁸ M.

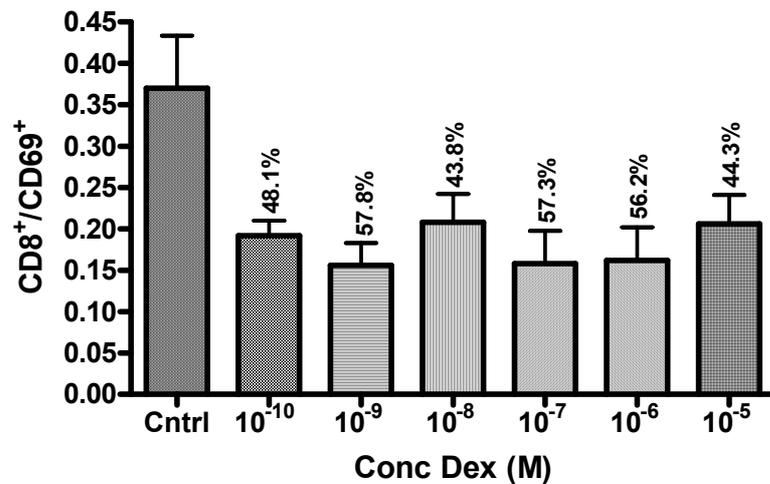
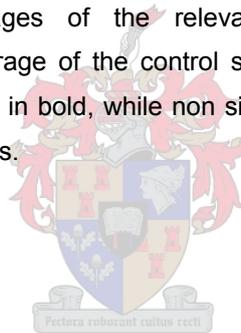


Figure 53. Reduction of activated CD8⁺ T cells in males by Dex at different concentrations, compared to non stimulated controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.



3.4 Discussion

The immunosuppressive and anti-inflammatory properties of GCs is a thoroughly described phenomenon and therefore they are widely used in the treatment of inflammatory and auto-immune states (Krakauer, 1995; Homo-Delarche *et al.*, 1991). It has previously been shown that Dex inhibited T cell proliferation after PBMCs were stimulated with staphylococcal enterotoxin (Krakauer, 1995). The inhibition of both proximal and distal events of T cells (Almawi *et al.*, 1991) and the suppression of cytokines and adhesion molecules (Haynesworth *et al.*, 1996; Munck & Náray Fejes Tóth, 1994; De Rijk & Berkenbosch, 1994) is evidence of the multi-faceted mechanism

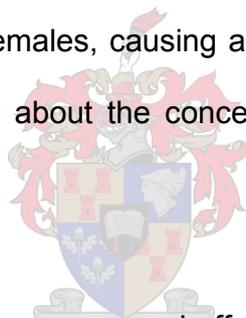
of action demonstrated by GCs. The trans-repression of target genes is the reason for the anti-inflammatory and immunosuppressive properties displayed by GCs (Wick *et al.*, 1993; Boumpas *et al.*, 1991; Bamberger *et al.*, 1996; Bamberger *et al.*, 1997).

The capacity of MPA to display certain GC activities is well known (Bamberger *et al.*, 1999) and this is further strengthened by findings demonstrating the immunosuppressive properties of this compound (Hulka *et al.*, 1965; Pelner & Rhoades, 1965; Jeremiah *et al.*, 1968). Little is known about the probable GC activity and immunosuppression of NET-A. It has been shown in the past that norethindrone and norethynodrel significantly and consistently prolonged skin allograft survival in castrated rabbits (Hulka *et al.*, 1965), thereby implying that these molecules inhibited the rejection crisis that normally follows transplantation. This clearly signifies an *in vivo* demonstration of CMI inhibition.

The function of CD4⁺ T cells in monitoring the extracellular environment for pathogens are well established and of great importance. These T cells require sufficient activation in order to function optimally. Therefore, hindering of this activation step will lead to the T cells not functioning properly. CD4⁺ T cells promote the responsiveness of other cells and play a major role in the activation of the bactericidal functions of macrophages. Additionally, CD4⁺ T cells are the foremost source of antigen-specific protection against viral infection and other intracellular infections. The major activity of the two helper cell subsets (T_H subsets) of CD4⁺ T cells is to release cytokines. These cytokines released from T_H cells determine whether the immune response will be driven in the direction of

antibody synthesis (T_H2 response) or in the direction of cell-mediated immunity (T_H1 response).

The first part of this study demonstrates the effects MPA, NET-A and the synthetic GC, Dex, had on the activation of $CD4^+$ T cells in an *in vitro* model. MPA and Dex had a minor effect on the combined gender group, while the presence of NET-A showed no interference in the activation of $CD4^+$ T cells in this combined group. However, MPA and Dex had a considerable effect on the activation of $CD4^+$ T cells in females and none whatsoever in males. Alarmingly, the overall effect of MPA on $CD4^+$ T cell activation in females was more potent than that of the synthetic GC Dex. MPA considerably reduced the activation of $CD4^+$ T cells in females, causing a 36.9% reduction at concentrations as low as 10^{-9} M, which is round about the concentration achieved during hormonal contraception.



MPA was the only compound to have a general affect on non-activated $CD4^+$ T cells in males, but this increase in activated $CD4^+$ T cells was only statistically significant at a concentration of 10^{-9} M. The significance of this finding is unknown at present but one could speculate that MPA could prevent the initial triggering of $CD4$ cells (in a resting/non-activated state) upon initial contact with a stimulus, rather than attempt to “reign in” on activated cells once they are triggered.

Protein vaccines are widely used in modern medicine. The efficiency and success of these vaccines depend on them being processed effectively for eventual presentation on MHC class II molecules to $CD4^+$ T Lymphocytes. Considering the effects MPA had

on the function of CD4⁺ T cells it is possible that females using contraceptives containing MPA are at a disadvantage concerning the effectiveness of these vaccines. The results of this study leans towards the possibility that any female subjected to any form of MPA treatment, whether it is HRT or contraception, might be poorly protected against challenges by extracellular pathogens. Furthermore, these findings demonstrate that regarding the influence on CD4⁺ T lymphocytes, NET-A might be the safer compound to use seeing that it had no significant influence on CD4⁺ T cells.

The main function of CD8⁺ T Lymphocytes is to deal with intracellular infections by killing virally infected cells. This killing function is achieved through activation signals that enable CD8⁺ T cells to differentiate into effector CTLs with the complete range of granular enzymes etc. required to kill a target cell. CTLs kill target cells by exchanging granule content and ultimately inducing apoptosis of these infected cells. The second part of this study demonstrated the effects MPA, NET-A and Dex had on CD8⁺ T cells and their activation. It appears from the results that MPA, NET-A and Dex had a far greater effect on CD8⁺ T Lymphocytes, compared to the influence these steroids had on CD4⁺ T Lymphocytes.

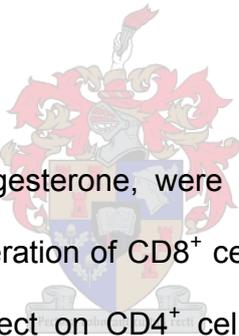
All three steroids demonstrated similar inhibiting effects on the activation of CD8⁺ T cells in the combined gender groups. Analysis on the separate gender groups revealed that all three steroids had a more marked effect on CD8⁺ T cell activation in females than in males. In female donors, the inhibiting effect MPA had on CD8⁺ T cell activation was statistically significant at all concentrations tested, while in male donors it was only statistically significant at three concentrations (10^{-7} M, 10^{-8} M, and 10^{-10} M). All six

concentrations of NET-A tested caused significant hindering of CD8⁺ T cell activation in female donors, while it only had a statistically significant effect in males at four concentrations (10^{-5} M, 10^{-6} M, 10^{-7} M, and 10^{-9} M). This difference in sensitivity between genders was more pronounced in donors treated with Dex. Activation of CD8⁺ T cells were significantly reduced in female donors by all six concentrations of Dex, while it had no statistically significant effect on male donors at any concentration tested.

As previously discussed in chapter 2, the gender sensitivity demonstrated by these results can be credited to a variety of factors. This includes the metabolism of these three steroids in our experimental model, the different types and concentrations of the receptors involved, the variation in EC₅₀ values amongst different individuals, the nature of the competition between the different receptors for the steroids that they have an affinity for, as well as the level of affinity each individual steroid has for its appropriate receptors. Collectively, all these factors give a clear indication of the complexity of the mechanisms involved by which these three steroids mediate their effects.

This multifaceted steroid-receptor-interaction is most likely also responsible for the observed variation in strength of the steroids when compared to each other in the same experiment. It has been shown that both MPA and NET bind to the PR and AR (Teulings *et al.*, 1980; Bentel *et al.*, 1999; Bergink *et al.*, 1983). MPA has been shown to have a high affinity for the GR and a low affinity for the MR (Bamberger *et al.*, 1999; Bojar *et al.*, 1979; Wambach *et al.*, 1979; Hapgood *et al.*, 2004), while NET displayed a much lower affinity for the GR and MR (Kontula *et al.*, 1983; Schoonen *et al.*, 2000;

Wambach *et al.*, 1979). Furthermore, both MPA and NET are known to bind to the PR (Teulings *et al.*, 1980; Bergink *et al.*, 1983). It has been demonstrated that MPA does not have any estrogenic activity (Teulings *et al.*, 1980; Bergink *et al.*, 1983), whereas NET demonstrated estrogenic activity by binding to the ER (Markiewicz & Gurpide, 1994; Mendoza-Rodriguez *et al.*, 1999), although conflicting findings also exist (Bergink *et al.*, 1983; Schoonen *et al.*, 2000). The purpose of this study was not to determine which receptors were involved in mediating the effects of the different steroids or which of these receptors were present in whole blood. Nevertheless, it is interesting to note that Dex, MPA and NET-A had a similar inhibiting effect on the activation of CD8⁺ T cells, despite the different receptors that they interact with as well as their affinities for these receptors.



Comparable results, but with progesterone, were demonstrated by Vassiliado *et al.* (1999). They found that the proliferation of CD8⁺ cells was significantly suppressed by progesterone, while it had no effect on CD4⁺ cells. They ascribed their observed inhibition of chemokine secretion by CD8⁺ cells as a result of the inhibiting action progesterone has on these cells. This suppressive effect exclusively occurred in activated T cells and not in resting T cells or macrophages, whereas in this study these three compounds did have an effect on resting CD8⁺ T cells.

Of the three steroids, MPA affected the basal levels of resting CD8⁺ T cells in male donors the least. NET-A significantly reduced the basal levels of resting CD8⁺ T cells in non-stimulated samples from male donors with as much as 60% at the lowest concentration of 10⁻¹⁰ M. Dex was unable to inhibit the activation of CD8⁺ T cells at all

in male donors, while it significantly reduced the basal levels of CD8⁺ T cells in this group at all concentrations tested. The fact that MPA, NET-A and Dex had variable significant effects on the basal levels of CD8⁺ T cells in male donors, but not at all in female donors, furthermore demonstrates the involvement of a variety of mechanisms and steroid receptors. Sexual dimorphism have been noted in GR affinity, binding capacity, nuclear translocation, and gene expression in the brain and anterior pituitary (Pfeiffer *et al.*, 1994; Pfeiffer *et al.*, 1991; Pfeiffer & Barden, 1987; Turner & Weaver, 1985), as well as during glucocorticoid-inhibition of thymocyte and splenocyte reponse in mice (Morale *et al.*, 1995).

It has long been a matter of dispute whether human lymphocytes expresses the PR (Vermeulen *et al.*, 2001; Schust *et al.*, 1996; Bamberger *et al.*, 1999). The bulk of evidence suggests that the PR is absent in human lymphocytes, leaving the involvement of the GR as an explanation. The multifaceted steroid hormone effect of MPA comes from its ability to interact with progesterone, androgen, mineralocorticoid and GC receptors. The GC activity of MPA was well described by Bamberger *et al.* (1999) where they demonstrated that MPA was able to suppress a human IL-2 promoter-luciferase construct to the same extent as the synthetic GC Dex in normal human lymphocytes. The expression studies of Bamberger *et al.* (1999) also revealed that the GR and not the PR was responsible for the effects of MPA, since human lymphocytes only express the GR. There are studies, although not widely accepted, that have demonstrated the existence of a second steroid-binding site (Svec *et al.*, 1989; Hollenberg *et al.*, 1989) which could have perhaps played a role in the observed differences amongst the tested compounds and their effects in different genders.

Further activation and differentiation is required by CD8⁺ T cells to develop into effector T cells that lyse virally infected target cells and tumor cells. The association of MPA and NET with an increased risk in cancer (Paul *et al.*, 1998; Skegg *et al.*, 1995; WHO Collaborative Study of Neoplasia and Steroid Contraceptives, 1991; Rossouw *et al.*, 2002; Million Women Study, 2003) could be due to the suppressive effects these progestins have on the activation of CD8⁺ T cells, thereby preventing them from killing targeted tumor cells. Strong CTL responses are vital in protecting the body from infections like HIV, Hepatitis B, Influenza, and EBV (Epstein-Barr virus). This crucial role of CD8⁺ cells in controlling intra-cellular pathogens was demonstrated by Jin *et al.* (1999). By depleting the CD8⁺ T cell population in macaques, they managed to show an increase in plasma viremia of SIV in these animals. Therefore, it might be possible that women using MPA or NET could be more prone to various infections. However, further infectivity studies are necessary in order to substantiate this hypothesis.

The results of this study suggests that MPA and NET-A, as far as activation of CD8⁺ T cells is concerned, can hold severe implications for CMI by preventing the immune system from mounting an effective immune response against intra-cellular pathogens. A remaining question that needs to be answered is whether our *in vitro* data reflects the behaviour of these progestins *in vivo*. If this is true then women who are using these progestins, whether for HRT or contraceptive purposes, will exhibit a deficient CMI response, since a significant reduction of CD8⁺ T cell activation occurred in our *in vitro* model at concentrations reached during HRT as well as during contraception. We can not extrapolate with certainty from our results *in vitro* using NET-A, that NET-En would have the same effect *in vivo* as a contraception. It is however worth mentioning that

both NET-A and NET-En are metabolized to NET, which together with their respective metabolites, have been shown to be the active compounds (Stanczyk & Roy, 1990). Therefore a great possibility exists that both these compounds might have the same influence *in vitro* and *in vivo*. Specifically, women who are using MPA might be at a greater risk, seeing that it inhibited the activation of CD4⁺ and CD8⁺ T cells in this specific group. This prevention of proper T lymphocyte function could also have repercussion on lymphocyte migration. However, no *in vivo* data exists that can support this hypothesis.

Due to the effects MPA had on CD4⁺ T Lymphocytes, it became a matter of interest to investigate the impact MPA and NET-A would have on the helper subsets of these cells, namely T_H1 and T_H2 cells (Chapter 4). Activation of T Lymphocytes ultimately leads to cytokine up regulation and secretion. Due to the observed interference of early cellular activation events and the suppressive effects that MPA and NET-A had on especially CD8⁺ T cells, it became the next logical step to investigate the effects these two compounds had on the CD8⁺ cell-specific chemokine, RANTES (Chapter 5).

CHAPTER 4

AN INVESTIGATION OF THE EFFECTS OF MPA AND NET-A ON THE CD4⁺ T CELL SUBSETS (T_H1 VERSUS T_H2)

Abstract

Aim: The existence of different subsets of CD4⁺ helper T lymphocytes forms a large part of the basis of an effective immune system. These helper subsets, termed T_H1 and T_H2 cells, differ in their cytokine secretion patterns and effector functions, while the ratio between these two subsets play an important physiological role. We attempted to determine the effects of MPA, NET-A and Dex on these subsets.

Method: The effects of MPA, NET-A and Dex on these subsets were determined *in vitro* by targeting the exclusive cytokines secreted by each subset.

Results: Dex significantly changed the T_H2:T_H1 ratio as well as significantly reduced T_H1 and T_H2 cells in donors at various concentrations. MPA and NET-A displayed a selective and weak activity on the T_H1 and T_H2 subsets as well as the ratio between them.

Conclusion: The activities of MPA and NET-A on the helper subsets were not as significant as that of Dex.

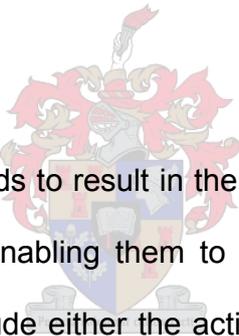
[KEY WORDS: MPA, NET-A, Dexamethasone, CD4⁺ T cells, T_H1 cells, T_H2 cells, T cell helper subsets, contraceptives]

4.1 Introduction

The heterogeneity of immune responses can be ascribed to the existence of different subsets of CD4⁺ helper T lymphocytes that differ in their cytokine secretion patterns and effector functions. It was Mosmann *et al.* (1986) who first demonstrated that mouse CD4⁺ T cell clones could be subdivided into two distinct populations based on their cytokine secretion profile. The two most readily noticeable populations were termed T_H1 and T_H2, with T_H1 clones being the ones that produced IL-2, IFN-γ and TNF-β, and T_H2 clones producing IL-4, IL-5, IL-6 and IL-13. Research on human CD4⁺ T helper clones later revealed a third subset, named T_H0, which produces both T_H1- and T_H2-type cytokines (Piccinni *et al.*, 1995; Wierenga *et al.*, 1990; Del Prete *et al.*, 1991; Haanen *et al.*, 1991). IL-10 was originally described as a product of T_H2 clones (Fiorentino *et al.*, 1989), but it is now well known that IL-10 is also secreted by T_H1 cells and activated macrophages (Sornasse *et al.*, 1996). These cytokines secreted by the subsets, together with CD4⁺ T cells, play an integral role in the initiation of acute inflammation and maintenance of chronic inflammatory responses. This interaction between the T_H clones, as well as the interaction with either macrophages or eosinophils, is referred to as the cytokine network.

The primary function of T_H1 cells is to bring forth phagocyte-mediated defence against intracellular infections, seeing that T_H1 cytokines promote the ability of macrophages to both phagocytose and destroy microbes. There are some T_H1 cells that acquire a cytolytic capacity, but the major cytolytic activity yields from the production of IL-2 and IFN-γ by T_H1 cells which in turn promote the differentiation of CD8⁺ T lymphocytes into

active cytotoxic cells. Several cytokines produced by T_H2 cells have immunosuppressive actions; therefore the principal function of T_H2 activation is to inhibit acute and chronic inflammation as well as delayed-type hypersensitivity reactions. The macrophage activating action of IFN- γ is antagonized by IL-4 and IL-13, while IL-10 suppresses various macrophage responses. The question has been raised whether T_H2 cells play an important physiological function in the regulation of immune responses rather than acting as effectors (Abbas *et al.*, 1996). This is seen in the appearance of T_H2 cells late in immune responses that serve to limit the injurious consequences of T_H1 -mediated protective immunity. It has been proposed that some type 2 cytokine secreting $CD8^+$ T cells have a similar regulatory function as T_H2 cells (Bloom *et al.*, 1992).



Infection by viruses or bacteria tends to result in the production of cytokines that favour the generation of T_H1 cells and enabling them to activate the appropriate protective responses. These responses include either the activation of CTLs, or the activation of the bactericidal action of macrophages through the release of IFN- γ . IFN- γ is the primary T_H1 effector cytokine and it has two key functions. Firstly it enhances the microbial actions of macrophages by activating them and, secondly, it stimulates the production of IgG antibodies. Some of the other major effects of IFN- γ include; increasing the expression of MHC on macrophages and other local cells, increasing macrophage antigen processing through proteosomes, inducing macrophage maturation, maintaining the expression of functional IL-12 receptors on $CD4^+$ T cells, increasing NK cell activity, inhibiting T_H2 cells, and causing mild antiviral effects. The IgG antibodies bind to high-affinity Fc γ receptors and complement proteins and

therefore serve as the principal antibodies involved in the phagocytosis of particulate microbes (Abbas *et al.*, 1996). In addition, macrophages also produce cytokines (which includes IL-1, IL-12 and IL-18) that act on T cells. IL-1, along with co-stimulatory molecules such as CD40, acts as general activators of all classes of T cells. T_H1 and NK cells are preferentially activated by IL-12 and IL-18 respectively, causing these cells to secrete IFN- γ and more TNF- α . Macrophages and dendritic cells produce IL-12 which is the principal T_H1-inducing cytokine. IL-12 activates three putative transcription factors, namely Stat1, Stat3 and Stat4 (Trinchieri, 1995). A strong positive feedback loop between T cells and macrophages is generated by this exchange of cytokines, thereby skewing the immune response towards a T_H1 pattern.

Parasitic infections (e.g., worms) generate cytokines that stimulates T_H2 cell production, thereby causing the activation of B cells and eosinophils. The activated B cells produce antibody which can neutralize the extracellular toxins or opsonize the extracellular bacteria. IL-4 and IL-5 are the signature cytokines of T_H2 cells. IL-4 is the foremost inducer of B-cell switching to IgE production, making it a key initiator of IgE-dependent mast-cell-mediated reactions (Galli, 1993). IL-4 signals through the activation of the transcription factor Stat6 and a protein first identified as an insulin-response substrate, called IRS-2 (Ryan *et al.*, 1996). The other signature cytokine of T_H2 cells, IL-5, serves as the principal eosinophil-activating cytokine (Wardlaw *et al.*, 1995). As previously mentioned, several cytokines produced by T_H2 cells have anti-inflammatory actions. It has also been shown that T_H1 and T_H2 clones may have distinct surface markers, where all T_H2 clones showed persistent expression of the CD30 molecule and T_H1 clones were consistently CD30-negative (Del Prete *et al.*, 1995).

It is worth mentioning that there are two other factors that are important in determining the balance between T_H1 and T_H2 subsets in immune responses. First, the dose or concentration of the antigen where, with a few exceptions, low antigen concentrations and low-dose infections tend to preferentially induce T_H1 responses and high doses induce T_H2 development (Bretscher *et al.*, 1992). The other factor is co-stimulation, which enhances specific T-cell responses through signals provided by APCs working together with antigen. Collectively, it can be said that each T cell subset produces cytokines that serve as their own autocrine growth factor which promotes differentiation of naive T cells to that subset and these two subsets also produce cytokines that cross-regulate each other's development and activity. Therefore, the net outcome of cytokine-mediated self-amplification and cross-regulation is that as soon as an immune response by T cells begins to develop along one of the two pathways, it tends to proceed progressively in that direction. However, the biochemistry of the development of T_H1 and T_H2 populations remains poorly understood, despite extensive research in this field. Little is known about the signals that steer the differentiation of these subsets other than the cytokine-induced STAT-pathways.

T_H1 and T_H2 subsets play a crucial role in the prevention and eradication of intra- and extracellular infections, but their malfunction is also associated with severe immune disorders. Considerable thought has been given to regulate the balance of T_H1 and T_H2 helper subsets as an immunotherapy for certain diseases, because these subsets play an important role in the health of an individual. Asthma is a disease largely mediated by T_H2 responses. The potential for treating this disease lies in converting the T cell balance to one in which T_H2 no longer dominates. The development and activation of

allergen-specific T_H2 cells are normally associated with allergic reactions involving IgE and mast cells (Romagnani, 1994). High levels of total and allergen-specific serum IgE are found in individuals with severe atopy. A strong correlation exists between serum IgE and frequencies of allergen-specific T_H2 cells that can be propagated from peripheral blood lymphocytes in some of these individuals (Romagnani, 1994). Elucidation of the genetic control of IgE-mediated allergy will provide valuable insight into the mechanisms responsible for the development of allergic diseases, and even more so, into the control of T cell differentiation pathways.

On the other hand, T_H1 cells are the main effector cells in several organ-specific autoimmune diseases. One such example is the inflammatory demyelinating disease of the central nervous system known as multiple sclerosis. The development of symptoms is caused by T cell-mediated autoimmune responses to central nervous system components and the lesions of many patients are infiltrated by macrophages and primarily T_H1 cells. The selective induction of T_H2 cells has been proposed as a treatment for tissue autoimmune diseases (Rocken *et al.*, 1996). Granulomatous inflammation, arthritis and colitis can arise from antimicrobial T_H1 responses and, in many cases, the host tissue damage is caused by the toxic side effects of cytokines and other inflammatory mediators released during the normal immune attack on the pathogen (Romagnani, 1994). This can also be the case with T_H2 responses, where the antihelminthic response of T_H2 cells can be detrimental to the host by contributing to granuloma formation and hypereosinophilia (Romagnani, 1994; Wynn & Cheever, 1995).

Pathogen growth and immunopathology are both influenced by the balance of T_H1 and T_H2 responses to an infectious agent. There are numerous examples of experimental models in which the administration of recombinant cytokines or cytokine antagonists are used to modify the T_H1/ T_H2 balance and ultimately alter the outcome of a disease. It is well accepted that the induction of T_H1 responses, in particular with the macrophage-activating cytokines, can be linked to a resistance against many intracellular microbes (Sher & Coffman, 1992; Kaufman, 1993). The administration of IL-12 at the time of infection enhances the resistance to many intracellular protozoan, pathogenic bacteria and fungi, and some viruses (Trinchieri, 1995). The resistance to infection can also be promoted by using IL-12 as a vaccine adjuvant with sensitizing doses of antigen, thereby converting the response from a T_H2 to a T_H1 pattern (Afonso *et al.*, 1994).

There are several ways to measure the T_H1 and T_H2 subsets in an individual. Reverse transcriptase polymerase chain reaction (RT-PCR) is used to measure the appropriate cytokine mRNA of T_H1 and T_H2 subset. An ELISA can also be used to measure the secreted protein in the supernatant of cultured T lymphocytes in order to determine the T_H1/ T_H2 balance. Probably the most accurate way of measuring the T_H1 and T_H2 subsets is by way of flow cytometry. Fluorescent antibodies directed against the major cytokines of each subset, namely IFN- γ and IL-4, are used in cytometric analysis. The role of steroid hormones in altering the T_H1/ T_H2 ratio has been documented previously. Glucocorticoids have been shown to reduce IL-2 and IFN- γ and increase IL-4 production (Dayens *et al.*, 1991; Rook *et al.*, 1994). Other studies showed that Dex negatively regulated IL-5 gene expression (Wang *et al.*, 1993), while progesterone induced the expression of the IL-5 gene (Piccinni *et al.*, 1995; Wang *et al.*, 1993) and

also IL-4 (Piccinni *et al.*, 1995). The synthetic progestins, MPA and NET-A, are able to bind to various steroid receptors. This interaction of MPA and NET-A with steroid receptors might lead to interference in the T_H1/ T_H2 balance. The aim of this study was therefore to determine the effects of these two hormones on T_H1 and T_H2 subsets using flow cytometric analysis.

4.2 Materials and Methods

4.2.1 Study design

Blood samples were collected from 10 healthy individuals, 5 females (between 20-34 yr of age) and 5 males (between 20-40 yr of age), who were not on any hormonal treatment, including any form of contraception. Medroxyprogesterone acetate (Sigma, Sigma Aldrich), Norethisterone acetate (Sigma, Sigma Aldrich) and Dexamethasone (Sigma, Sigma Aldrich) were used at 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} and 10^{-10} M final concentrations. A 70% ethanol solution was used to dissolve as well as dilute all three steroids. These concentrations were chosen to represent different physiologic states these steroids are found in, where higher concentrations e.g. 10^{-7} M to 10^{-9} M are found in hormone replacement therapy (Adams *et al.*, 1997; Stanczyk, 2003) and lower concentrations e.g. 10^{-8} M and 10^{-9} M are reached in hormonal contraception (Mishell, 1996; Zalanyi *et al.*, 1984; Smit *et al.*, 2004).

4.2.2 Lymphocyte separation

Venous blood samples were collected in EDTA tubes after which the anticoagulated blood was double diluted with sterile PBS. Respectively, 3mL of Histopaque-1077 (Sigma Diagnostics) was added to 15mL conical tubes and the diluted blood was carefully layered onto the Histopaque-1077. The tubes were centrifuged at 1800 rpm for 25 minutes at 4°C. The top layer above the mononuclear cell layer (buffycoat) was removed and the buffycoat was carefully transferred with a sterile Pasteur pipette to clean 15mL conical tubes. The cells were washed with sterile PBS and centrifuged at 1500 rpm for 15 minutes at 4°C. The supernatants were discarded and the pellet was resuspended in PBS and centrifuged at 1800 rpm for 10 minutes at 4°C. The supernatant fluid was removed and the cells were resuspended in 1mL Complete medium (RPMI 1640, Bio Whittaker, Sterilab; 10% fetal calf serum; 0.02% PEN/STREP). A 1:10 dilution of the cells was made in TURKS white cell counting fluid (0.0002% Gentian Violet and 7.41% of 5% Glacial Acetic Acid in distilled H₂O) and the cells were counted using a Neubauer counting chamber. The cells were diluted with complete medium to a final concentration of 1x10⁶ cells/mL.

4.2.3 Determination of T_H1 and T_H2 subsets

Thirty-eight (38) Facs tubes (Falcon[®], Becton Dickinson, Scientific Group) were set up as follow: Five-hundred (500) µL of the diluted cells were added to each tube and 19 of the tubes were stimulated with 0.25µg/mL TPA and 2µg/mL Ionomycin. Ten (10) µg/mL Brefeldin A was added to all 38 tubes. Eighteen (18) tubes of the stimulated samples and 18 tubes of the non-stimulated samples were divided into three groups of six tubes each. One group out of each set was made up with six different concentrations of each

steroid (MPA, NET-A or Dex) in order to reach final concentrations of 10^{-5} to 10^{-10} M of each steroid. An equivalent volume (10 μ L of a 70% ethanol solution) to the volume of steroids added to the tubes was added to the stimulated, with no steroid, tube and the non-stimulated, with no steroid, tube to compensate for the 70% ethanol solution that was used to dilute the steroids. All the tubes were made up to 1mL with complete medium and incubated overnight at 37°C and 98% humidity in a CO₂ incubator (NuAir CO₂ Water-Jacketed Incubator, Centrotec).

After incubation the tubes were centrifuged for 5 minutes at 1800 rpm and the supernatants were discarded. Twenty (20) μ L of CD3 PerCP (Becton Dickinson, Scientific Group) were added to all the tubes, whereafter the tubes were vortexed and incubated for 15 minutes in the dark at room temperature. Thereafter 2mL of lysing solution (FACS lysing solution, Becton Dickinson, Scientific Group) was added to all the tubes and the tubes were incubated for 10 minutes. The samples were then centrifuged for 5 minutes at 1800 rpm and the supernatants were discarded, whereafter 500 μ L of a 0.3% Saponin-PBS solution with 30 g/L PEG 4000 was added to all the tubes and incubated for 25 minutes at room temperature. The tubes were centrifuged again for 5 minutes at 1800 rpm and the supernatants were poured off. After this, the cells were washed by resuspending them in 2mL of a 0.1% Saponin-PBS solution and centrifuging for 5 minutes at 1800 rpm.

The supernatants were then discarded and 20 μ L of a 1:2 dilution of IFN- γ -FITC/IL-4-PE monoclonal antibodies (FastImmune Interferon- γ /IL4, Becton Dickinson, Scientific Group) and 0.1% Saponin was added to each tube and incubated in the dark for 20

minutes. The cells were washed again by adding 2mL of 0.1% Saponin-PBS solution to each tube, centrifuging for 5 minutes at 1800 rpm and discarding the supernatants. The cells were then resuspended in 500µL of fixative (5% Formaldehyde-PBS solution) and analyzed on flow cytometer (FacsCaliber, Becton Dickinson, Scientific Group) using CellQuest Software (Becton Dickinson, Scientific Group). Figure 54 is an example of our flow cytometric analysis on the effects MPA, NET-A and Dex have on the helper subsets of CD4⁺ T cells. Experiments were performed on the blood of 5 female donors and 5 male donors, thereby assuring reproducibility.

4.2.4 Data analysis

The background, represented by the corresponding non-activated value, of each activated subset was first deducted from each corresponding value. Primary analysis was done on values represented by T_{H2}:T_{H1} ratios, which was achieved by dividing each T_{H2} value with its corresponding T_{H1} value. Secondary analysis was done on the results of each individual subset, namely T_{H1} and T_{H2}. This analysis was done to determine which subset was responsible for an observed change in ratio or whether both subsets were influenced to the same extent and thereby causing no change in ratio. Analysis of datasets were achieved by repeated measures ANOVA followed by post testing using Dunnett's test. Post testing was only done when there was significant pairing ($p < 0.05$) among the groups. Analysis was done on the combined male and female groups (n=10), as well as on the male and female groups separately (n=5). All results were considered statistically significant when $p < 0.05$.

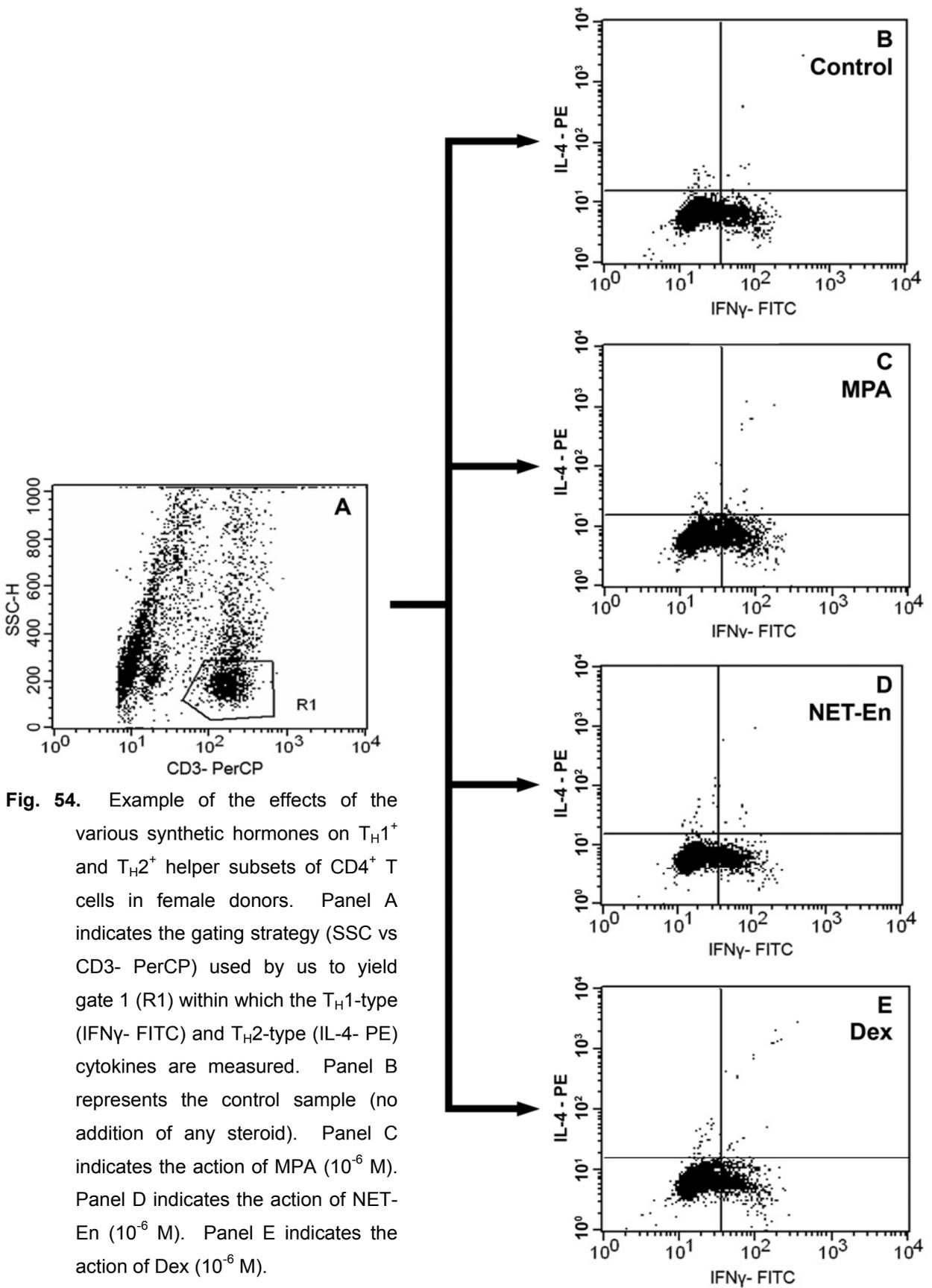
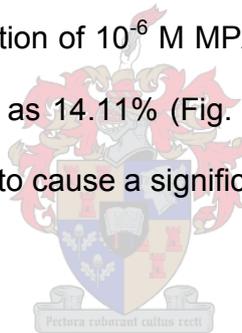


Fig. 54. Example of the effects of the various synthetic hormones on T_H1^+ and T_H2^+ helper subsets of $CD4^+$ T cells in female donors. Panel A indicates the gating strategy (SSC vs CD3- PerCP) used by us to yield gate 1 (R1) within which the T_H1 -type (IFN γ - FITC) and T_H2 -type (IL-4- PE) cytokines are measured. Panel B represents the control sample (no addition of any steroid). Panel C indicates the action of MPA (10^{-6} M). Panel D indicates the action of NET-En (10^{-6} M). Panel E indicates the action of Dex (10^{-6} M).

4.3 Results

Monoclonal antibodies (mAbs) directed against CD3 were used to identify T lymphocytes, while mAbs directed against γ IFN and IL-4 were used to differentiate between T_H1 and T_H2 subsets respectively. Once again, the data was analysed according to the gender of the blood donors in case the biological effect was more pronounced on a particular gender.

Analysis of the combined group showed that MPA did not have a significant effect on the $T_H2:T_H1$ ratio (Data not shown). However, analysis on the separate T helper subsets indicated that a concentration of 10^{-6} M MPA significantly ($p < 0.05$) reduced the amount of T_H1 cells with as much as 14.11% (Fig. 55). This change in the amount of T_H1 cells by MPA was not enough to cause a significant change in the $T_H2:T_H1$ ratio.



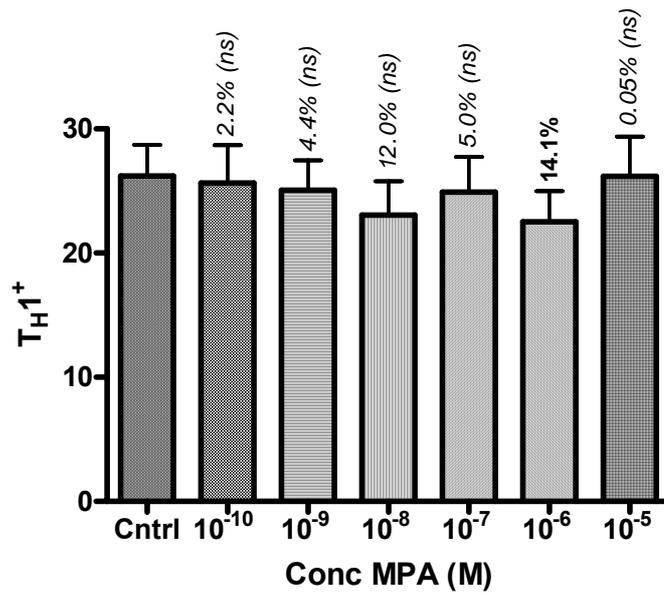


Figure 55. Reduction of T_H1^+ cells by different concentrations of MPA in the combined group, compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

NET-A caused a general decrease in the $T_H2:T_H1$ ratio, but the only concentration of NET-A that had a statistically significant effect on the $T_H2:T_H1$ ratio in the combined group was 10^{-5} M, which caused a 54.6% decrease in this ratio (Fig. 56). Secondary analysis on the individual subsets revealed that a 55.3% decrease of T_H2^+ cells by 10^{-5} M NET-A was responsible for this change in $T_H2:T_H1$ ratio (Fig. 57).

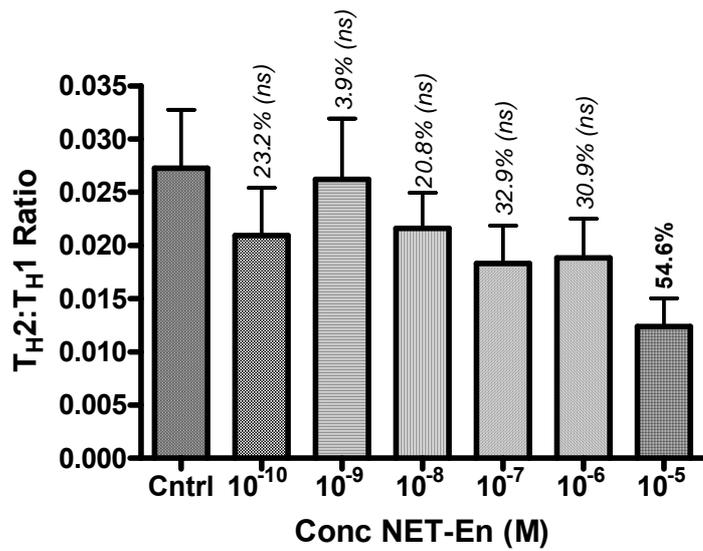


Figure 56. Change in $T_H2:T_H1$ ratio in the combined group caused by different concentrations of NET-A, compared to controls (Cntrl) ($n=10$). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.



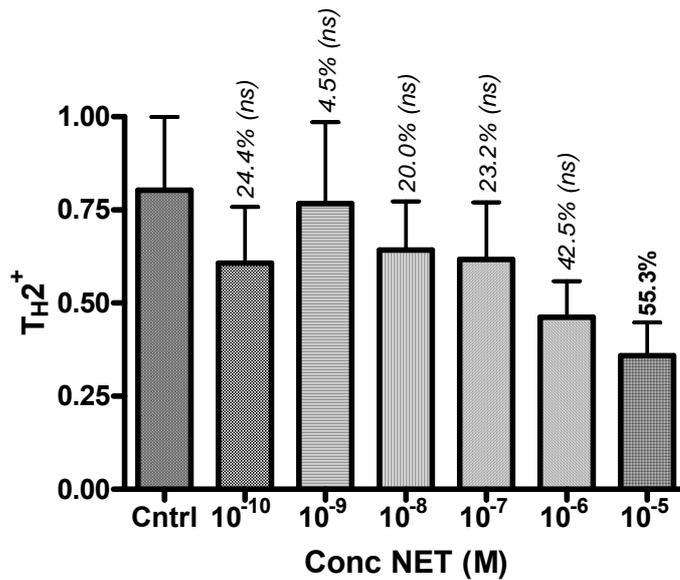


Figure 57. Reduction of T_{H2}⁺ cells by different concentrations of NET-A in the combined group, compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

The T_{H2}:T_{H1} ratio of combined group was significantly affected by all concentrations of Dex tested (Fig. 58). Dex at a concentration of 10⁻⁷ M caused the most inhibition of 62.8%, while 10⁻⁵ M Dex caused the least change. Analysis on the influence of Dex on the separate helper subsets of the combined group showed that the effects of Dex was more pronounced on the T_{H2}⁺ cells than the T_{H1}⁺ cells (Fig. 59 & 60).

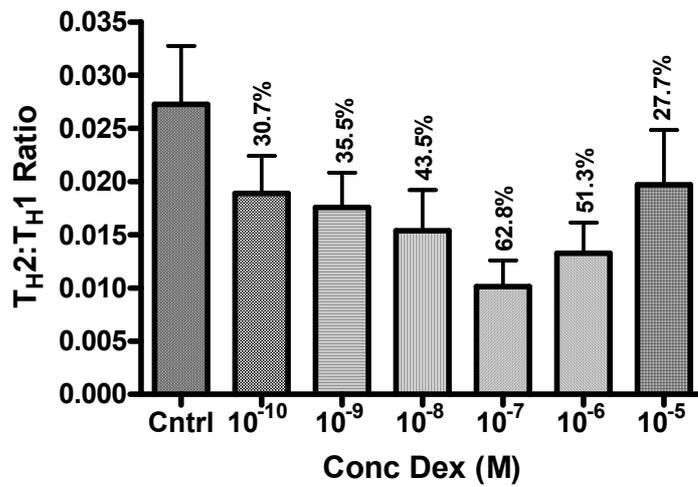


Figure 58. Change in $T_H2:T_H1$ ratio in the combined group caused by different concentrations of Dex, compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

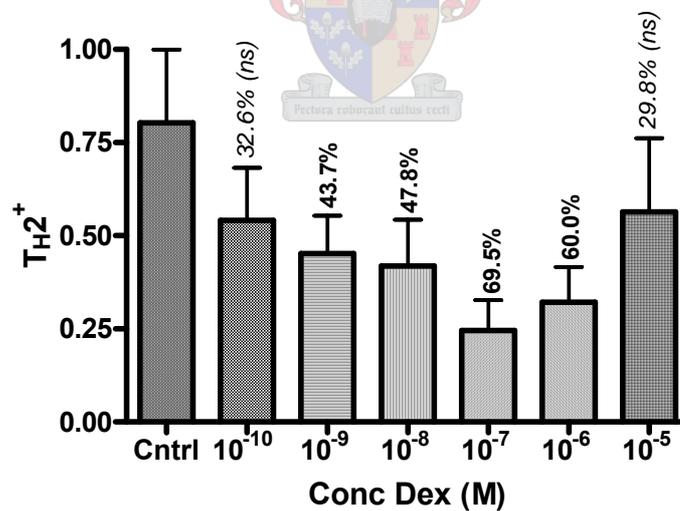


Figure 59. Reduction of T_H2^+ cells by different concentrations of Dex in the combined group, compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

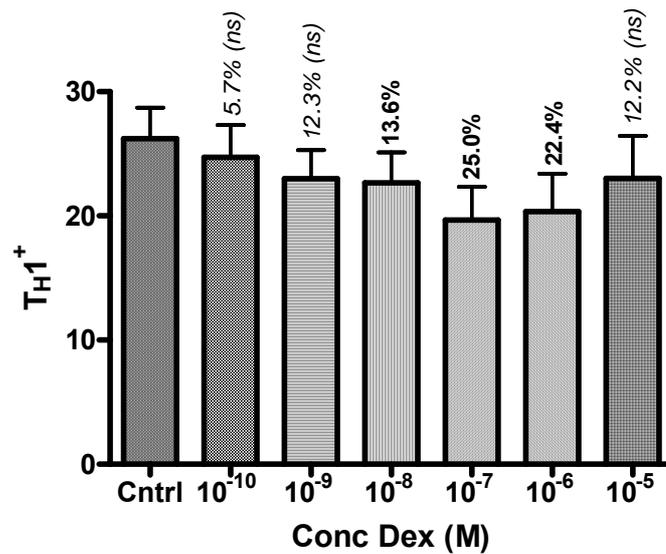


Figure 60. Reduction of T_H1^+ cells by different concentrations of Dex in the combined group, compared to controls (Cntrl) ($n=10$). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.



When the data was analyzed according to gender, MPA demonstrated no significant effect on the $T_H2:T_H1$ ratio in female donors and this was supported by MPA having no effect on either of the two helper subsets (Data not shown). Similar results were demonstrated for NET-A in female donors by it not having a significant effect on the $T_H2:T_H1$ ratio or any of the two subsets (Data not shown). In female donors, 10^{-6} M – 10^{-9} M Dex caused a statistically significantly change in the $T_H2:T_H1$ ratio (Fig. 61). Analysis on the separate helper subsets revealed that Dex did not have a significant effect on T_H1^+ cells (Data not shown), but that it significantly reduced T_H2^+ cells in females at 10^{-6} M and 10^{-7} M (Fig. 62).

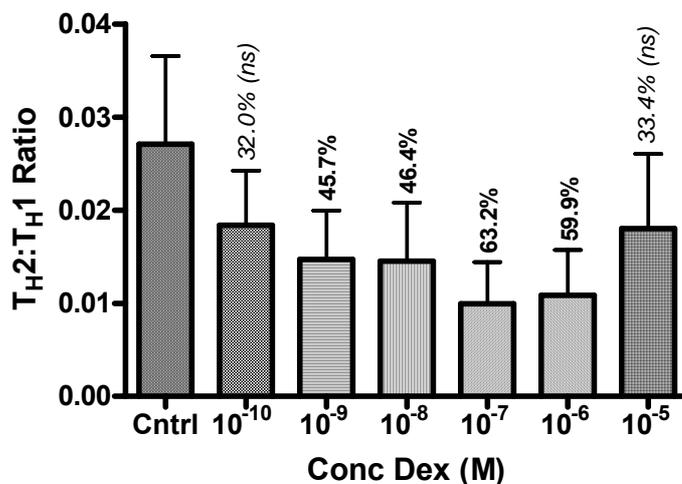


Figure 61. Change in $T_{H2}:T_{H1}$ ratio in female donors caused by different concentrations of Dex, compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

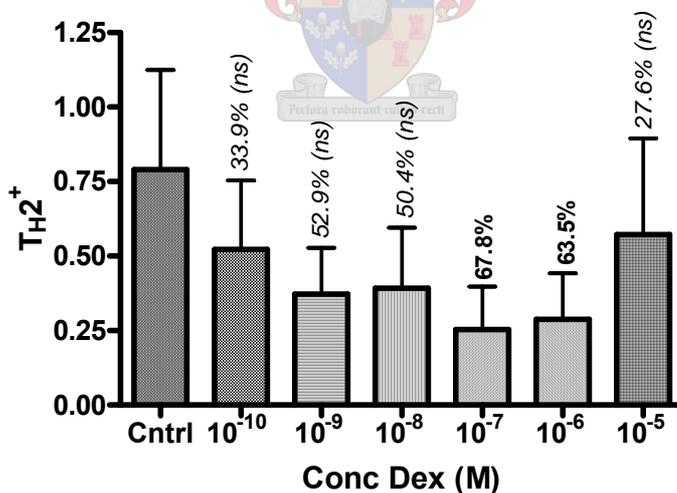


Figure 62. Reduction of T_{H2}^+ cells by different concentrations of Dex in female donors, compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

MPA did not have a significant effect on the ratio of T_H1^+ and T_H2^+ cells in male donors and this was further verified by the fact that it did not have a significant effect on either one of the two helper subsets (Data not shown). NET-A caused a general change in the ratio of the male donor group, however, the only statistical significant ($p < 0.05$) change in the ratio of T_H1^+ to T_H2^+ cells occurred at a concentration of 10^{-5} M (Fig. 63), which also explains the observed effect at 10^{-5} M NET-A in the combined group. Analysis on the separate subsets revealed however that neither of the T-helper subsets was significantly affected by NET-A (Data not shown).

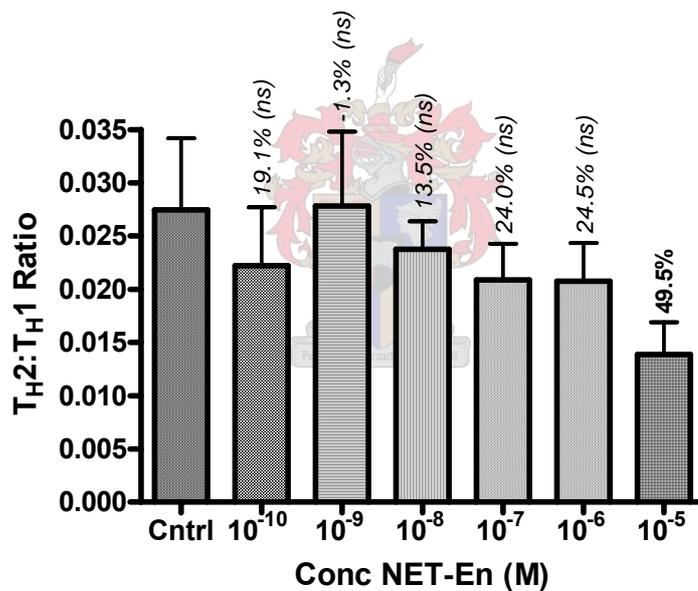


Figure 63. Change in $T_H2:T_H1$ ratio in male donors caused by different concentrations of NET-A, compared to controls (Cntrl) ($n=5$). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. A negative value indicates an increase. Significant values are represented in bold, while non significant (ns) values are represented in italics.

The ratio of T_H1^+ to T_H2^+ cells in male donors was significantly affected by concentrations of Dex ranging from 10^{-6} M to 10^{-8} M (Fig. 64). Dex at a concentration of 10^{-7} M brought about the highest significant degree of change (62.4%; $p < 0.01$), while the lowest significant change of 40.7% ($p < 0.05$) occurred at 10^{-8} M of Dex. Analysis on the separate T-helper subsets revealed that 10^{-6} M and 10^{-7} M Dex significantly reduced both T_H1^+ and T_H2^+ cells in male donors (Fig. 65 & 66).

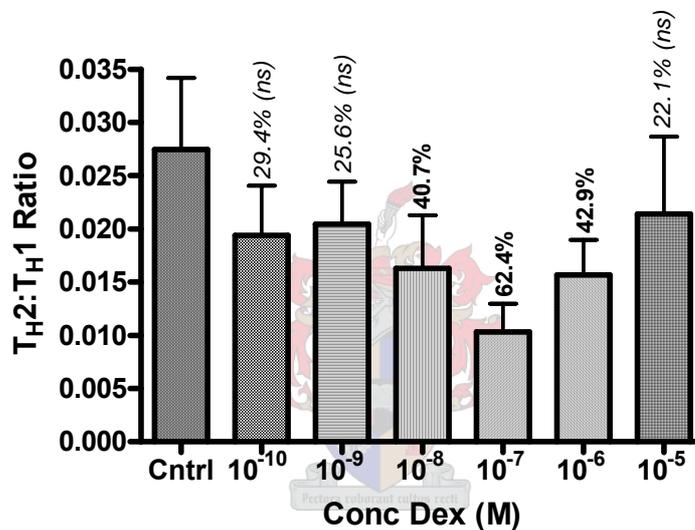


Figure 64. Change in $T_H2:T_H1$ ratio in male donors caused by different concentrations of Dex, compared to controls (Cntrl) ($n=5$). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

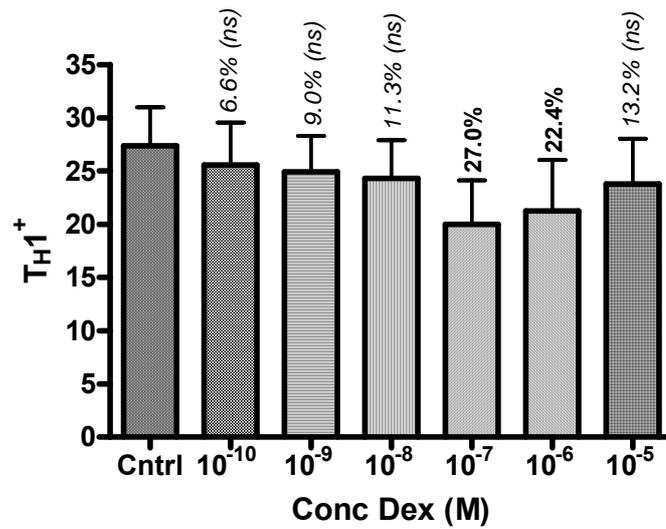


Figure 65. Reduction of T_H1⁺ cells by different concentrations of Dex in male donors, compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.



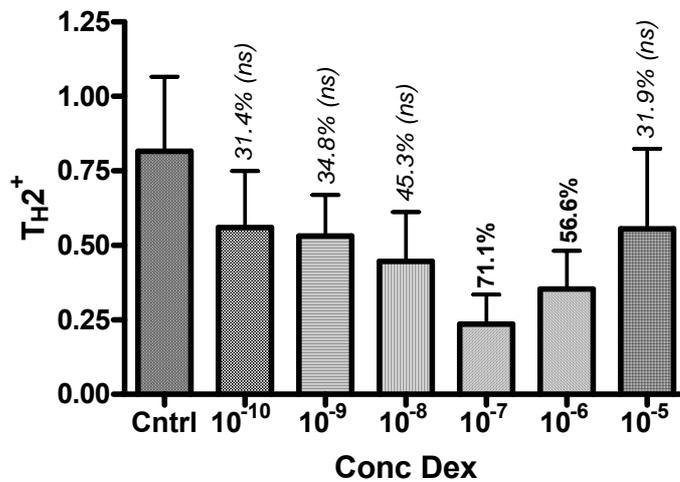


Figure 66. Reduction of T_{H2}^+ cells by different concentrations of Dex in male donors, compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

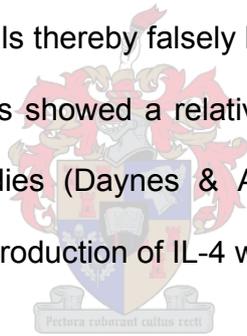


4.4 Discussion

Glucocorticoids have been shown to negatively regulate IL-5 gene expression and therefore have an impact on T_{H1}^+ subsets (Wang *et al.*, 1993). In our study, Dex significantly changed the $T_{H2}:T_{H1}$ ratio in the combined gender group at all concentrations tested compared to controls. An increase in the $T_{H2}:T_{H1}$ ratio can be caused by either an increase in T_{H2}^+ cells, or a decrease in T_{H1}^+ cells or both of these events. Conversely a decrease in the $T_{H2}:T_{H1}$ ratio will be caused by a decrease in T_{H2}^+ cells, or an increase in T_{H1}^+ cells, or the occurrence of both these events. When the effect of Dex on the separate helper subsets was analysed it was discovered that Dex had a slightly more potent inhibitory effect on T_{H2}^+ cells than on T_{H1}^+ cells. These

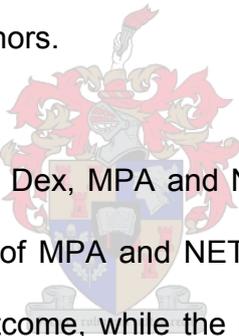
findings coincides with previous studies, one demonstrating that Dex inhibited IL-4 and IL-5 release from murine T_H2-type cell clones (Schmidt *et al.*, 1994), while other studies demonstrated that Dex decreased IFN- γ levels (Agarwal & Marshall, 2001; Krakauer, 1995; Kunicka *et al.* 1993).

However, there are also studies that demonstrate an increase in IL-4 production caused by GCs (Daynes & Araneo, 1989; Rook *et al.*, 1994). Most of these studies were based on ELISA results (i.e. secreted proteins). It is known that such results are not accurate and/or sensitive due to the autocrine nature of most of these cytokines. This would mean that as the cytokines are produced some bind to the receptors on the producing cells as well as on the adjacent cells thereby falsely lowering the measured levels. This could explain why previous studies showed a relative decline in IFN- γ production. As previously mentioned, some studies (Daynes & Araneo, 1989; Rook *et al.*, 1994) showed a relative increase in the production of IL-4 which is contrary to our findings.



This influence of Dex on T_H1 and T_H2 subsets was also demonstrated in the analysis of the separate gender groups. Dex (10^{-6} M, 10^{-7} M, 10^{-8} M and 10^{-9} M) caused a statistically significant change in T_H2:T_H1 ratio of up to 63.2% at 10^{-7} M in female donors, while it had more or less the same effect in male donors. Separate analysis on the subsets revealed that Dex (10^{-6} M and 10^{-7} M) significantly reduced T_H2⁺ cells in female donors, but had no significant effect on the T_H1 subset. Dex had a similar effect on the T_H2:T_H1 ratio in males, but conversely, Dex significantly reduced both T_H1⁺ and T_H2⁺ cells in male donors. This could have been caused by a difference in receptor levels existing in males and females.

MPA and NET-A had a diminished and selective effect on T_H1 and T_H2 cells, despite the significant effects MPA had on $CD4^+$ T lymphocyte activation (Chapter 3). The only statistical significant value was seen at 10^{-6} M MPA where it significantly decreased the $T_H2:T_H1$ ratio in the combined group, while analysis on the separate helper subsets revealed that neither of the subsets was affected significantly by MPA. Furthermore, MPA demonstrated no significant effects on the $T_H2:T_H1$ ratio of either of the two helper subsets in female as well as male donors. NET-A only had a statistically significant decreasing effect on the $T_H2:T_H1$ ratio in the combined group at the highest concentration tested (10^{-5} M) and this was due to the 55.3% decrease it caused in T_H2^+ cells. Similar to MPA, NET-A had no significant effect on the $T_H2:T_H1$ ratio or either helper subset in female or male donors.



The observed differences between Dex, MPA and NET-A could have been caused by the probability that the interaction of MPA and NET-A with their various receptors did not translate into the expected outcome, while the receptor interaction of Dex had an influence on T_H1^+ and T_H2^+ cells. However, this statement cannot be substantiated in case MPA and/or NET-A may require longer incubation periods than what we used (longer than 12 hrs) in order to mediate the end-effect we were measuring. Once again, a complex network of receptor interactions and steroid behaviour has to be taken into consideration when attempting to clarify our results. The dynamics of this network include the concentrations of the various receptors involved in our system, the competition between these receptors for the different steroids, the degree of metabolism of the compounds in our system, as well as the various receptors that are involved. Any

one, or several, of these factors could have been responsible for the results obtained by us.

The cytokine-mediated self-amplification and cross-regulation of T cells are responsible for the occurrence where as soon as an immune response begins to develop along one of the two pathways, it tends to advance progressively in that direction. Therefore it cannot be excluded that we might have been looking at already committed cells which were possibly not susceptible to the effects of MPA or NET-A. If this was the case, and taking into consideration the effect Dex had on T_H1^+ and T_H2^+ cells, the potent effect of this GC becomes apparent. Another matter that has to be taken into consideration is from the studies showing the existence of at least three distinct functional subsets of human $CD4^+$ T helper clones based on their cytokine secretion profile (Del Prete *et al.*, 1991; Yssel *et al.*, 1991; Haanen *et al.*, 1991; Mosmann *et al.*, 1986; Street *et al.*, 1990). The existence and cytokine secretion patterns of T_H1 and T_H2 cells have already been presented in the introduction of this chapter, while a third subset termed T_H0 exists that produces both T_H1 - and T_H2 -type cytokines. Seeing that we used whole blood cultures, thereby including all three helper subsets, it is possible that the cytokine secretion pattern of the T_H0 subsets could have influenced the outcome of our experiments and ultimately have lead to our observed results.

Furthermore, we investigated the direct effects of MPA and NET-A on T helper cells, but not their effects on monocytes. Macrophages produce cytokines, including IL-1, IL-12 and IL-18, which act on T cells (Trinchieri, 1995). Dendritic cells, together with macrophages, also produce IL-12 which is the primary T_H1 -inducing cytokine. T_H1 cells

are preferentially activated by IL-12, therefore it would be of value to investigate the effects MPA and NET-A would have on IL-12 production and release from macrophages. It is also possible that our results are indicative of an “end result” of the effects of the hormones on monocytes/dendritic cells which may be more sensitive when compared to committed T cells. This also requires further experimentation.

Despite extensive research, knowledge about the biochemistry behind the development of T_{H1} and T_{H2} populations remains inadequate. Besides the cytokine-induced pathways, very little is known about the other signals that are responsible for the differentiation of these subsets. Further research on the interaction and differentiation of these T helper subsets and the pathways they follow is imperative and can possibly be very beneficial in our understanding and manipulation of the immune system.



CHAPTER 5

INVESTIGATION OF THE EFFECTS OF MPA AND NET-A ON THE PRODUCTION AND SECRETION OF THE CD8⁺ T CELL-SPECIFIC CHEMOKINE, RANTES.

Abstract

Aim: The cells of the immune system rely on a large family of chemotactic cytokines, called chemokines, for intercellular communication. We previously demonstrated that MPA, NET-A and Dex significantly inhibited the activation of CD8⁺ T lymphocytes. Based on these findings, it became of interest to determine the effects of these three synthetic hormones on one of the CD8⁺ T cell-specific chemokines, RANTES.

Method: RANTES production was measured by means of ELISA in the supernatants of whole blood samples with and without activation in the presence of various concentrations of the three hormones.

Results: Surprisingly, despite the suppressive effects MPA, NET-A and Dex had on CD8⁺ T cell activation, we found that all three hormones caused a general increase in RANTES production. Dex displayed the least significant effects on RANTES production, while the effects of MPA and NET-A were more pronounced.

Conclusion: Various factors were taken into consideration in our effort to determine the reason behind our findings.

[KEY WORDS: RANTES, chemokines, MPA, NET-A, Dexamethasone, contraceptives, HRT]

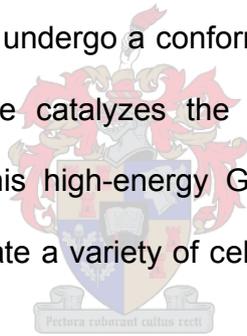
5.1 Introduction

The cells of the immune system rely on intercellular communications to mount an effective immune response. These intercellular communications are made possible by a large family of structurally homologous chemotactic cytokines called chemokines. Members of the chemokine family are 8 – 12 kDa polypeptides containing two internal disulfide loops and they interact specifically with certain leukocyte groups. Almost 50 different chemokines have already been identified and many more may be discovered. Various cells may produce chemokines in response to inflammatory stimuli thereby recruiting leukocytes to sites of inflammation, while other chemokines are produced constitutively in a variety of tissues in order to recruit leukocytes (mainly lymphocytes) to these tissues in the absence of inflammation.

The chemokines are classified into subfamilies on the basis of the conservation of cysteine (C) residues within the aminyl-terminal region of each subfamily. The two major subfamilies are the C-C chemokines, in which the cysteine residues are adjacent, and the C-X-C subfamily, in which these residues are separated by a single amino acid (X). Two other subfamilies consist of a small number of chemokines that have a single cysteine (C family) or two cysteines separated by three amino acids (C-X₃-C). These differences between the subfamilies correlate with their organization into separate gene clusters. The chemokines of the C-C and C-X-C subfamilies are produced by leukocytes and several types of tissue cells, including endothelial cells, epithelial cells, and fibroblasts. Microbes and inflammatory cytokines, mainly TNF and IL-1, are responsible for the secretion of chemokines by many of these cells. Antigen-stimulated

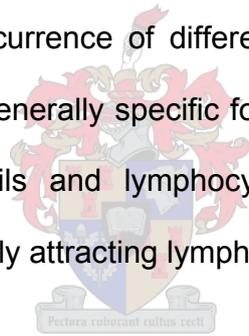
T cells also produce several C-C chemokines, thereby forming a link between the trafficking of inflammatory leukocytes and adaptive immunity. Furthermore, lymphoid organs produce certain chemokines constitutively, thereby enabling physiologic trafficking of lymphocytes through the organs.

At present, there are eleven distinct receptors for C-C chemokines, termed CCR1 to CCR11, and six for C-X-C chemokines, termed CXCR1 to CXCR6. These receptors are expressed on leukocytes, with T cells displaying the highest density of distinct chemokine receptors. All known chemokine receptors are members of the rhodopsin receptor superfamily and have a characteristic structure with seven transmembrane α -helical domains. These receptors undergo a conformational change when occupied by a ligand. This structural change catalyzes the replacement of bound guanosine diphosphate (GDP) by GTP. This high-energy GTP-associated form is capable of transmitting signals that can activate a variety of cellular enzymes, including some that stimulate cellular locomotion.



A likely mechanism of termination of their responses can be seen by the rapid down-regulation of chemokine receptors after exposure to the chemokine. Functional and molecular cloning has elucidated four general categories of chemokine receptors, namely specific, shared, promiscuous, and viral (Schall & Bacon, 1994). Specific receptors bind a single type of chemokine, while shared receptors bind several related chemokines within a subclass. Promiscuous receptors bind chemokines of both the C-C and C-X-C subclass, while some receptors are encoded within a viral genome.

The activities of chemokines as leukocyte chemoattractants were originally the basis for their discovery, but it is now clear that they serve many vital functions in the immune system as well as other systems. Leukocyte recruitment by chemokines to sites of infection occurs as a result of several sequential actions that chemokines have on these cells. Chemokines are expressed on endothelial cells by binding to heparan, where they exert their actions on leukocytes rolling down the endothelium. Chemokines also increase the affinity of leukocyte integrins for their ligands, thereby causing integrin activation which is crucial for firm leukocyte adherence to the endothelium. After this adherence step, chemokines, working synergistically with cytokines, induce the migration of leukocytes into extravascular tissue. The nature of an inflammatory response is controlled by the occurrence of different chemokines acting on different cells. The C-C chemokines are generally specific for monocytes and lymphocytes (but not neutrophils), while neutrophils and lymphocytes are attracted by the C-X-C chemokines and C chemokines only attracting lymphocytes.



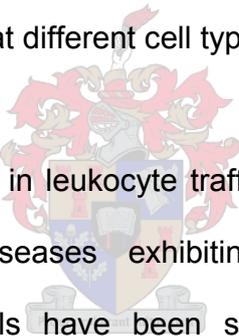
Another important function of chemokines is their role in the normal migration of leukocytes through peripheral lymphoid tissues. Migration of previously activated effector and memory T cells to nonlymphoid tissues, like mucosal organs and skin, are also promoted by a range of chemokines. The association of different cell types with different tissues is largely due to which chemokine receptors are expressed on the cell types and which chemokines are secreted by the tissues. An unexpected role of chemokines, where they are involved in development of diverse nonlymphoid organs, raises the possibility of the existence of numerous undiscovered functions of chemokines in morphogenesis.

RANTES (regulated on activation, normal T cell expressed and secreted) is an 8 kDa protein and a member of the C-C chemokine subfamily (Schall & Bacon, 1994). The mature 8 kDa secreted protein is formed after a 23 amino acid signal peptide is cleaved from the protein. The resulting 68 amino acid protein contains four cysteine residues, where the C-C chemokine namesake is represented by Cys10 and Cys11. Disulfide bonds are formed respectively between Cys10-Cys34 and Cys11-Cys50. The RANTES gene has been localized in humans to chromosome 17, a location shared with other C-C chemokines (Nelson *et al.*, 1993).

Since different receptors have an affinity for RANTES, a receptor that is specific for RANTES has not yet been discovered, while the human C-C chemokine receptor 1 (C-C CKR-1) shares its preference predominantly between RANTES and MIP-1 α (Neote *et al.*, 1993a). The only chemokine receptor identified so far that binds both C-C and C-X-C chemokines is the Duffy blood group antigen-erythrocyte chemokine receptor (DFA-ECKR) (Neote *et al.*, 1993b; Neote *et al.*, 1994). This receptor is present primarily on red blood cells and is recognized by antibodies defining the Duffy blood group antigen. A C-C chemokine receptor is encoded within the genome of CMV and its presumable function is to augment viral evasion of the host immune antiviral response (Neote *et al.*, 1993a; Schall *et al.*, 1994). The major receptors that bind RANTES are CCR1 and CCR5, thereby constituting the systemic name, CCL5, given to RANTES.

RANTES has been shown to be a chemoattractant (and in some cases an activator) for a variety of leukocytes, including lymphocytes (Schall *et al.*, 1988), natural killer (NK) cells (Maghazachi *et al.*, 1994), eosinophils (Rot *et al.*, 1992; Kameyoshi *et al.*, 1994),

basophils (Bacon *et al.*, 1994; Alam *et al.*, 1992), and monocytes (Schall *et al.*, 1990). The fact that RANTES could exhibit marked discrimination between closely related cell types was demonstrated by the ability of the protein to be chemotactic for the migration of CD4⁺ T cells of the memory (CD45 RO⁺) phenotype but not for the naive phenotype (CD45 RA⁺) (Schall *et al.*, 1988). RANTES mRNA is expressed constitutively in T lymphocytes (Schall *et al.*, 1988), NK lines, and NK clones (Nelson *et al.*, 1993), while it is expressed upon stimulation in activated peripheral blood lymphocytes (Schall *et al.*, 1988), fibroblast cells (Schall, 1991), macrophages, and endothelial cells (Devergne *et al.*, 1994). These cells can be stimulated with various agents, such as antigen, the T cell mitogen phytohemagglutinin, TNF- α , IFN- γ , IL-1 β , or a combination of these agents. A very interesting observation is that different cell types respond to different inducers.



The important role RANTES plays in leukocyte trafficking reflects the potential clinical significance of RANTES in diseases exhibiting an inflammatory component. Eosinophils, T-cells and basophils have been shown to be chemoattracted and activated by RANTES, while these cells are also the cells most often associated with chronic inflammatory or autoimmune processes, including asthma and allergy (Schall & Bacon, 1994). It has been shown that long-term MPA treatment could suppress the inflammatory response within endometrial implants by inhibiting RANTES gene expression via a progesterone-mediated suppression of gene transcription (Zhao *et al.*, 2002). Progesterone has been shown to inhibit chemokine secretion (including MIP-1 α , MIP-1 β and RANTES) by CD8⁺ T lymphocytes, possibly by inhibition of CD8⁺ cell proliferation (Vassiliadou *et al.*, 1999).

In chapter 3, MPA and NET-A inhibited the activation of CD8⁺ T lymphocytes and in some cases decreased the basal level of activated CD8⁺ T lymphocytes in experiments on male and female donors. Consequently, the aim of this chapter was to determine the effects MPA and NET-A has on RANTES release from leukocytes in an *in vitro* model. An ELISA was used to determine the concentrations of RANTES in the supernatants of stimulated cells.

5.2 Materials and Methods

5.2.1 Study design

Blood samples were collected from 10 healthy individuals, 5 females (between 20-34 yr of age) and 5 males (between 20-40 yr of age), who were not on any hormonal treatment, including any form of contraception. Medroxyprogesterone acetate (Sigma, Sigma Aldrich), Norethisterone acetate (Sigma, Sigma Aldrich) and Dexamethasone (Sigma, Sigma Aldrich) were used at 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} and 10^{-10} M final concentrations. A 70% ethanol solution was used to dissolve as well as dilute all three steroids. These concentrations were chosen to represent different physiologic states these steroids are found in, where higher concentrations e.g. 10^{-7} M to 10^{-9} M are found in hormone replacement therapy (Adams *et al.*, 1997; Stanczyk, 2003) and lower concentrations e.g. 10^{-8} M and 10^{-9} M are reached in hormonal contraception (Mishell, 1996; Zalanyi *et al.*, 1984; Smit *et al.*, 2004).

5.2.2 Preparation and incubation of samples

The following were added into sterile FACScan (Falcon[®], Becton Dickinson, Scientific Group) tubes:

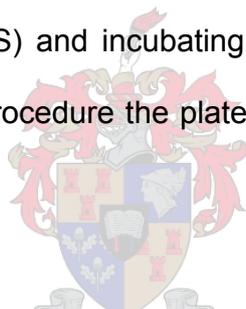
1. 200µL whole heparinized blood per tube.
2. Two sets of each steroid (MPA, NET-A or Dex) at six different concentrations (10^{-5} to 10^{-10} M).
3. Each tube was filled up to a final volume of 350µL with RPMI 1640 (Bio Whittaker, Sterilab)

A positive (stimulated) and a negative (non-stimulated) control tube was also set up with an equal volume (4µL) added, as the volume of steroids added, of a 70% ethanol solution to compensate for the 70% ethanol solution used to dilute the steroids. The tubes were then incubated at 37°C and 98% humidity in a CO₂ incubator (NuAir CO₂ Water-Jacketed incubator, Centrotec) for 40 minutes. After this incubation, one set of concentrations of each steroid was stimulated with TPA (0.005 mg/mL) (Sigma). The positive control tube was also stimulated with 0.005mg/mL TPA. A dose response experiment was performed with TPA and whole blood to determine optimal RANTES production (data not shown). The second non-stimulated set of concentrations of each steroid represented the effect the steroids would have on RANTES production without any stimuli. The tubes were then incubated for 6 hours at 37°C and 98% humidity in a CO₂ incubator. The tubes were then centrifuged for 5 minutes at 2000 rpm and 130µL supernatant of each well was drawn off and double diluted with RPMI. The samples were kept overnight in eppendorf tubes at -20°C.

5.2.3 RANTES ELISA

The human RANTES/CCL5 DuoSet[®] ELISA Development kit by R&D Systems was used to assay the samples.

Ninety-six (96) well plates (Nunc-Immunoplate, Maxisorp, AEC Amersham) were coated with 100 μ L of a 1.0 μ g/mL, in PBS, working concentration of the capture antibody and incubated sealed overnight at ambient temperature. The plates were then washed three times with wash buffer (0.05% Tween[®] 20 in PBS, pH 7.2-7.4) using an autowasher (Denley Wellwash 4, Alltech Medical Services). Any remaining wash buffer was removed after the wash by inverting the plate and blotting it against clean paper towels. The plates were blocked by adding 200 μ L of block buffer (1% HSA, 5% Sucrose, and 0.05% NaN₃ in PBS) and incubating for a minimum of 1 hour at room temperature. After the blocking procedure the plates were washed again as described previously with wash buffer.



The standards were set up with a final volume of 100 μ L/well in duplicate as follow: A seven point 2-fold serial dilution (diluted with reagent diluent – 1% HSA in PBS, pH 7.2 – 7.4, 0.2 μ m filtered) of the standard with a high standard of 1000pg/mL was used. The blanks were filled with 100 μ L of reagent diluent per well. One hundred (100) μ L of each sample was loaded in duplicate on the plates and the plates were incubated sealed for 2 hours at room temperature. Thereafter the plates were washed as previously described and 100 μ L of detection antibody, with a working concentration of 10ng/mL diluted with reagent diluent, was added to each well. The plates were sealed and incubated for another 2 hours at room temperature, where after the plates were washed again as previously described. One hundred (100) μ L of the working dilution of

Streptavidin-HRP (1:200 dilution in reagent diluent) was added to each well and the plates were incubated in the dark for 20 minutes at room temperature. The plates were washed again as described previously and 100 μ L of substrate solution (BM Blue Pod substrate, Roche Diagnostics) was added to each well. The plates were incubated in the dark at room temperature for 10-15 minutes or until a light-blue colouration occurred. The reaction was stopped by adding 50 μ L of stop solution (2M H₂SO₄) to each well and gently tapping the plates to ensure thorough mixing. The plates were read directly after the reaction was stopped on a microplate reader (Organon Teknika Microwell system, Alltech Medical Services) at an optical density of 450nm and a reference wavelength of 690nm using Organon Teknika MIMS software. Sterile techniques and materials were used throughout the whole experiment and the experiments were performed on the blood of 5 female donors and 5 male donors.

5.2.4 Data analysis

Analysis of datasets were achieved by repeated measures ANOVA followed by post testing using Dunnett's test. Post testing was only done when there was significant pairing ($p < 0.05$) among the groups. Analysis was done on the combined male and female groups ($n=10$), as well as on the male and female groups separately ($n=5$). All results were considered statistically significant when $p < 0.05$.

5.3 Results

Analysis of the results generated from the combined gender groups revealed surprising results. An overall increase in RANTES levels occurred when whole blood samples were stimulated with TPA, in the presence of different concentrations of MPA (Fig. 67). This enhancing characteristic of MPA was statistically significant at all concentrations tested except 10^{-6} M. At a concentration of 10^{-10} M, MPA significantly increased RANTES levels by 33.5% ($p<0.01$) and had the least effect of 20.7% ($p<0.05$) at 10^{-7} M.

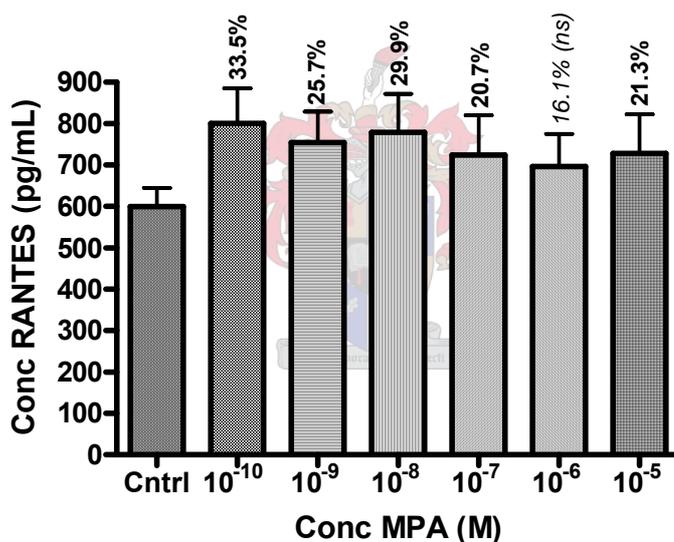


Figure 67. Enhancing effects of MPA on RANTES levels, compared to controls (Cntrl), in whole blood samples stimulated with TPA (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

NET-A also significantly increased RANTES levels in the combined gender group, although the two lowest concentrations did not show any statistical significance (Fig. 68). A maximum effect of 39.4% ($p < 0.01$) was achieved at a concentration of 10^{-8} M, while 10^{-7} M NET-A caused the least significant increase of 29.2% ($p < 0.01$) in RANTES levels.

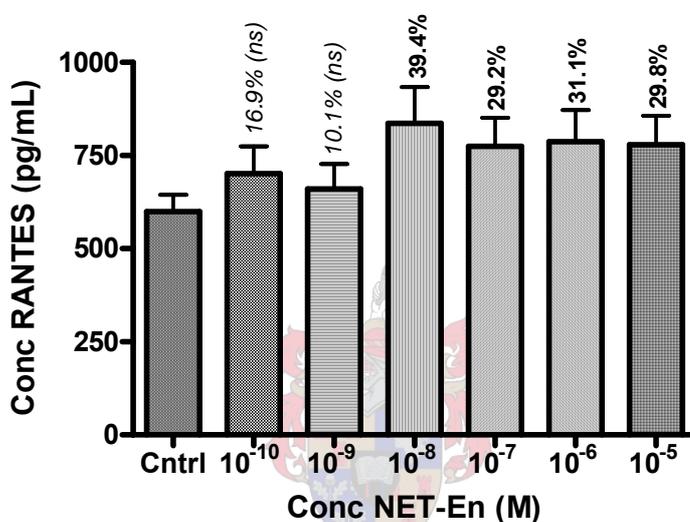


Figure 68. Enhancing effects of NET-A on RANTES levels, compared to controls (Cntrl), in whole blood samples stimulated with TPA ($n=10$). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

Dex generally increased RANTES levels in the combined gender group, while this increase was only statistically significant at two of the concentrations tested (Fig. 69). An increase of 18.6% ($p < 0.05$) and 18.3% ($p < 0.05$) occurred at 10^{-10} M and 10^{-8} M respectively.

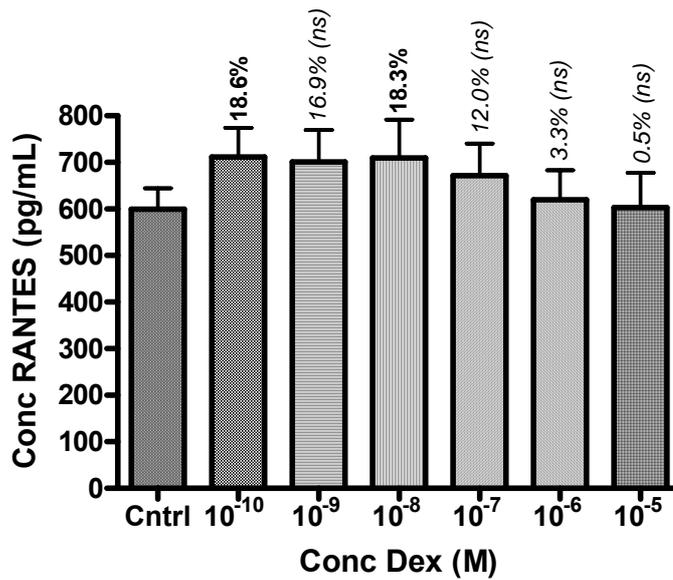


Figure 69. Enhancing effects of Dex on RANTES levels, compared to controls (Cntrl), in whole blood samples stimulated with TPA (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

Statistically, MPA significantly increased RANTES levels in whole blood samples obtained from female donors, stimulated with TPA, at 10⁻⁵ M, 10⁻⁸ M and 10⁻¹⁰ M (Fig. 70). A maximum increase of 36% (p<0.05) occurred at 10⁻⁸ M, while 10⁻⁵ M MPA had the weakest significant effect (33.9%; p<0.05) on RANTES levels.

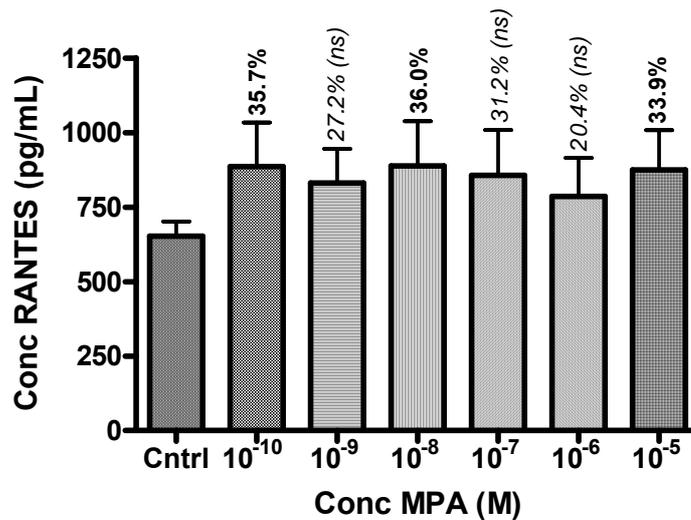


Figure 70. Enhancing effects of MPA on RANTES levels in female donors, compared to controls (Cntrl), in whole blood samples stimulated with TPA (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

NET-A had a more pronounced effect on RANTES levels in female donors by presenting a statistical significant increase at the four highest concentrations tested (Fig. 71). NET-A at a concentration of 10⁻⁸ M had the most potent effect on RANTES levels, causing a 44% (p<0.01) increase, while 10⁻⁵ M NET-A affected RANTES levels the least, causing a 28.5% (p<0.05) increase. Unlike MPA or NET-A, Dex had no significant effect on RANTES levels in females (Data not shown).

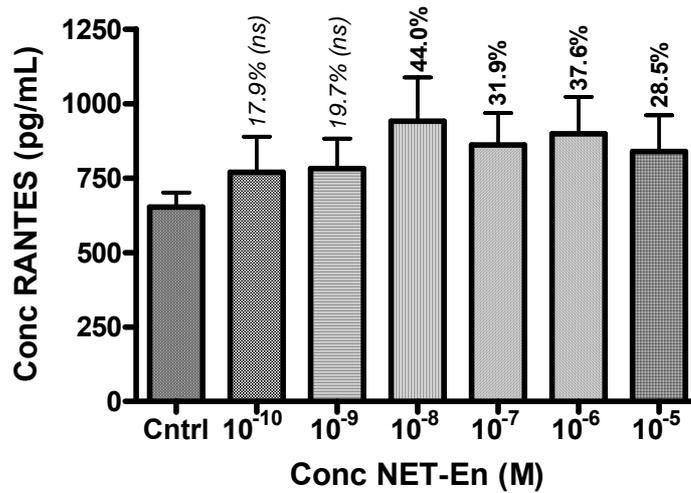


Figure 71. Enhancing effects of NET-A on RANTES levels in female donors, compared to controls (Cntrl), in whole blood samples stimulated with TPA (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

Statistically, MPA only affected RANTES levels significantly in male donors at the two lowest concentrations tested, although a general increase can be seen (Fig. 72). MPA at a concentration of 10⁻¹⁰ M resulted in a 30.9% (p<0.01) increase, while 10⁻⁹ M caused a 23.9% (p<0.05) increase in RANTES levels.

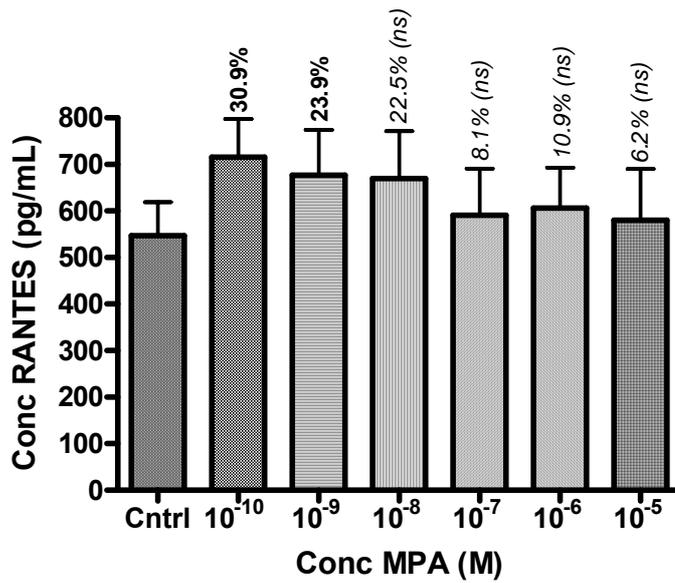


Figure 72. Enhancing effects of MPA on RANTES levels in male donors, compared to controls (Cntrl), in whole blood samples stimulated with TPA (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

The potency of NET-A was slightly less in male donors than in female donors (Fig. 73). It only had a statistical significant effect at 10⁻⁵ M, 10⁻⁷, and 10⁻⁸ M with a maximum increase of 33.9% (p<0.01) occurring at the latter concentration.

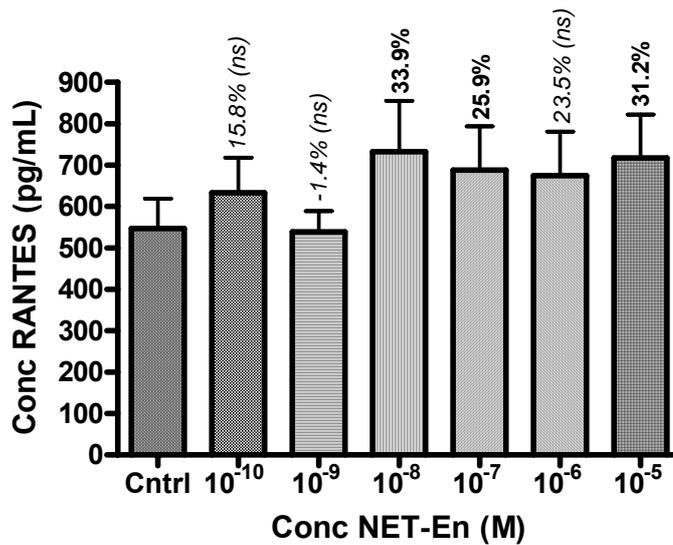
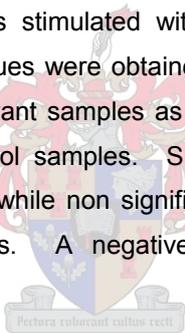


Figure 73. Enhancing effects of NET-A on RANTES levels in male donors, compared to controls (Cntrl), in whole blood samples stimulated with TPA (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics. A negative value indicates a decrease.



Statistically, the only significant increase of RANTES in the Dex treated male samples was a 22.1% ($p < 0.05$) increase and this occurred at the lowest concentration tested (Fig. 74). This again confirms the little interaction Dex has on RANTES.

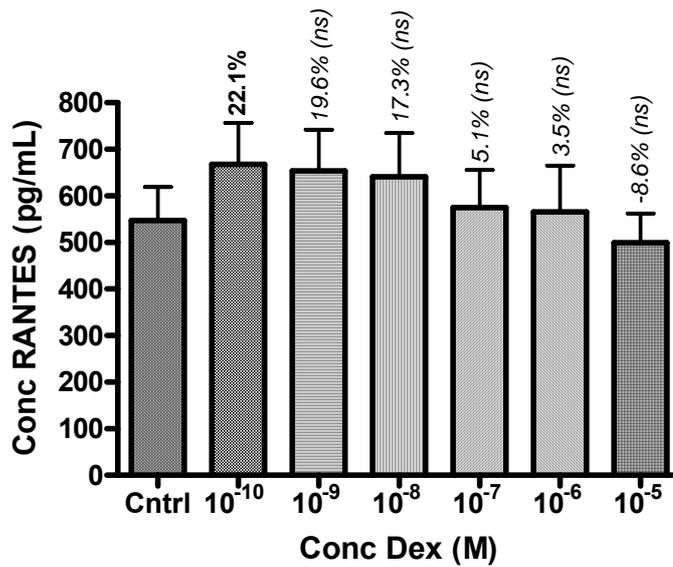


Figure 74. Enhancing effects of Dex on RANTES levels in male donors, compared to controls (Cntrl), in whole blood samples stimulated with TPA (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics. A negative value indicates a decrease.

MPA and NET-A, but not Dex, affected the basal levels of RANTES (non stimulated samples) far greater in the combined gender group when compared to its influence on stimulated samples in the same group. MPA significantly increased the basal levels of RANTES at all concentrations tested, ranging from 78.8% ($p < 0.01$) at 10^{-5} M to 38.3% ($p < 0.05$) at 10^{-7} M (Fig. 75).

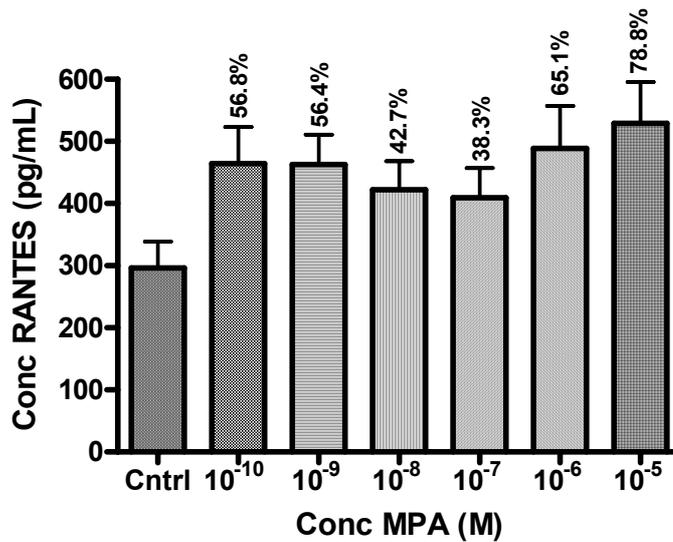


Figure 75. Influence of MPA on the basal levels of RANTES in non stimulated samples compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

NET-A also significantly ($p < 0.01$ for all concentrations) increased the basal levels of RANTES at all concentrations tested (Fig. 76). An increase of 73.7% by 10^{-10} M NET-A was the highest, while the 10^{-6} M NET-A had the least effect on the basal levels of RANTES.

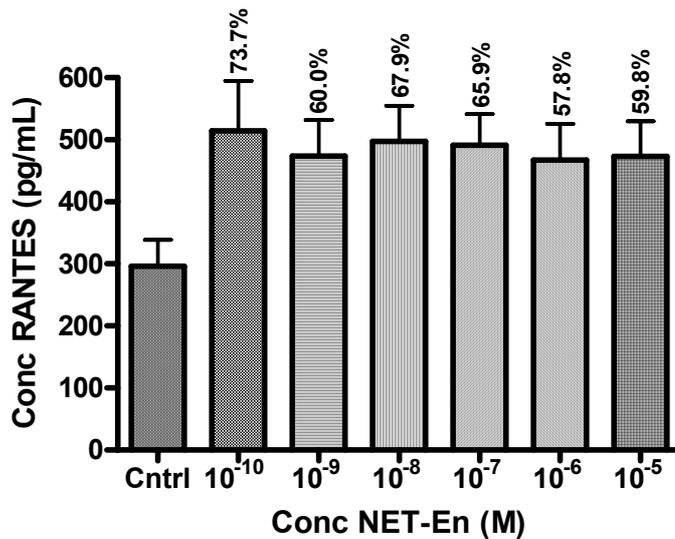


Figure 76. Influence of NET-A on the basal levels of RANTES in non stimulated samples compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

The basal levels of RANTES were significantly raised by 10⁻⁵ M and 10⁻⁶ M Dex (Fig. 77). Dex at 10⁻⁵ M and 10⁻⁶ M respectively, brought about a 68.1% ($p < 0.01$) and 55.4% ($p < 0.01$) elevation in basal RANTES levels.

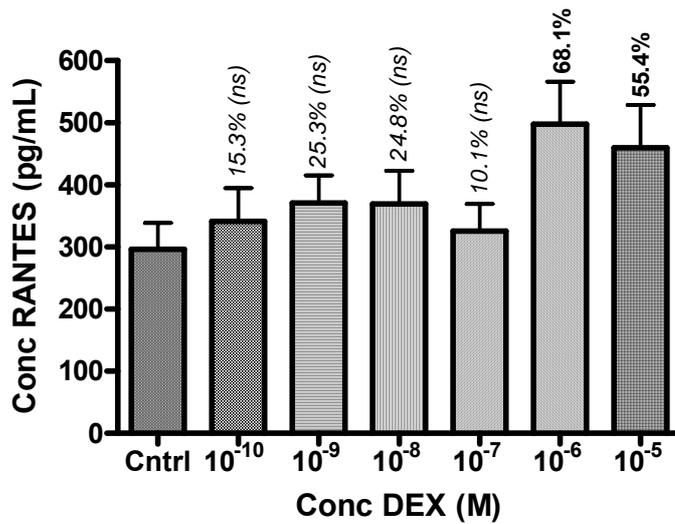


Figure 77. Influence of Dex on the basal levels of RANTES in non stimulated samples compared to controls (Cntrl) (n=10). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

The increase in basal levels of RANTES by MPA and NET-A was less pronounced in female donors than in the combined gender group. MPA only caused a statistical significant increase of 69.9% ($p < 0.05$) in RANTES levels in non stimulated samples obtained from female donors at a concentration of 10^{-5} M (Fig. 78).

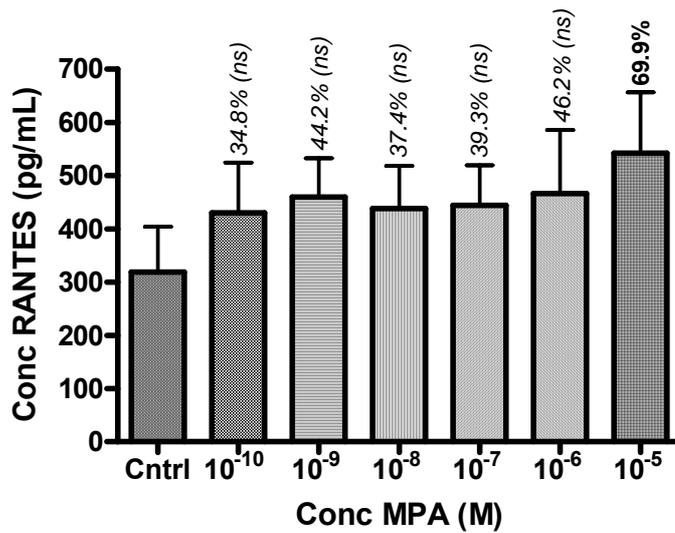


Figure 78. Influence of MPA on the basal levels of RANTES in non stimulated samples of female donors, compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

Only 10⁻⁷ M (49.2%; p<0.05) and 10⁻¹⁰ M (53.9%; p<0.05) NET-A displayed a statistical significant effect on RANTES levels in female donors, although a general increase in RANTES levels can be seen (Fig. 79).

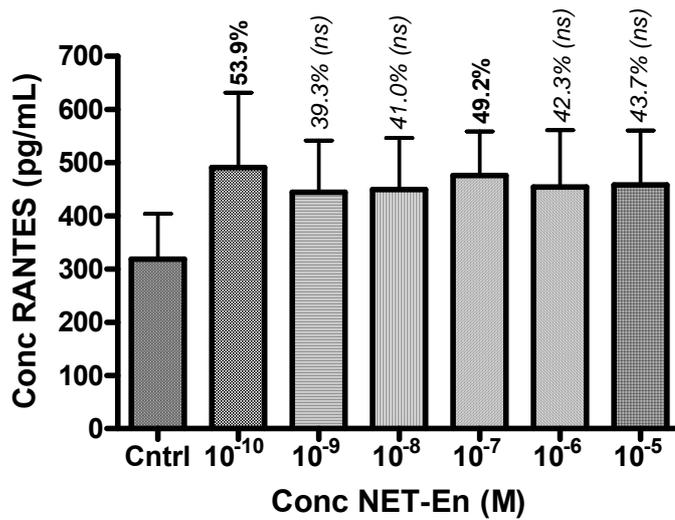


Figure 79. Influence of NET-A on the basal levels of RANTES in non stimulated samples of female donors, compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

The two highest concentrations of Dex used were the only ones to affect the basal levels of RANTES in female donors significantly (Fig. 80). An increase of 55.6% ($p < 0.05$) was caused by 10^{-6} M of Dex and 10^{-5} M Dex caused a 53.5% ($p < 0.05$) increase in RANTES levels.

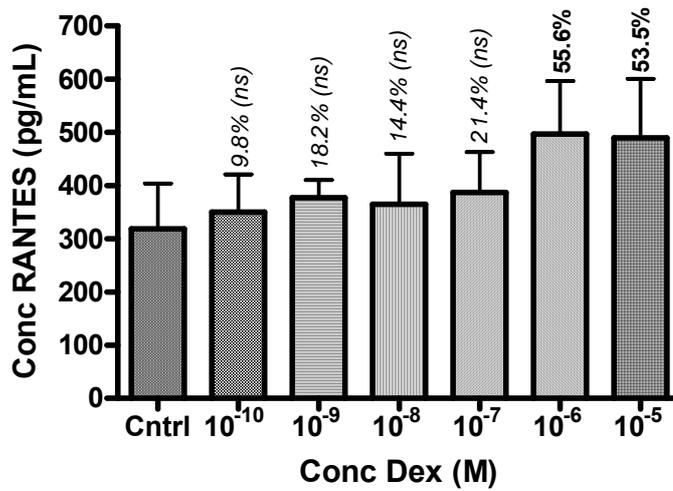


Figure 80. Influence of Dex on the basal levels of RANTES in non stimulated samples of female donors, compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

The effects of MPA and NET-A on the basal levels of RANTES were more exaggerated in male donors. MPA significantly increased RANTES levels at all concentrations tested, while the increase at 10⁻⁷ M was the only value that was not statistically significant (Fig. 81). The greatest effect of 89.2% (p<0.01) was found at 10⁻⁵ M MPA, while the lowest increase of 48.9% (p<0.05) occurred at 10⁻⁸ M.

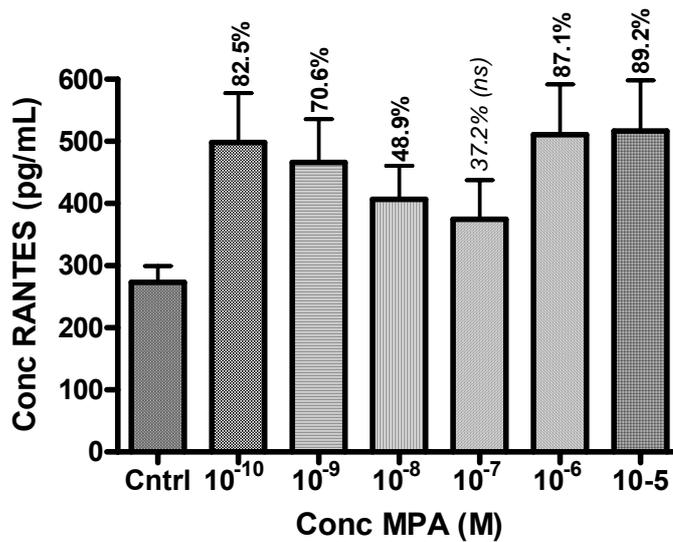


Figure 81. Influence of MPA on the basal levels of RANTES in non stimulated samples of male donors, compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (*ns*) values are represented in italics.

NET-A at all concentrations caused a very significant ($p < 0.01$) elevation of basal RANTES levels in male donors (Fig. 82). An up most increase of 99.5% occurred in the presence of 10^{-8} M NET-A, while 10^{-6} M NET-A affected RANTES levels the least (75.9%).

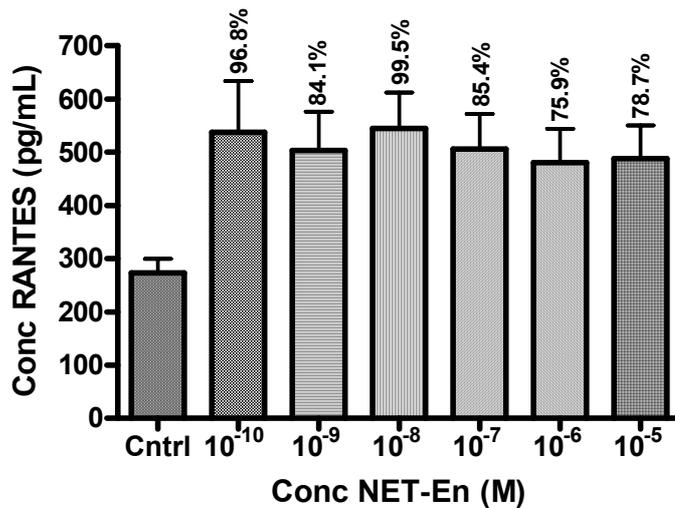


Figure 82. Influence of NET-A on the basal levels of RANTES in non stimulated samples of male donors, compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics.

Only 10⁻⁶ M Dex had an effect in male donors that was statistically significant, increasing basal RANTES levels by 82.6% (p<0.05) (Fig. 83).

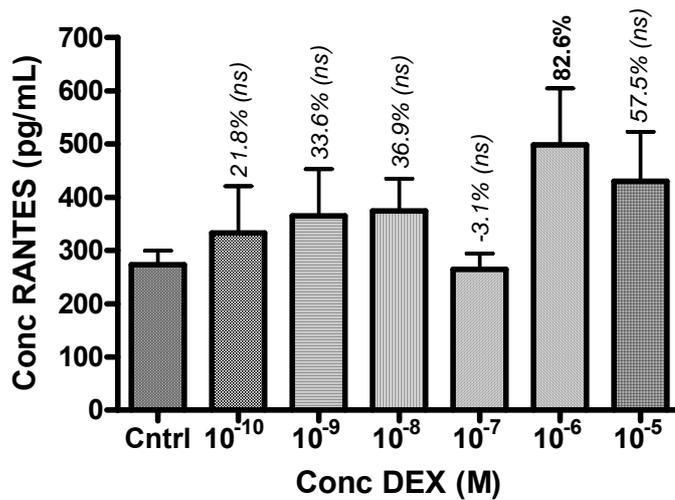
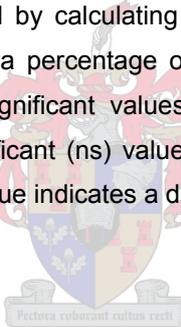


Figure 83. Influence of Dex on the basal levels of RANTES in non stimulated samples of male donors, compared to controls (Cntrl) (n=5). The given percentage values were obtained by calculating the averages of the relevant samples as a percentage of the average of the control samples. Significant values are represented in bold, while non significant (ns) values are represented in italics. A negative value indicates a decrease.

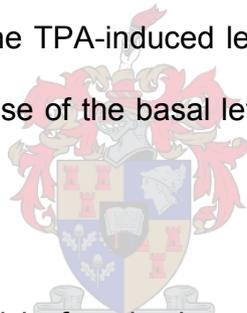


5.4 Discussion

Intercellular communications between the various immune cells form an important part of an effective immune response. Chemokines, like RANTES, make these intercellular communications possible. The effects of MPA and NET-A on RANTES release in this study revealed some unpredictable results in the form of a general increase in RANTES levels caused by these hormones.

In samples of the combined group where RANTES release was induced by TPA, Dex had the least effect on RANTES levels followed by NET-A, with MPA affecting RANTES levels the most. This observed effect also applied to the effects of these three hormones on the basal levels of RANTES in non-induced samples of the combined group.

In female donors, the majority of the concentrations of MPA and NET-A had a significant effect on the TPA-induced levels of RANTES than what it had on the basal levels of RANTES in this group. However, the overall enhancing effects of these two hormones were higher in the non-induced samples than in the TPA-induced samples. Dex had no significant effect on the TPA-induced levels of RANTES in female donors, while it caused a significant increase of the basal levels of RANTES at the two highest concentrations tested.



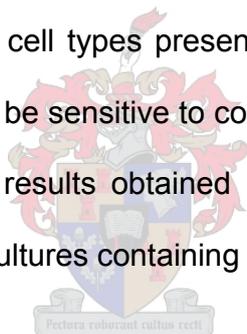
A converse effect from that found in female donors occurred in male donors. More concentrations of MPA and NET-A had a significant effect on basal RANTES levels than concentrations that had a significant effect on TPA-induced RANTES levels. Corresponding to the overall effect in female donors, the overall enhancing effects of these two hormones in male donors were higher in the non-induced samples than in the TPA-induced samples. As was the case with female donors, Dex did not really have any significant effect on both the basal as well as the TPA-induced levels of RANTES in male donors. Dex only had a significant effect at one concentration in both the TPA-induced and non-induced samples and the effect in non-induced samples were more pronounced than the effect in TPA-induced samples.

We found that MPA exhibited a more pronounced effect on the basal levels of RANTES in males than in females, while the effect of NET-A on the basal levels of RANTES was more or less the same between the genders. The differences we found amongst genders and type of hormones implies the involvement of different receptors as well the levels of the receptors present in our experimental model. Furthermore, none of the hormones studied exhibited a clear cut dose response.

It has previously been shown that progesterone significantly inhibited the expression of chemokine receptors on activated T cells and also inhibited mitogen-induced chemokine secretion by CD8⁺ T lymphocytes (Vassiliadou *et al.*, 1999). Contradictory to the findings of our study, Zhao *et al.* (2002) demonstrated that MPA could inhibit RANTES production in isolated endometrial stromal cells and stated that this observed inhibition was mediated via the progesterone receptor. However, the cell type used in our study was lymphocytes whereas the results of Zhao *et al.* (2002) were demonstrated in endometrial stromal cells. They also found that short-term treatment with MPA (2-4 days) had no effect on RANTES production, while long-term exposure (8 days) to MPA produced significant result. Similar observations were made previously (Tseng & Zhu, 1997). The requirement for long-term exposure to MPA could possibly explain why the expected inhibition of RANTES did not occur in our study, seeing that samples were only incubated in the presence of the different hormones for no more than 6 hours. Considering that, for basically all the experiments and different hormones, the overall average increase of the basal levels of RANTES was more than the overall average increase of RANTES in TPA-stimulated samples, it can be seen that the effects of the

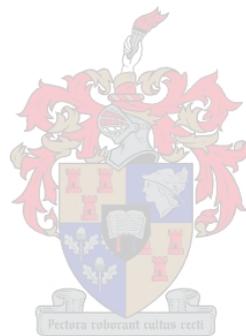
different hormones could be predisposed towards a possible state of inhibition. This however, can only be confirmed in a study containing longer incubation periods.

It is possible that the results obtained by us were due to the use of an inappropriate stimulus, considering that TPA is not really a physiological activator. TPA is a potent activator of T cells *in vitro*, but it could be questioned whether this depicts an appropriate *in vivo* situation concerning the sought after effects on RANTES release from these cells. Another possible criticism of this study could be the fact that we used whole blood. The stimulus used (TPA) is a polyclonal activator which targets monocytes, lymphocytes as well as neutrophils. Hence, the RANTES secretion could have been contributed by several cell types present in the culture. It is possible that these different cell types might not be sensitive to control by the synthetic progestins we used, thereby contributing to the results obtained by us. This problem can only be eradicated if assays are done on cultures containing only CD8 T cells.



Furthermore, it could be possible that we pursued the wrong factor after the observed effect MPA and NET-A had on CD8⁺ T lymphocyte activation (Chapter 3). Other CD8⁺ T cell-specific chemokines that provide potential investigative possibilities include MIP-1 α and MIP-1 β . However, due to time constraints and limitations of this study it was not possible to explore these possibilities further. If our observed enhancing effect of MPA and NET-A on RANTES release from lymphocytes *in vitro* did indeed turn out to be a true reflection of their actions within *in vivo* conditions, it would imply that an increased number of lymphocytes will be recruited throughout the body. However, the functions of

these T lymphocytes would be inhibited due to the demonstrated effects MPA and NET-A had on CD8⁺ T cells (Chapter 3).

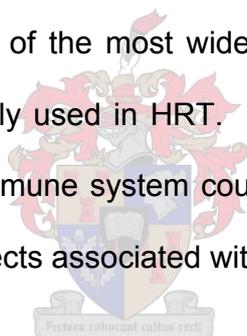


CHAPTER 6

GENERAL CONCLUSION AND FUTURE PERSPECTIVE

6.1 General conclusion

The aim of this study was to determine whether the synthetic progestins, MPA and NET-A, would have any significant effects on certain important immunological parameters. This was of importance because these two synthetic progestins are not only the active substances in two of the most widely used hormonal-based injectable contraceptives, but also extensively used in HRT. Determining the influence of these two synthetic hormones on the immune system could help us to better understand, or even explain, some of the side-effects associated with the use of these two progestins.



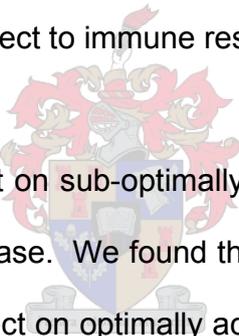
We began with a thorough literature review on GCs, MPA as well as NET (Chapter 1). During this literature review it became clear that MPA and NET possessed some immunosuppressive properties, while MPA displayed certain GC activities. However, most studies focussed on supra-physiological concentrations (Jeremiah *et al.*, 1968; Brunelli *et al.*, 1996) and not on concentrations reached from contraceptive doses. Therefore we decided to investigate a wide range of concentrations of each hormone, ranging from 10^{-5} M to 10^{-10} M, thereby including contraceptive and HRT doses. The evidence that an association exists between contraceptives, STD's and the incidence of HIV-1 infection raises concern (Bulterys *et al.*, 1994; Rehle *et al.*, 1992; Martin *et al.*,

1998; Baeton *et al.*, 2001). The findings of the two *in vivo* studies on female macaques (Smith *et al.*, 2000; Marx *et al.*, 1996) further strengthened our determination in elucidating the effects of MPA and NET at a cellular level.

We first investigated the effects of the two synthetic hormones on IL-6 and TNF- α release from monocytes *in vitro*, due to well documented anti-inflammatory properties of GCs and specifically Dex (Munck & Naray Fejes Toth, 1994; Chrousus, 1995; De Rijk & Berkenbosch, 1994). This was achieved by looking at the effects on sub-optimally, moderately as well as optimally activated monocytes under the control of either MPA or NET-A. Thereafter we investigated the implications of the two synthetic hormones on CMI by looking at the activation of CD4⁺ and CD8⁺ T cells *in vitro*. This was followed by investigating the possible effects MPA and NET-A could have on the CD4⁺ T cell helper subsets. Finally we investigated the possible effects of MPA and NET-A on the CD8⁺ T cell-specific chemokine, RANTES. The effects of MPA and NET-A were compared to the known GC, Dex, throughout all the different experiments. We found discrepancies amongst genders in initial experiments and therefore analysed our data according to a combined gender group as well as separate gender groups.

A point worth mentioning is that we did not take any steps to ensure equal numbers of cells per assay throughout our study. Although each culture was not normalized as far as protein and cell concentration is concerned, we assumed that most of the healthy donors we used had normal white blood cell counts and that the relative variation of each cell type in the blood used would be minimal. Whole blood (or rather the differential cell count) has a narrow range of normality, for instance, lymphocytes are

described as “normal” should the count be between 30-35% of whole blood (similarly, granulocytes account for 60-65 of whole blood). In this study we did not attempt to “normalize” cell counts in any way for the reason that we felt that the results obtained would reflect a physiological response measured *in vitro* and that this may reflect the reality *in vivo*. We do realize that this is an assumption, especially since not all individuals may show identical dose-responses vis-à-vis the compound used. However, the aim of this pilot study was to determine general trends *in vitro* and future studies may have to consider the normalization of the culture conditions (namely, exact cell numbers, etc.). We feel that by using whole blood (which obviates the need for intricate, laborious cell preparation), the end result measured would reflect a possible scenario of *in vivo* activity with respect to immune responses.



MPA displayed no significant effect on sub-optimally activated monocytes in any of the different groups regarding IL-6 release. We found that all concentrations of MPA tested had a very significant inhibiting effect on optimally activated monocytes in the combined group by inhibiting IL-6 release from these cells. This inhibiting effect of MPA on IL-6 release correlated with findings from other studies (Yamashita *et al.*, 1996; Wakatsuki *et al.*, 2002). The effect of MPA on IL-6 from optimally activated monocytes differed amongst genders. Although the inhibitory effect of MPA was less potent in female donors, it was still potent enough to cause statistically significant inhibition at 10^{-6} M, 10^{-9} M and 10^{-10} M. This anti-inflammatory effect of MPA on females was almost as strong as that of Dex, where the inhibitory effect of Dex was only 7.2% stronger than that of MPA at a contraceptive dose of 10^{-9} M. We also found that MPA exhibited a very strong inhibitory effect on IL-6 release from optimally activated monocytes obtained from male

donors. When this effect of MPA was compared to that of Dex in the same group it was seen that the effect of MPA was sometimes stronger than that of Dex. This indicates the possible consideration of using MPA, rather than Dex, as an anti-inflammatory agent in males.

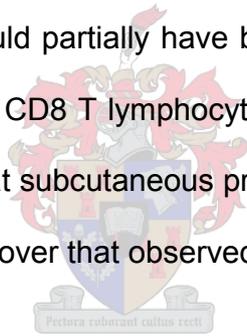
MPA also had a significant effect on TNF- α release from monocytes. Moderately activated monocytes were activated with 50 μ g/mL LPS. Only the two lowest concentrations of MPA displayed a statistical significant effect on TNF- α release from moderately activated monocytes in the combined group. This slight effect of MPA still mounted to an astounding average decrease of 81.3% at 10⁻¹⁰ M MPA. This same effect on moderately activated monocytes was seen in the male donor group, while MPA had no significant influence on the female donor group. The effects of MPA on TNF- α release from optimally activated monocytes (stimulated with 100 μ g/mL LPS) were more potent than in the moderately activated group. This inhibiting effect of MPA on TNF- α in the combined group was comparable to the effects of Dex on the same group and coincided with the findings from another study (Koh *et al.*, 2002).

The effects of MPA on TNF- α release from optimally activated monocytes varied amongst genders. We found that more concentrations of MPA had statistical significant inhibitory effects on TNF- α release in female donors, although MPA had a more pronounced inhibitory effect in male donors. This variation amongst genders, together with the observed gender variation of MPA on IL-6 release, clearly indicates that there are differences in the receptor-mediated action of MPA. This gender dependent activity could possibly play an important role where MPA is used therapeutically. The average

inhibition of MPA at a contraceptive concentration of 10^{-9} M was 48.8% in females, compared to the 66.6% inhibition of Dex at the same concentration in the same group. Taking into consideration the effects that MPA had at a contraceptive dose (10^{-9} M) on IL-6 and TNF- α release in females, it becomes evident that there is a good possibility that women who are on hormonal contraceptives containing MPA might not be able to respond effectively to a severe inflammatory episode. This might also be the case for women undergoing HRT. However, thorough *in vivo* investigations still need to be done, since our results are based on an *in vitro* model.

We discovered that MPA significantly inhibited the activation of both CD4⁺ and CD8⁺ T cells. MPA significantly inhibited the activation of CD4⁺ T cells at all concentrations tested in the combined group. The overall average inhibition of CD4⁺ T cell activation in females by MPA was more than that caused by Dex in the same group. In females, MPA demonstrated a 1.5% stronger average inhibitory effect at a concentration of 10^{-9} M when compared to the effect Dex had on the activation of CD4⁺ T cells at the same concentration. MPA did not have a significant effect on CD4⁺ T cell activation in males. Bearing in mind the effects MPA had on CD4⁺ T cell activation in female donors and male donors, it can be postulated that the interaction of MPA with its appropriate receptors differs between females and males. This can occur due to a gender-dependent variation in the levels of these different receptors. The answer to our observed gender variation may also lie in the high affinity MPA has for the AR (Teulings *et al.*, 1980; Bentel *et al.*, 1999).

The effects of MPA on the activation of CD8⁺ T cells were more pronounced than the effect it had on CD4⁺ T cells. Comparable to the gender variation found during analysis of CD4⁺ T cell activation, the inhibiting effect of MPA on CD8⁺ T cell activation was more pronounced in females than in males. Unlike the more potent (when compared to Dex) effect MPA had on CD4⁺ T cell activation at contraceptive doses, we found that the effect of MPA on CD8⁺ T cell activation was less potent than that of Dex. MPA still managed to induce an average inhibition of 48.8% in female donors, compared to the 66.6% average inhibition caused by Dex. The immunosuppressive property of MPA that was demonstrated by us was also demonstrated in other studies (Hulka *et al.*, 1965; Jeremiah *et al.*, 1968; Pelner & Rhoades, 1965). Furthermore, this demonstrated effect of MPA on CD8⁺ T cells could partially have been responsible for the findings of Marx *et al.* (1996), due to the role CD8 T lymphocytes play in protecting the body from infections like HIV. They found that subcutaneous progesterone implants enhanced SIV vaginal transmission almost 8-fold over that observed in placebo controls.



The efficient activation of CD4⁺ T cells is required for these cells to successfully monitor the extracellular environment for pathogens. Furthermore, CD4⁺ T cells promote the responsiveness of other cells and they play a major role in the activation of the bactericidal functions of macrophages. They also act as the foremost source of antigen-specific protection against viral infection and other intracellular infections. The main function of CD8⁺ T Lymphocytes is to deal with intracellular infections by differentiating into CTLs that kill virally infected cells. By taking the various effects MPA had on the activation of CD4⁺ and CD8⁺ T cells into consideration, the possibility arises that MPA could possess the ability to suppress the immune response of an individual to

more or less the same extend Dex would at the same concentration. If our results are an indication of the effects MPA would have *in vivo*, then there is a possibility that this progestin would also have this detrimental effect when used as a contraceptive.

Studies have shown that GCs reduces IL-2 and IFN- γ and increase IL-4 production (Dayens *et al.*, 1991; Rook *et al.*, 1994). The evidence of these studies, together with our observed effects of MPA on CD4⁺ T cell, lead us to investigate the possible effects MPA and NET-A could have on the CD4 helper subsets. In our study, Dex acted as was previously described (Wang *et al.*, 1993) by reducing IFN- γ production which translated into a decrease in T_H1⁺ cells. Contradictory to other findings (Rook *et al.*, 1994), we discovered that Dex reduced IL-4 production as well. We also investigated the effects the three synthetic hormones would have on the ratio of T_H1 and T_H2 cells. The T_H2:T_H1 ratio in the combined group was significantly decreased by all concentrations of Dex tested. We found that this reduction of the T_H1: T_H2 ratio by Dex was caused by its overall inhibiting effect on both T_H1 and T_H2 cells.

Despite the positive results we found with Dex, MPA hardly displayed a significant effect on the T_H2:T_H1 ratio or on any of the two helper subsets. We found that only the highest concentration of MPA tested displayed a statistical significant effect by decreasing the T_H2:T_H1 ratio in the combined group, while it had no significant effect on any of the two helper subsets. In our study MPA had no significant effect in any of the gender groups. Dex (10⁻⁶ M – 10⁻⁹ M) significantly reduced the T_H2:T_H1 ratio in female donors and this observed change in ratio was caused more by the effect Dex had on T_H2 cells than what it had on T_H1 cells. On the other hand, Dex (10⁻⁶ M – 10⁻⁸ M)

significantly reduced the T_H1 : T_H2 ratio in male donors while this observed change was caused by the equal effect Dex had on both helper subsets.

It is possible that the difference between MPA and Dex regarding their effects on helper subsets could have been caused by a difference in their interaction with various receptors. However, we cannot be entirely sure bearing in mind that we might have used the incorrect incubation periods. It is possible that MPA and/or NET-A may require longer incubation periods than what we used (longer than 12 hrs). As was previously discussed, each T cell subset produces cytokines that serve as their own autocrine growth factor which promotes differentiation of naive T cells to that subset and these two subsets also produce cytokines that cross-regulate each other's development and activity. This would imply that the net outcome of cytokine-mediated self-amplification and cross-regulation is such that, as soon as an immune response by T cells begins to develop along one of the two pathways, it tends to proceed progressively in that direction. Therefore, we cannot predict whether the results would have been different had we managed to exclude these already committed cells. What we did manage to determine was that a slight difference exists in the activity of Dex on T helper subsets amongst genders.

After obtaining the results on the effects of MPA on $CD8^+$ T cells, we investigated the effects of MPA on the $CD8$ T cell-specific chemokine, RANTES. The fact that our results of MPA on RANTES production surprised us was duly attested by the effects Dex had on the production of this chemokine. We discovered that Dex, although not as strong as MPA, exhibited an enhancing tendency in the combined group when

compared to controls in the same group. A general increase in RANTES production occurred in TPA-induced samples under the control of different concentrations of MPA. We found that this enhancing ability of RANTES was more pronounced in TPA-induced samples of female donors, while the enhancing effect on basal RANTES levels was more pronounced in non-induced samples of male donors.

A study using progesterone showed that it significantly inhibited the expression of chemokine receptors on activated T cells and also inhibited mitogen-induced chemokine secretion by CD8⁺ T lymphocytes (Vassiliadou *et al.*, 1999). Furthermore, it has been shown that MPA inhibited RANTES production in isolated endometrial stromal cells and that this observed effect was mediated by the PR (Zhao *et al.*, 2002). If this observed effect of MPA was accurate and seeing that we did not observe an inhibition of RANTES production, it would mean that MPA did not bind to the PR in our study, thereby indicating the possibility that the PR is absent in T lymphocytes. Whether we used the correct incubation periods is of interest, since Zhao *et al.* (2002) only found significant results after long-term exposure (8 days) to MPA. Another matter of interest was whether we used the appropriate stimulus as well as the correct concentration of the stimulus. We cannot exclude this possibility, even though we performed dose-response experiments between TPA and RANTES release. Further research is required in several fields, including the appropriate stimulus required, the necessary concentration of the stimulus, as well as the correct incubation periods needed. RANTES was the most logical chemokine to investigate but our results indicated that it may have been the wrong parameter to measure, therefore it would be of interest to investigate the effects of MPA on other chemokines, like MIP-1 α and MIP-1 β .

The effects that MPA exerted on the six different immunologic parameters studied by us are summarized as follow. Regarding the anti-inflammatory properties of MPA, we demonstrated that concentrations of MPA reached during contraception were able to inhibit the production of IL-6 and TNF- α to almost the same extend as Dex at the same concentration during a severe inflammatory episode. When we investigated the effect of MPA on CD4⁺ and CD8⁺ T cells, we found that the suppressive effect of MPA on both CD4⁺ and CD8⁺ T cells were comparable to that of Dex and in some cases even stronger. Even at contraceptive doses (10^{-9} M), MPA caused a 36.9% decrease in the activation of CD4⁺ T cells of female donors and this suppression was 1.5% more than that of Dex at the same concentration.

Furthermore, we found that although the suppressive effect of MPA at contraceptive doses on the activation of CD8⁺ T cells in female donors was slightly less than that of Dex, it still mounted to a significant 42.6% suppression. Despite the significant effect MPA had on CD4⁺ T cells, we discovered that it hardly had a significant effect on the T helper subsets of CD4 cells that secrete the discrete regulatory cytokines IFN- γ and IL-4. We highlighted various factors that could have been responsible for this observed effect. Although MPA significantly affected the activation of CD8⁺ T cells in our experiments, we were unable to demonstrate any significant reduction in the CD8 T cell-specific chemokine, RANTES. In contrast, we discovered that MPA caused a general increase in RANTES production. We could not entirely explain why this enhancing effect was observed in our experiments, but we were able to speculate on a few contributing factors that may perhaps have been responsible for our findings.

The collective thought of this thesis on MPA, in particular at contraceptive doses, and its effect on the immune system are as follow. The CMI of women, who are using MPA for either contraceptive purposes or HRT, might be severely affected by the inhibition of their intra-cellular as well as extra-cellular immune responses by MPA. In addition to this immunosuppressive property, MPA may influence the ability of women on this progestin to deal optimally with a severe inflammatory episode, due to the suppressive effects it has on the essential inflammatory markers. We, however, need to point out that our conclusions are based on *in vitro* findings, which could differ from *in vivo* situations.

Another interesting phenomenon that arose from our research was the variation in activity displayed by MPA amongst different genders throughout the different experiments. Several reasons have been given throughout this study as to why this variation amongst genders could have occurred in our study. The majority of these arguments were based on steroid receptor theory and action, although the aim of this study did not include this interesting area. Although not a widely accepted theory, it is worth mentioning the existence of a second steroid-binding site (Svec *et al.*, 1989; Hollenberg *et al.*, 1989) which can not be excluded when trying to determine the basis of the observed gender differentiation displayed by MPA. However, further research is required in the field of receptors before this hypothesis can be confirmed since this was beyond the scope of this study.

The effect of NET-A on IL-6 production differed from that of MPA. This could have been due to the fact that these two progestins vary greatly in their activity towards the AR,

MR and GR. Adding to this is that NET and its A-ring metabolites can be metabolized to a compound that has ER activity (Bergink *et al.*, 1983; Mendoza-Rodriguez *et al.*, 1999; Markiewicz & Gurpide, 1994), while MPA does not have any ER activity (Teulings *et al.*, 1980). NET-A did not have a significant effect on IL-6 release from sub-optimally activated monocytes in any group. Although the inhibitory effect of NET-A on IL-6 release from optimally activated monocytes in the combined group was less potent than that of MPA, it was still potent enough to cause a statistical significant decrease at the four highest concentrations tested. We found that NET-A did not significantly affect the release of IL-6 from the optimally activated monocytes of female donors, while the effect that it had on male donor monocytes was responsible for the observed effect in the combined group. When we compared the effects of Dex to the effects of NET-A in the male donor group, we found that the suppressive effects of NET-A were less potent than that of Dex.



Unlike the findings of MPA, we found that NET-A did not have a significant effect on TNF- α release from moderately activated monocytes in any of the genders or the combined group. This lack of NET-A to inhibit TNF- α release was further substantiated by the effect it had on optimally activated monocytes. We found that the only concentration of NET-A that caused a statistically significant decrease in TNF- α release from optimally activated monocytes in the combined group, was the highest concentration of 10^{-5} M. This decreased potency of NET-A was further demonstrated by the fact that it did not have a significant effect on optimally activated monocytes of either one of the gender groups. Again, steroid receptor interactions come into play when trying to unravel this behaviour of NET-A. Whether the ER plays a role is unclear

and needs further investigation, since NET and its metabolites possess intrinsic estrogenic activity (Bergink *et al.*, 1983; Mendoza-Rodriguez *et al.*, 1999; Markiewicz & Gurpide, 1994).

The lack of NET-A having anti-inflammatory properties could be ascribed to the pro-inflammatory nature it displayed. In the combined group, 10^{-7} M NET-A was able to increase IL-6 release from non-activated monocytes, while 10^{-9} M NET-A increased the release of TNF- α from non-activated monocytes. Although these were the only concentrations of NET-A which was of statistical significance, the general pro-inflammatory tendency of this hormone could be seen by looking at the appropriate graphs. Smit *et al.* (2002) reported on the common experience of vaginal wetness amongst South African users of progestogen-only injectable contraceptives which included NET-En. This *in vitro* pro-inflammatory tendency of NET-A could possibly explain the observed *in vivo* effect of a non-infective/non-specific vaginal discharge associated with the use of NET-En. This pro-inflammatory activity of NET-A could have clinical implications if responsible for the observed side-effects that will be discussed later.

Regarding the effect of NET-A on the activation of CD4⁺ T cells, we found that NET-A had no significant effect in either of the genders as well as the combined group. NET-A, however, significantly affected the activation of CD8⁺ T cells at all concentrations tested in the combined group. This inhibition of activation was more pronounced in female donors than in male donors, while, surprisingly, the overall average suppressive effect of NET-A in the female group was stronger than that of MPA in the same group. This

occurrence where NET-A was able to have a similar effect on the activation of CD8⁺ T cells is quite fascinating, since MPA had the advantage of its high affinity for the GR while NET-A does not bind to the GR. Comparing contraceptive doses, we discovered that 10⁻⁹ M NET-A had an average inhibition of 52.4% on the activation of CD8⁺ T cells in females while the average inhibitory effect of Dex at the same concentration was slightly more (55.1%).

Although we can not extrapolate with certainty from an *in vitro* model to an *in vivo* system, our results indicate that there is a possibility that women using NET-En might not be able to mount an effective immune response against the attack of intra-cellular pathogens due to the inhibiting effect NET-A has on the activation of CD8⁺ T cells. Therefore, the tendency of NET-A to inhibit the activation of CD8⁺ T cells needs urgent attention, since NET is widely used in contraception and HRT.

Just as the effects of MPA were studied, we investigated the effects of NET-A on the T_H2:T_H1 ratio as well as on the separate helper subsets. Only the highest concentration of NET-A tested displayed a statistically significant decrease in the T_H2:T_H1 ratio in the combined group, while the reduction of T_H2⁺ cells by NET-A at the same concentration was responsible for this observed change. The effects of NET-A on the separate gender groups were even less significant. Statistically, the only significant effect occurred at a concentration of 10⁻⁵ M NET-A, where it was able to cause a 49.5% decrease in the T_H2:T_H1 ratio in male donors.

Similar to our statement regarding the effects of MPA on T_H1 and T_H2 subsets, we can also argue here that the difference in receptor interactions of NET-A and the levels of these receptors might be responsible for our observed results. Furthermore, we can also question our incubation periods as well as what the results would have been if we had managed to exclude the already committed helper cells.

Included with our research on the effects of MPA on RANTES production was the possible effects of NET-A on this chemokine. Similar to the effects caused by MPA, NET-A also caused a general increase in RANTES production upon TPA stimulation of the combined group. By analysing the effects of NET-A on the separate gender groups, we discovered that the enhancing effect on TPA-induced RANTES production was more pronounced in female donors than in male donors. Unlike the variable effect of MPA on the basal RANTES levels in the separate gender groups, the enhancing effect on the basal levels of RANTES by NET-A was more or less the same between genders. The same arguments that were used concerning the observed effect of MPA on RANTES production regarding the appropriate stimulus and the correct incubation periods can also apply to the observed effect NET-A had in our study. Here too, the question whether we targeted the correct chemokine can be asked. If in reality we did not target the correct chemokine, it would be appropriate to investigate the effects of NET-A on the two other significant chemokines, MIP-1 α and MIP-1 β .

The following can be concluded by summarizing the collective evidence of the effects NET-A had on the six different immunologic parameters studied by us. After investigating whether NET-A possessed certain anti-inflammatory and/or pro-

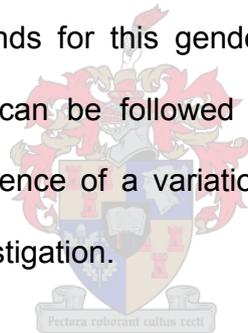
inflammatory properties, we found that NET-A did not have a significant effect on sub-optimally activated monocytes and that the anti-inflammatory effect of NET-A during a severe inflammatory response was less potent than that displayed by MPA. The significant anti-inflammatory effect we found of NET-A, regarding IL-6 production, in the combined group was caused by the fact that NET-A had a significant effect on male donors, but not on female donors. The effect of NET-A on TNF- α production during a severe inflammatory episode was even less than the effect it had on IL-6 production. We found that NET-A had no significant effect on any of the genders regarding TNF- α production, while it only had a statistically significant effect at the highest concentration tested in the combined group.

Furthermore, we found that NET-A had no significant effect on moderately activated monocytes. However, we did discover a general pro-inflammatory tendency of NET-A regarding IL-6 and TNF- α production, but we can not be sure whether this *in vitro* event could relate back to the observed *in vivo* side-effect of vaginal discharge. If this observed *in vitro* pro-inflammatory activity of NET-A is a proper reflection of *in vivo* conditions it would mean that an increase in the recruitment of inflammatory cells would occur throughout the body, including at mucosal surfaces. This non-infective pro-inflammatory activity of NET-A will result in inflammatory cells that cannot detect bacteria, but their increased presence may result in an increased possibility of infection and transmission of HIV and other intracellular pathogens.

Regarding the investigated effects of NET-A on the activation of CD4⁺ and CD8⁺ T cells, we discovered that NET-A did not significantly affect the activation of CD4⁺ T cells.

However, the effects of NET-A on the activation of CD8⁺ T cells was stronger than that of MPA and was comparable to that of Dex, even at contraceptive doses. We suspected that NET-A would not have a significant effect on the T helper subsets, due the effect it had on CD4⁺ T cells. This was confirmed by the fact that NET-A did not really have a significant effect on the T_H1: T_H2 ratio as well as the separate helper subsets in the combined group and no effect on either of the genders. Concerning the effects of NET-A on the chemokine RANTES, we found that the enhancing effect of NET-A was less pronounced than that of MPA.

We found that the activity of NET-A amongst genders also differed throughout the different experiments. The grounds for this gender discrepancy are not completely clear, but the same arguments can be followed for that given for the differences observed with MPA. This occurrence of a variation in the effect of NET-A between genders, also calls for further investigation.



Regarding the effects of NET-A in particular at contraceptive doses, our collected thoughts are that the effects associated with NET-A was not as severe as that of MPA. Although we found that NET-A significantly suppressed the activation of CD8⁺ T cells, it displayed no significant suppressive effects on the production of IL-6, the production of TNF- α or the activation of CD4⁺ T cells. However, the pro-inflammatory tendency of NET-A demonstrated by us is a matter of concern and requires further investigation.

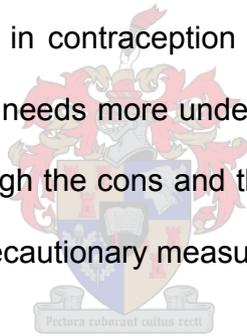
In addition to the results of MPA and NET-A, we demonstrated that the response to Dex varied amongst genders. We found that Dex had a more potent suppressive effect on

both IL-6 and TNF- α production in females during a severe inflammatory episode. The effect of Dex on IL-6 production during a mild inflammatory episode was also more potent in female donors, while its effect on TNF- α production during a moderate inflammatory episode was more potent in male donors. This indicates that the gender of an individual should be taken into consideration when Dex is used as an anti-inflammatory agent. We found that this gender difference displayed by Dex also occurred in our investigations on CD4⁺ and CD8⁺ T cells. The suppressive effects of Dex on the activation of CD4⁺ as well as CD8⁺ T cells were more pronounced in females than in males. This was especially true for the effect it had on CD8⁺ T cells, since it had a very significant inhibitory effect in female donors and no significant effect at all in male donors. Surprisingly, Dex significantly reduced the number of basal CD8⁺ T cells in male donors, but had no significant effect on the basal levels of CD8⁺ T cells of female donors. These gender differences demonstrated by Dex cannot be entirely explained by us. We can only speculate that this was due to a difference in receptor levels (EC50 values) between genders.

The prevalence of STD's and the frequency of sexual transmission of HIV-1 are high in South Africa and the rest of the developing world. According to the latest statistics, there are currently 37.8 million people that are infected with HIV in the world of which approximately 25 million are found in Sub-Saharan Africa (<http://www.avert.org>). Furthermore, it is estimated that 4.73 million people were newly infected with HIV in 2003 despite the availability of antiretroviral therapy and the implementation of extensive awareness programs. There are a number of factors that are responsible for HIV reaching pandemic proportions and such factors include malnutrition, lack of proper

sex education, lack of family planning, religious believes etc. The evidence of this study indicates that the immunosuppressive and anti-inflammatory effects associated with MPA and NET-A could be one of these contributing factors, seeing that injectable contraceptives containing MPA or NET-En are used extensively in South Africa and the rest of the developing world.

We conclude by stating that this study is not suggesting the complete discontinuation of injectable contraceptives containing either MPA or NET-En. What can be derived from the results of this study is that injectable contraceptives containing NET-En is the safer route to follow. What this *in vitro* study hopes to accomplish is to create awareness around the use of MPA and NET in contraception and HRT, thereby inspiring further research in this field which clearly needs more understanding. At the moment the pros of injectable contraceptives outweigh the cons and this equation can be improved if the cons are known and the proper precautionary measures are taken.



6.2 Future perspective

Suggested future research should involve the investigation of these two synthetic progestins in an *in vivo* environment and the effects they have on certain aspects of the immune response. New epidemiological studies in women using these contraceptives should also be planned. These studies should focus specifically on whether the incidence of HIV transmission/seroconversion differs in women using these contraceptives compared to non-users, although it would be difficult to obtain a

comparison group. Furthermore, it is vital that these studies are closely monitored seeing that this difficult task might have been responsible for inconsistencies experienced in similar studies done previously (Mati *et al.*, 1995; Bulterys *et al.*, 1994; Martin *et al.*, 1998; Beaten *et al.*, 2001). Some of these studies found a positive association between the use of hormonal contraception and HIV infection, while others found no association.

Regarding the effects of these synthetic progestins on the production of chemokines, it would also be of importance to investigate the effects of these steroids on other important chemokines, like MIP-1 α and MIP-1 β . Further studies can also be done to compare these two synthetic progestins with progesterone in order to see which progestin acts differently to progesterone. More studies in the field of steroid receptors should be done, since it was at least partially responsible for the interesting results obtained by us. One way to determine which receptors are involved would be to repeat the experiments with and without steroid receptor-specific antagonists.

REFERENCES

Aarnisalo, P., Palvimo, J.J., Janne, O.A. CREB-binding protein in androgen receptor-mediated signaling. *Proc Natl Acad Sci U S A*. 1998; 95(5): 2122-2127.

Abbas, A.K. and Lichtman, A.H. *Cellular and molecular immunology*. 5th. edition. Saunders. 2003.

Abbas, A.K., Murphy, K.M., Sher, A. Functional diversity of helper T lymphocytes. *Nature*. 1996; 383: 787-793.

Adams, M.R., Register, T.C., Golden, D.L., Wagner, J.D., Koudy Williams, J. Medroxyprogesterone acetate antagonizes inhibitory effects of conjugated equine estrogens on coronary artery atherosclerosis. *Arterioscl Thromb Vascul Biol*. 1997; 17: 217-221.

Achari, K. Effects of norethisterone enanthate (SH 393) on the structure of the endometrium and on the cervical mucus. *J Obstet Gynec India*. 1969; 19: 731-736.

Adcock, I.M., Brown, C.R., Gelder, C.M., Shirasaki, H., Peters, M.J., Barnes, P.J. Effects of glucocorticoids on transcription factor activation in human peripheral blood mononuclear cells. *Am J Physiol*. 1995; 268: C331-C338.

Affandi, B. Long-acting progestogens. *Best Practise Res Clin Obstet Gynaecol*. 2002; 16: 169-179.

Afonso, L.C., Scharon, T.M., Vieira, L.Q., Wysocka, M., Trinchieri, G., Scott, P. The adjuvant effect of interleukin-12 in a vaccine against *Leishmania major*. *Science*. 1994; 263: 235-237.

Agarwal, S.K. and Marshall, G.D. Dexamethasone promotes type 2 cytokine production primarily through inhibition of type 1 cytokines. *J Interferon Cytokine Res.* 2001; 21 (3): 147-155.

Aggarwal, B.B. and Gutterman, J.U. *Human cytokines: handbook for basic and clinical research II.* Blackwell Science. 1996.

Akira, S., Hirano, T., Taga, T. *et al.* Biology of multifunctional cytokines: IL-6 and related molecules. *FASEB J.* 1990; 4: 2860-2867.

Alam, R., Stafford, S., Forsythe, P. *et al.* RANTES is a chemotactic and activating factor for human eosinophils. *J Immunol.* 1993; 3442-3448.

Almawi, W.Y. Molecular mechanisms of glucocorticoid effects. *Mod Asp Immunobiol.* 2001; 2 (2): 78-82.

Almawi, W.Y., Hess, D.A., Rieder, M.J. Multiplicity of glucocorticoid action in inhibiting allograft rejection. *Cell Transplant.* 1998; 7(6): 511-523.

Almawi, W.Y., Lipman, M.L., Stevens, A.C., Zanker, B., Hadro, E.T., Strom, T.B. Abrogation of glucocorticoid-mediated inhibition of T cell proliferation by the synergistic action of IL-1, IL-6, and IFN-gamma. *J Immunol.* 1991; 146(10): 3523-3527.

Amatayakul, K. Safety and hazards of injectable hormonal contraceptives. *Singapore J Obstet Gynec.* 1979; 9: 57.

Amatayakul, K., Petpoo, W., Ratanawanukul, N., Tanthayaphinant, O., Tovanabutra, S., Suriyanon, V. A study of adrenal cortical function and its reserve activity in long-acting injectable contraceptive users. *Contraception.* 1988; 37: 483-492.

Anderson, G.P. and Coyle, A.J. TH2 and 'TH2-like' cells in allergy and asthma: pharmacological perspectives. *Trends Pharmacol Sci.* 1994; 15(9): 324-332.

Ang, Y.S., Mahmud, N., White, B., Byrne, M., Kelly, A., Lawler, M., McDonald, G.S., Smith, O.P., Keeling, P.W. Randomized comparison of unfractionated heparin with corticosteroids in severe active inflammatory bowel disease. *Aliment Pharmacol Ther.* 2000; 14(8): 1015-1022.

Auphan, N., DiDonato, J.A., Rosette, C., Helmborg, A., Karin, M. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science.* 1995; 270(5234): 286-290.

Babcock, J.C., Gutsell, E.S., Herr, M. *et al.* Six methyl seven hydroxyprogesterone seventeen acylates: a new class of potent progestins. *J Amer Chem Soc.* 1958; 80: 2092.

Bacon, K.B., Flores, R.L., Aubry, J.P. *et al.* Interleukin-8 and RANTES induce the adhesion of the human basophilic cell line KU-812 to human endothelial cell monolayers. *Immunol.* 1994; 82: 473-481.

Baeten, J.M., Nyange, P.M., Richardson, B.A., Lavreys, L., Chohan, B., Martin, H.L., Mandaliya, K., Ndinya-Achola, J.O., Bwayo, J.J., Kreiss, J.K. Hormonal contraception and risk of sexually transmitted disease acquisition: Results from a prospective study. *Am J Obstet Gynecol.* 2001; 185 (2): 380-385.

Baker, D.A., Philips, C.A., Roessner, K., Albertini, R.J., Mann, L.I. Suppression by progesterone of nonspecific stimulation in mice as a mechanism for the enhancement of herpes simplex virus type 2 vaginal infection. *Am J Obstet Gynecol.* 1980; 136 (4): 440-445.

Bamberger, A-M., Erdmann, I., Bamberger, C.M., Jenatschke, S., Schulte, H.M. Transcriptional regulation of the human leukemia inhibitory factor gene: modulation by glucocorticoids and estradiol. *Molec Cell Endocrinol.* 1997; 127: 71-79.

Bamberger, C.M. and Schulte, H.M. Molecular mechanisms of dissociative glucocorticoid activity. *Eur J Clin Invest.* 2000; 30(Suppl. 3): 6-9.

Bamberger, C.M., Else, T., Bamberger, A-M., Ulrich Beil, F., Schulte, H.M. Dissociative glucocorticoid activity of medroxyprogesterone acetate in normal human lymphocytes. *J Clin Endocr Metab.* 1999; 84 (11): 4055-4061.

Bamberger, C.M., Schulte, H.M., Chrousos, G.P. Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. *Endocr Rev.* 1996; 17 (3): 245-261.

Banks, E., Berrington, A., Casabonne, D. Overview of the relationship between use of progestogen-only contraceptives and bone mineral density. *BJOG.* 2001; 108: 1214-1221.

Bassol, S., Cravioto, M.C., Durand, M., Bailon, R., Carranza, S., Fugarolas, J. *et al.* Mesigyna once-a-month combined injectable contraceptive: experience in Latin America. *Contraception.* 2000; 61: 309-316.

Batuman, O.A., Ferrero, A.P., Diaz, A., Berger, B., Pomerantz, R.J. Glucocorticoid-mediated inhibition of interleukin-2 receptor alpha and -beta subunit expression by human T cells. *Immunopharmacology.* 1994; 27(1): 43-55.

Baumann, H. and Gauldie, J. The acute phase response. *Immunol Today.* 1994; 15(2): 74-80.

Baus, E., Andris, F., Dubois, P.M., Urbain, J., Leo, O. Dexamethasone inhibits the early steps of antigen receptor signaling in activated T cells. *J Immunol.* 1996; 156 (12): 4555-4561.

Beato, M. Gene regulation by steroid hormones. *Cell.* 1989; 56: 335-344.

Beato, M., Herrlich, P., Schütz, G. Steroid hormone receptors: many actors in search of a plot. *Cell*. 1995; 83: 851-857.

Becker, P.B., Gloss, B., Schmid, W., Strähle, U., Schütz, G. *In vivo* protein-DNA interactions in a glucocorticoid response element require the presence of the hormone. *Nature*. 1986; 324 (6098): 686-688.

Bell, P.A, Weatherill, P.J., Jones, T.R. Influence of hydrophobic interactions on the structure and steroid-binding properties of glucocorticoid receptors. *J Steroid Biochem*. 1986; 24: 57-62.

Belvisi, M.G., Wicks, S.L., Batram, C.H., Bottoms, S.E., Redford, J.E., Woodman, P., Brown, T.J., Webber, S.E., Foster, M.L. Therapeutic benefit of a dissociated glucocorticoid and the relevance of an *in vitro* separation of transrepression from transactivation activity. *J Immunol*. 2001; 166 (3): 1975-1982.

Benagiano, G., Pera, A., Primiero, F.M. The endometrium and hormonal contraceptives. *Hum Reprod*. 2000; 15 (Suppl 1): 101-118.

Beral, V., Banks, E., Reeves, G. Evidence from randomised trails on the long-term effects of hormone replacement therapy. *Lancet*. 2002; 360 (9337): 942-944.

Berenson, A.B., Radecki, C., Grady, J.J., V.I., Thomas, A. A prospective, controlled study of the effects of hormonal contraception on bone mineral density. *Obstet Gynecol*. 2001; 98: 576-582.

Bentel, J.M., Birrell, S.N., Pickering, M.A., Holds, D.J., Horsfall, D.J., Tilley, W.D. Androgen receptor agonist activity of the synthetic progestin, medroxyprogesterone acetate, in human breast cancer cells. *Mol Cell Endocrinol*. 1999; 154(1-2): 11-20.

Berg, J.M. DNA binding specificity of steroid receptors. *Cell*. 1989; 57(7): 1065-1068.

Bergink, E.W., van Meel, F., Turpijn, E.W., van der Vies, J. Binding of progestagens to receptor proteins in MCF-7 cells. *J Steroid Biochem.* 1983; 19(5): 1563-1570.

Blanchard, D.K., Michelini-Norris, M.B., Pearson, C.A., Freitag, C.S., Djeu, J.Y. Mycobacterium avium-intracellular induced interleukin-6 from human monocytes and large granular lymphocytes. *Blood.* 1991; 77: 2218-2224.

Bloom, B.R., Modlin, R.L., Salgame, P. Stigma variations: observations on suppressor T cells and leprosy. *Annu Rev Immunol.* 1992; 10: 453-488.

Bloom, H.J. Medroxyprogesterone acetate (Provera) in the treatment of metastatic renal cancer. *Brit J Cancer.* 1971; 25: 250-265.

Blossey, H.C., Wander, H.C., Koebberling, J., Nagel, G.A. Pharmacokinetic and pharmacodynamic basis for the treatment of metastatic breast cancer with high-dose medroxyprogesterone acetate. *Cancer.* 1984; 54: 1208-1215.

Bojar, H., Maar, K., Staib, W. The endocrine background of human renal cell carcinoma. IV. Glucocorticoid receptors as possible mediators of progestogen action. *Urol Int.* 1979; 34: 330-338.

Bonte, J. Beneficial effects of contraceptive doses and large doses of medroxyprogesterone in the prevention and treatment of uterine hyperplasia. *Gynak Rdsch.* 1978; 18: 172-182.

Boumpas, D.T., Anastassiou, E.D., Older, S.A., Tsokos, G.C., Nelson, D.L., Balow, J.E. Dexamethasone inhibits human interleukin 2 but not interleukin 2 receptor gene expression in vitro at the level of nuclear transcription. *J Clin Invest.* 1991; 87(5): 1739-47.

Boumpas, D.T., Paliogianni, F., Anastassiou, E.D., Balow, J.E. Glucocorticosteroid action on the immune system: molecular and cellular aspects. *Clin Exp Rheumatol.* 1991; 9: 413-423.

Branham, J. Oral contraceptives and depression. *Brit Med J.* 1970; 1: 237.

Brasier, A.R. and Li, J. Mechanisms for inducible control of angiotensinogen gene transcription. *Hypertension.* 1996; 27 (3 Part 2): 465-475.

Braunsberg, H., Coldham, N.G., Leake, R.E., Cowan, S.K., Wong, W. Actions of a progestogen on human breast cancer cells: mechanisms of growth stimulation and inhibition. *Eur J Cancer Clin Oncol.* 1987; 23: 563-571.

Brenner, P.F. Long-acting progestin-only contraception. *Salerni Collegium Medicine Update.* University of Southern California, Los Angeles. 1995.

Bretscher, P.A., Wei, G., Menon, J.N. Bielefeldt-Ohmann, H. Establishment of stable, cell-mediated immunity that makes "susceptible" mice resistant to *Leishmania major*. *Science.* 1992; 257: 539-542.



Briggs, M.H. and Briggs, M. Glucocorticoid properties of progestagens. *Steroids.* 1973; 22: 555-559.

Brooke, S.M. and Sapolsky, R.M. The effects of steroid hormones in HIV-related neurotoxicity: A mini review. *Biol Psychiatry.* 2000; 48: 881-893.

Brunelli, R., Frasca, D., Perrone, G., Pioli, C., Fattorossi, A., Zichella, L., Doria, G. Hormone replacement therapy affects various immune cell subsets and natural cytotoxicity. *Gynecol Obstet Invest.* 1996; 41 (2): 128-131.

Bullock, J.L., Massey, F.M., Gambrell, R.D. Use of medroxyprogesterone acetate to prevent menopausal symptoms. *Obstet Gynecol.* 1975; 46: 165-168.

Bulterys, M., Chao, A., Habimana, P., Dushimimana, A., Nawrocki, P., Saah, A. Incident HIV-1 infection in a cohort of young women in Butare, Rwanda. *AIDS*. 1994; 8: 1585-1591.

Camanni, F., Massaraf, F., Molinatti, G.M. The cortisol-like effect of 6alpha-methyl-17alpha-acetoxypregesterone in the adrenalectomized man. *Acta Endocrinol (Copenh)*. 1963; 43: 477-483.

Chen, C.Y., Del Gatto-Konczak, F., Wu, Z., Karin, M. Stabilization of interleukin-2 mRNA by the c-jun NH2-terminal kinase pathway. *Nature (Lond)*. 1998; 280: 1945-1949.

Chikanza, I.C., Petrou, P., Kingsley, G., Chrousos, G., Panayi, G.S. Abnormal cortisol response to surgery in rheumatoid arthritis. *Clin Rheumatol*. 1992; 11: 138-139.

Chinnatamby, S. A comparison of the long-acting contraceptive agents norethisterone oenanthate and medroxyprogesterone acetate. *Aust NZ J Obstet Gynaec*. 1971; 11: 233-236.

Chiu, L., Nishimure, M., Ishii, Y. *et al*. Enhancement of the expression of progesterone receptor on progesterone-treated lymphocytes after immunotherapy in unexplained spontaneous abortion. *Am J Reprod Immunol*. 1996; 35: 552-527.

Chotnopparatpattara, P. and Taneepanichskul, S. Use of medroxyprogesterone in Thai adolescents. *Contraception*. 2000; 62: 137-140.

Chrousos, G.P. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. *N Engl J Med*. 1995; 332: 1351-1362.

Clerici, M., Hakim, F.T., Venzon, D.J., Blatt, S., Hendrix, C.W., Wynn, T.A., Shearer, G.M. Changes in interleukin-2 and interleukin-4 production in asymptomatic human immunodeficiency virus-seropositive individuals. *J Clin Invest*. 1993; 91: 759-765.

Conca, W., Kaplan, P.B., Krane, S.M. Increase in levels of procollagenase messenger RNA in cultures fibroblasts induced by human recombinant interleukin 1 β or serum follow c-jun expression and are dependent on new protein synthesis. *J Clin Invest.* 1989; 83: 1753-1757.

Connell, E.B. Depot medroxyprogesterone acetate: clinical experience in the United States. *The Female Patient.* 1994; 18: 56-62.

Corsini, G. and Puppo, F. Effects of medroxyprogesterone acetate upon PHA, Con A and PWM stimulated lymphocytes and on E-rosette function. *J Immunopharmacol.* 1983; 4 (3): 247-253.

Costas, M.A., Muller Igaz, L., Holsboer, F., Arzt, E. Transrepression of NF-kappaB is not required for glucocorticoid-mediated protection of TNF-alpha-induced apoptosis on fibroblasts. *Biochim Biophys Acta.* 2000; 1499: 122-129.

Cottingham, J. and Hunter, D. Chlamydia trachomatis and oral contraceptive use: a quantitative review. *Genitourin Med.* 1992; 68: 209-216.

Coutinho, E.M. and De Souza, J.C. Conception control by monthly injections of medroxyprogesterone suspension and a long-acting oestrogen. *J Reprod Fertil.* 1966; 15: 209-214.

Croft, M., Carter, L., Swann, S.L., Dutton, R.W. Generation of polarized antigen-specific CD8 effector populations: reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 vs. type 1 cytokine profiles. *J Exp Med.* 1994; 180: 1715-1728.

Csapo, A., De Souza-Filho, M.B., De Souza, J.C., De Souza, O. The effects of massive progestational hormone treatment on the human uterus. *Fertil Steril.* 1966; 17: 621-636.

Cua, D.J., Coffman, R.L., Stohlman, S.A. Exposure to T helper 2 cytokines in vivo before encounter with antigen selects for T helper subsets via alterations in antigen-presenting cell function. *J Immunol.* 1996; 157: 2830-2836.

Cummings, J.A. and Brizendine, L. Comparison of physical and emotional side effects of progesterone or medroxyprogesterone in early postmenopausal women. *Menopause.* 2002; 9: 253-263.

Da Silva, J.A.P., Peers, S.H., Perretti, M., Willoughby, D.A. Sex steroids affect glucocorticoid response to chronic inflammation and to interleukin-1. *J Endocrinol.* 1993; 136: 389-397.

Dabancens, A., Prado, R., Larraguiebel, R., Zanartu, J. Intraepithelial cervical neoplasm in women using intrauterine devices and long-acting injectable progestogens as contraceptives. *Amer J Obstet Gynec.* 1974; 119: 1052-1056.

Dayens, R.A., Meikle, A.W., Araneo, B.A. Locally active steroid hormones may facilitate compartmentalization of immunity by regulating the types of lymphokines produced by helper T cells. *Res Immunol.* 1991; 142: 40-45.

Dayer, J-M., Krane, S.M., Russel, G.G., Robinson, D.R. Production of collagenase and prostaglandins by isolated adherent rheumatoid synovial cells. *Proc Natl Acad Sci.* 1976; 73: 945-949.

Daynes, R.A. and Araneo, B.A. Contrasting effects of glucocorticoids on the capacity of T cells to produce the growth factors interleukin 2 and interleukin 4. *Eur J Immunol.* 1989; 19 (12): 2319-2325.

De Bosscher, K., Schmitz, M.L., Vanden Berghe, W., Plaisance, S., Fiers, W., Haegeman, G. Glucocorticoid-mediated repression of nuclear factor- κ B-dependent transcription involves direct interference with transactivation. *Proc Natl Acad Sci USA.* 1997; 94: 13504-13509.

De Ceulaer, K., Gruber, C., Hayes, R., Serjeant, G.R. Medroxyprogesterone and homozygous sickle cell disease. *Lancet*. 1982; 2: 229-231.

De Rijk, R. and Berkenbosch, F. in. *Bilateral Communication between the endocrine and immune system* (Grossman, C.J., ed.). pp. 73-95. Springer-Verlag.

Del Prete, G.F., De Carli, M., Almerigogna, F., Daniel, C.K., D'Elios, M.M., Zancuoghi, G., Vinante, F., Pizzolo, G., Romagnani, S. Preferential expression of CD30 by human CD4⁺ T cells producing Th2-type cytokines. *FASEB J*. 1995; 8: 81-86.

Del Prete, G.F., De Carli, M., Mastromauro, C., Macchia, D., Bagiotti, R., Ricci, M., Romagnani, S. Purified protein derivative of *mycobacterium tuberculosis* and excretory/secretory antigen(s) of *Toxocara canis* expand in vitro human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production. *J Clin Invest*. 1991; 88: 346-350.

Dentener, M.A., Bazil, V., Von Asmuth, E.J., Ceska, M., Buurman, A. Involvement of CD14 in lipopolysaccharide-induced tumor necrosis factor-alpha, IL-6 and IL-8 release by human monocytes and alveolar macrophages. *J Immunol*. 1993; 150: 2885-2891.

Deslypere, J.P., Thiery, M., Vermeulen, A. Effect of long-term hormonal contraception on plasma lipids. *Contraception*. 1985; 31: 633-642.

Devergne, O., Marfaing, K.A., Schall, T.T. *et al*. Production of the RANTES chemokine in delayed-type hypersensitivity reactions: involvement of macrophages and endothelial cells. *J Exp Med*. 1994; 179: 1689-1694.

Dodds, G.H. The use of sterile medroxyprogesterone suspension as a contraceptive during a three year period. *Contraception*. 1975; 11: 15-23.

Duax, W.L., Cody, V., Griffin, J. *et al.* Steroid structure and function: II. Conformational transformation and receptor binding of medroxyprogesterone acetate. *J Steroid Biochem.* 1978; 9: 901-907.

Ebrecht, M., Buske-Kirschbaum, A., Hellhammer, D., Kern, S., Rohleder, M., Walker, B., Kirschbaum, C. Tissue specificity of glucocorticoid sensitivity in healthy adults. *J Clin Endocrinol Metab.* 2000; 85 (10): 3733-3739.

Ettiene, M.C., Milano, G., Frenay, M., Renee, N., Francois, E., Thyss, A., Schneider, M., Namer, M. Pharmacokinetics pharmacodynamics of medroxyprogesterone acetate in advanced breast cancer patients. *J Clin Oncol.* 1992; 10: 1167-1182.

Ettinger, B. and Golditch, I.M. Medroxyprogesterone acetate for the evaluation of hyper testosterone in hirsute women. *Fertil Steril.* 1977; 28: 1285-1288.

Evans, R.M. The steroid and thyroid hormone receptor superfamily. *Science.* 1988; 240 (4854): 889-895.

Fawcett, D.W. Bloom and Fawcett: A textbook of histology. 11th edition. Philadelphia: WB Saunders. 1986.

Feghali, C.A., Wright, T.M. Cytokines in acute and chronic inflammation. *Front Biosc.* 1997; 2: d12-d26.

Ferguson, M.M., McKay-Hart, D., Lindsay, R., Stephen, K.W. Progestogen therapy for menstrually related aphthae. *Int J Oral Surg.* 1978; 7: 463-470.

Fessler, B.J., Paliogianni, F., Hama, N., Balow, J.E., Boumpas, D.T. Glucocorticoids modulate CD28-mediated pathways for interleukin 2 production in human T cells: evidence for posttranscriptional regulation. *Transplantation.* 1996; 62 (8): 1113-1118.

Fine, E., Levin, H.M., McConnel, E.L. Masculinization of female infants associated with norethisterone acetate. *Obstet Gynecol.* 1962; 22: 210-213.

Fiorentino, D.F., Bond, M.W., Mosmann, T.R. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med.* 1989; 170(6): 2081-2095.

Fotherby, K. Oral contraceptives, lipids and cardiovascular disease. *Contraception.* 1985; 31: 367-394.

Fotherby, K., Howard, G., Shrimanker, K. *et al.* Occurrence of ovulation in women receiving the injectable contraceptive norethindrone-oenanthate. *Contraception.* 1978; 18: 535-542.

Franchimont, D., Galon, J., Gadina, M., Visconti, R., Zhou, Y., Aringer, M., Frucht, D.M., Chrousos, G.P., O'Shea, J.J. Inhibition of Th1 immune response by glucocorticoids: dexamethasone selectively inhibits IL-12-induced Stat4 phosphorylation in T lymphocytes. *J Immunol.* 2000; 164 (4): 1768-1774.

Fraser, I.S. A comprehensive review of injectable contraception with special emphasis on depot medroxyprogesterone acetate. *Med J Aust.* 1981; 1 (Spec. Suppl.1): 1-20.

Freedman, L.P. Increasing the complexity of coactivation in nuclear receptor signaling. *Cell.* 1999; 97(1): 5-8.

Fujisawa, H., Wang, B., Sauder, D.N., Kondo, S. Effects of interferons on the production of interleukin-6 and interleukin-8 in human keratinocytes. *J Interferon Cytokine Res.* 1997; 17: 347-353.

Furth, P.A., Westphal, H., Hennighausen, L. Expression from the HIV-LTR is stimulated by glucocorticoids and pregnancy. *AIDS Res Hum Retrovir.* 1990; 6: 553-560.

Gallagher, C.J., Cairnduff, F., Smith, I.E. High dose vs. low dose medroxyprogesterone acetate: a randomized trial in advanced breast cancer. *Eur J Cancer Clin Oncol.* 1987; 23:1895-1900.

Galli, S.J. New concepts about the mast cell. *N Engl J Med.* 1993; 328(4): 257-265.

Galve-de Rochemonteix, B., Wiktorowicz, K., Kushner, I. *et al.* C-reactive protein increases production of IL-1 α , IL-1 β , and TNF- α , and expression of mRNA by human alveolar macrophages. *J Leukoc Biol.* 1993; 53: 439-445.

Garza-Flores, J., Alba, V.M., Cravioto, M-C., Hernandez, L., Perez-Palacios, G. Estrogen-progestogen once-a-month injectible contraceptives and serum prolactin. *Contraception.* 1989; 39: 519-529.

Gerhards, E., Hecker, W., Bellmann, O. Studies of the kinetics and metabolism of 17 beta-heptanoyl-17-alpha-ethinyl-4-oestren-3-one-7-3H (norethisterone enanthate) in humans following intramuscular injection. *Arzneimittelforschung.* 1976; 26: 1611-1614.

Gerretsen, G., Kremer, J., Bleumink, E., Nater, J.P., de Gast, G.C., The, T.H. Immune reactivity of women on hormonal contraceptives: Phytohemagglutinin and Concanavalin-A induced lymphocyte response. *Contraception.* 1980; 22 (1): 25-29.

Gerretsen, G., Kremer, J., Nater, J.P., Bleumink, E., de Gast, G.C., The, T.H. Immune reactivity of women on hormonal contraceptives: Dinitrochlorobenzene sensitization test and skin reactivity to irritants. *Contraception.* 1979; 19 (1): 83-89.

Gershberg, H., Zarrilla, E., Hernandez, A., Hulse, M. Effects of medroxyprogesterone acetate on serum insulin and growth hormone levels in diabetics and potential diabetics. *Obstet Gynec.* 1969; 33: 383-389.

Gessani, S., Borghi, P., Fantuzzi, L., Varano, B., Conti, L., Puddu, P., Belardelli, F. Induction of cytokines by HIV-1 and its gp120 protein in human peripheral blood monocytes/macrophages and modulation of cytokine response during differentiation. *J Leukoc Biol.* 1997; 62 (1): 49-53.

Ghosh, D. Glucocorticoid receptor-binding site in the human immunodeficiency virus long terminal repeat. *J Virol.* 1992; 66: 586-590.

Giwa-Osagie, O.F. Metabolic effects of once-a-month combined injectable contraceptives. *Contraception.* 1994; 49: 421-433.

Giwa-Osagie, O.F., Savage, J., Newton, J. Norethisterone oenanthate as an injectable contraceptive: a study of patients discontinuing treatment. *Contraception.* 1978; 18: 517-526.

Goebelsmann, U., Stanczyk, F.Z., Brenner, P. *et al.* Serum norethindrone concentrations following intramuscular norethindrone oenanthate injection: effect upon serum LH, FSH, estradiol and progesterone. *Contraception.* 1979; 19: 283-313.

Gomez, F., Ruiz, P., Briceño, F., Lopez, R., Michan, A. Treatment with progesterone analogues decreases macrophage Fcγ receptors expression. *Clin Immunol Immunopathol.* 1998; 89: 231-239.

Gonzalez, M.V., Jimenez, B., Berciano, M.T., Gonzalez-Sancho, J.M., Caelles, C., Lafarga, M., Munoz, A. Glucocorticoids antagonize AP-1 by inhibiting the Activation/phosphorylation of JNK without affecting its subcellular distribution. *J Cell Biol.* 2000; 150(5): 1199-1208.

Goppelt-Struebe, M., Rehm, M., Schaefers, H.J. Induction of cyclooxygenase-2 by platelet-derived growth factor (PDGF) and its inhibition by dexamethasone are independent of NF-kappaB/IkappaB transcription factors. *Naunyn Schmiedebergs Arch Pharmacol.* 2000; 361(6): 636-645.

Goswami, R., Lacson, R., Yang, E., Sam, R., Unterman, T. Functional analysis of glucocorticoid and insulin response sequences in the rat insulin-like growth factor-binding protein-1 promoter. *Endocrinology*. 1994; 134(2): 736-743.

Haanen, J.B.A.G., de Waal Malefyt, R., Res, P.C.M., Kraakman, E.M., Ottenhof, T.H.M., de Vries, R.R.P., Spits, H. Selection of human T helper type 1-like T cell subset by *Mycobacteria*. *J Exp Med*. 1991; 174: 583-592.

Haiba, N.A., El-Habashy, M.A., Said, S.A., Darwish, E.A., Abdel-Sayed, W.S., Nayel, S.E. Clinical evaluation of two monthly injectable contraceptives and their effects on some metabolic parameters. *Contraception*. 1989; 39: 619-632.

Hall, P.E. New once-a-month injectable contraceptives, with particular reference to Cyclofem/Cyclo-Provera. *Int J Gynaecol Obstet*. 1998; 62 (Suppl 1): s43-s56.

Hapgood, J.P., Koubovec, D., Louw, A., Africander, A. Not all progestins are the same: implications for usage. *Trends Pharmacol Sci*. 2004; 25: 554-557.

Hatcher, R.A., Trussel, J., Stewart, F., Stewart, G.K., Kowal, D., Guest, F., Gates, W., Polikar, M.S. *Contraceptive technology* (16th ed.). New York: Irvington Publishers, Inc. 1994.

Haukkamaa, M. and Luukkainen, T. The cytoplasmic progesterone receptor of human endometrium during the menstrual cycle. *J Steroid Biochem*. 1974; 5: 447-452.

Hausmann, U., Goebel, K.M., Klohir, D., Haffoenick, H. *Insulin und Proinsulin Sekretion bei Anwendung antikonzeptioneller Steroide. Klin Wochenschr*. 1975; 53: 853-860.

Haynesworth, S.E., Baber, M.A., Caplan, A.I. Cytokine expression by human marrow-derived mesenchymal progenitor cells in vitro: effects of dexamethasone and IL-1 alpha. *J Cell Physiol*. 1996; 166(3): 585-592.

Helfgott, D.C., Tatter, S.B., Santhanam, U., Clarick, R.H., Bhardwaj, N., May, L.T., Seghal, P.B. Multiple forms of IFN- β / IL-6 in serum and body fluids during acute bacterial infection. *J Immunol.* 1989; 142 (3): 948-953.

Henry, S.C., Walsh, P.C., Rotner, M.B. Choriocarcinoma of the testes. *J Urol.* 1974; 112: 105-108.

Herkert, O., Kuhl, H., Sandow, J., Busse, R., Schini-Kerth, V.B. Sex steroids used in hormonal treatment increase vascular procoagulant activity by inducing thrombin receptor (PAR-1) expression: role of the glucocorticoid receptor. *Circulation.* 2001; 104: 2826-2831.

Hess, S., Rensing-Ehl, A., Schwabe, R., Bufler, P., Engelmann, H. CD40 function in non-hematopoietic cells. Nuclear factor kappaB mobilisation and induction of IL-6 production. *J Immunol.* 1995; 155 (10): 4588-4595.

Hibi, M., Nakajima, K., Hirano, T. Il-6 cytokine family and signal transduction: a model of the cytokine system. *J Mol. Med.* 1996; 74 (1): 1-12.

Hillier, S.L. and Lau, R.J. Vaginal microflora in postmenopausal women who have not received estrogen replacement therapy. *Clin Infect Dis.* 1997; 25 (Suppl 2): S123-126.

Hirano, T., Tsuboi, N., Homma, M., Oka, K., Takekoshi, T., Tahara, K., Takanashi, H., Abe, H., Urata, Y., Hayashi, T. Comparative study of lymphocyte-suppressive potency between prednisolone and methylprednisolone in rheumatoid arthritis. *Immunopharmacology.* 2000; 49(3): 411-417.

Hirano, T., Yasukawa, K., Harada, H., Taga, T., Watanabe, Y., Matsuda, T., Kashiwamura, S., Nakajima, K., Koyama, K., Iwamatsu, A. *et al.* Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature.* 1986; 324: 73-76.

Hollenberg, S.M., Giguere, V., Evans, R.M. Identification of two regions of the human glucocorticoid receptor hormone binding domain that block activation. *Cancer Res.* 1989; 49 (8 suppl): 2292s-2294s.

Hollows, F.C. and Beaumont, P. Types of diabetic retinopathy and treatment. *Trans Ophthalmol Soc NZ.* 1973; 25: 50-55.

Homo-Delarche, F., Fitzpatrick, F., Christeff, N., Nunez, E.A., Bach, J.F., Dardenne, M. Sex steroids, glucocorticoids, stress and autoimmunity. *J Steroid Biochem Molec Biol.* 1991; 40:619-637.

Howard, G., Warren, R.J., Fotherby, K. Plasma levels of norethisterone in women receiving norethisterone oenanthate intramuscularly. *Contraception.* 1975; 12: 45-52.

Hricik, D.E., Almawi, W.Y., Strom, T.B. Trends in the use of glucocorticoids in renal transplantation. *Transplantation.* 1994; 57(7): 979-989.

<http://www.avert.org/worldstats.htm>, Accessed September 2004.

Hulka, J.F., Mohr, K., Liebermann, M.W. Effects of synthetic progestational agents on allograft rejection and circulating antibody production. *Endocrinology.* 1965; 77: 897-901.

Irahara, M., Uemura, H., Yasui, T., Kinoshita, H., Yamada, M., Tezuka, M., Kiyokawa, M., Kamada, M., Aono, T. Efficacy of every-other-day administration of conjugated equine estrogen and medroxyprogesterone acetate on gonadotropin-releasing hormone agonists treatment in women with endometriosis. *Gynecol Obstet Invest.* 2001; 52: 217-222.

Ishida, Y., Ishida, Y., Heersche, J.N. Pharmacologic doses of medroxyprogesterone may cause bone loss through glucocorticoid activity: a hypothesis. *Osteoporos Int.* 2002; 13 (8): 601-605.

Izquierdo, J.A., Savini, C., Borghi, E. *et al.* Role of ACTH on the effects of medroxyprogesterone on brain stem serotonin. *Pharmacol Res Commun.* 1978; 10: 643-656.

Jeppsson, S., Johansson, E.D.B., Ljungberg, O., Sjoberg, N. Endometrial histology and circulating levels of of medroxyprogesterone acetate (MPA), estradiol, FSH and LH in women with MPA-induced amenorrhoea compared with women with secondary amenorrhoea. *Acta Obstet Gynec Scand.* 1977; 56: 43-48.

Jeremiah, G., Turcotte, M.D., Haines, R.F., Brody, G.L., Meyer, T.J., Schwartz, S.A. Immunosuppression with medroxyprogesterone acetate. *Transplantation.* 1968; 6 (2): 248-260.

Jiang, H.Y., Yang, H., Zhang, M.Y., Lu, F.Y., Gu, L.J. Effects of long-term use of two monthly injectable contraceptives on carbohydrate metabolism and liver function. *Chin J Fam Plann.* 1992; 1: 28-33.

Jin, X., Bauer, D.E., Tuttleton, S.E., Lewin, S., Gettie, A., Blanchard, J., Irwin, C.E., Safrit, J.T., Mittler, J., Weinberger, L. *et al.* Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med.* 1999; 189 (6): 991-998.

Jones, K.P. and Wild, R.A. Contraception for patients with psychiatric or medical disorders. *Amer J Obstet Gynec.* 1994; 170: 1575-1580.

Jones, T.R. and Bell, P.A. Glucocorticoid receptor interactions: studies of the negative co-operativity induced by steroid interactions with a secondary hydrophobic binding site. *Biochem J.* 1980; 188: 237-245.

Kameyoshi, Y., Schroder, J.M., Christophers, E. *et al.* Identification of the cytokine RANTES released from platelets as an eosinophil chemotactic factor. *Int Arch Allergy Immunol.* 1994; 266: 1395-1399.

Kamischke, A., Diebacker, J., Nieschlag, E. Potential of norethisterone enanthate for male contraception: pharmacokinetics and suppression of pituitary and gonadal function. *Clin Endocrinol.* 2000; 53: 351-358.

Kamischke, A., Heuermann, T., Kruger, K., von Eckardstein, S., Schellschmidt, I., Rubig, A., Nieschlag, E. An effective hormonal male contraceptive using testosterone undecanoate with oral or injectable norethisterone preparations. *J Clin Endocrinol Metab.* 2002; 87: 530-539.

Kamischke, A., Venherm, S., Ploger, D., von Eckardstein, S., Nieschlag, E. Intramuscular testosterone undecanoate and norethisterone enanthate in a clinical trial for male contraception. *J Clin Endocrinol Metab.* 2001; 86: 303-309.

Karim, M., Ammar, A., El Mahgoub, S. *et al.* Injected progestogen and lactation. *Brit Med J.* 1971; 1 (742): 200-203.

Kaufmann, S.H. Immunity to intracellular bacteria. *Annu Rev Immunol.* 1993; 11: 129-163.

Kaunitz, A.M. Injectable contraception: New and existing options. *Obstet Gynecol Clin North Am.* 2000; 27: 741-780.

Kaunitz, A.M. Long-acting injectable contraception with medroxyprogesterone acetate. *Amer J Obstet Gynec.* 1994; 170:1543-1549.

Keller, E.T., Waganat, E., Ershler, W.B. Molecular and cellular biology of interleukin-6 and its receptor. *Frontiers Biosc.* 1996; 1: d340-d357.

Kemppainen, J.A., Langley, E., Wong, C., Bobseine, K., Kelce, W.R., Wilson, E.M. Distinguishing androgen receptor agonists and antagonists: Distinct mechanisms of activation by medroxyprogesterone acetate and dihydrotestosterone. *Mol Endocrinol.* 1999; 13: 440-454.

Kesserü, E., Albornoz, H., Diaz, M., Socolsky, R. Serum lipid variation in women on copper intrauterine devices, triphasic oral contraceptives and monthly injectable contraceptives. *Contraception*. 1991; 44: 235-244.

Kesseru-Koos, E., Hurtado-Koo, H., Larranaga-Leguia, A., Scharff, H.J. Fertility control with norethindrone enanthate: a long standing parenteral progestogen. *Acta Eur Fertil*. 1973; 4: 203-221.

Khoiny, F.E. Use of depo-provera in teens. *J Ped Health Care*. 1996; 10 (5): 195-201.

Kim, C., Seidel, K.W., Begier, E.A., Kwok, Y.S. Diabetes and depot medroxyprogesterone contraception in Navajo women. *Arch Intern Med*. 2001; 161: 1766-1771.

Kino, T. and Chrousos, G.P. Glucocorticoid and mineralocorticoid resistance/hypersensitivity syndromes. *J Endocrinol*. 2001; 169 (3): 437-445.

Kino, T., Kopp, J.B., Chrousos, G.P. Glucocorticoids suppress human immunodeficiency virus type-1 long terminal repeat activity in a cell type-specific, glucocorticoid receptor-mediated fashion: direct protective effects at variance with clinical phenomenology. *J Steroid Biochem Mol Biol*. 2000; 75: 283-290.

Kinter, A.L., Biswas, P., Alfano, M., Justement, J.S., Mantelli, B., Rizzi, C., Gatti, A.R., Vicenzi, E., Bressler, P., Poli, G. Interleukin-6 and glucocorticoids synergistically induce human immunodeficiency virus type-1 expression in chronically infected U1 cells by a long terminal repeat independent post-transcriptional mechanism. *Molec Med*. 2001; 7 (10): 668-678.

Kinter, A.L., Biswas, P., Alfano, M., Justement, J.S., Mantelli, B., Rizzi, C., Gatti, A.R., Vicenzi, E., Bressler, P., Poli, G. Interleukin-6 and glucocorticoids synergistically induce human immunodeficiency virus type-1 expression in chronically infected U1 cells by a long terminal repeat independent post-transcriptional mechanism. *Mol Med*. 2001; 7: 668-678.

Kobayashi, K., Mimura, N., Fujii, H., Minami, H., Sasaki, Y., Shimada, N., Chiba, K. Role of human cytochrome p450 3A4 in metabolism of medroxyprogesterone acetate. Clin Cancer Res. 2000; 6: 3297-3303.

Koetsawang, S., Bhiraleus, P., Chiemprasert, T. Effects of oral contraceptives on lactation. Fertil Steril. 1972; 23: 24-28.

Koh, K.K., Ahn, J.Y., Jin, D.K., Yoon, B.K., Kim, H.S., Kim, D.S., Shin, M.S., Son, J.W., Choi, I.S., Shin, E.K. Effects of continuous combined hormone replacement therapy on inflammation in hypertensive and/or overweight postmenopausal women. Arterioscler Thromb Vasc Biol. 2002; 22: 1459-1464.

Kolesnitchenko, V. and Snart, R.S. Regulatory elements in the human immunodeficiency virus type 1 long terminal repeat LTR (HIV-1) responsive to steroid hormone stimulation. AIDS Res Hum Retrovir. 1992; 8: 1977-1980.

Kontula, K., Paavonen, T., Luukkainen, T., Anderson, L.C. Binding of progestins to the glucocorticoid receptor. Correlation to their glucocorticoid-like effects on *in vitro* functions of human mononuclear leukocytes. Biochem Pharmacol. 1983; 32: 1511-1518.

Kopf, M., Baumann, H., Freer, G., Freudenberg, M., Lamers, M., Kishimoto, T., Zinkernagel, R., Bluethmann, H., Kohler, G. Impaired immune and acute-phase responses in interleukin-6-deficient mice. Nature. 1994; 368: 339-342.

Koryntova, D., Cepicky, P., Zivny, J. Contraception with depot gestagens. Ceska Gynekol. 2001; 66: 202-208.

Koubovec, D., Berghe, W.V., Vermeulen, L., Haegman, G., Hapgood, J.P. Medroxyprogesterone acetate downregulates cytokine gene expression in mouse fibroblast cells. Mol Cell Endocrinol. 2004; 221: 75-85.

Krakauer, T. Differential inhibitory effects of interleukin-10, interleukin-4, and dexamethasone on staphylococcal enterotoxin-induced cytokine production and T cell activation. *J Leukoc Biol.* 1995; 57 (3): 450-454.

Krane, S.M. Some molecular mechanisms of glucocorticoid action. *Brit J Rheum.* 1993; 32 (suppl 2): 3-5.

Kunicka, J.E., Talle, M.A., Denhardt, G.H., Brown, M., Prince, L.A., Goldstein, G. Immunosuppression by glucocorticoids: inhibition of production of multiple lymphokines by in vivo administration of dexamethasone. *Cell Immunol.* 1993; 149: 39-49.

Ladisich, W. Influence of progesterone on serotonin metabolism: a possible causal factor for mood changes. *Psychoneuroendocrinol.* 1977; 2: 257-266.

Lang, I., Zielinski, C.C., Templ, H., Spona, J., Geyer, G. Medroxyprogesterone acetate lowers plasma corticotropin and cortisol but does not suppress anterior pituitary responsiveness to human corticotropin releasing hormone. *Cancer.* 1990; 66: 1949-1953.

Lanza, L., Scudeletti, M., Monaco, E., Monetti, M., Puppo, F., Filaci, G., Indiveri, F. Possible differences in the mechanism(s) of action of different glucocorticoid hormone compounds. *Ann N Y Acad Sci.* 1999; 876: 193-197.

Leis, D., Bottermann, P., Ermler, R., Henderkott, U., Gluck, H. The influence of high doses of oral medroxyprogesterone acetate on glucose tolerance, serum insulin levels and adrenal response to ACTH. A study of 17 patients under treatment for endometrial cancer. *Arch Gynecol.* 1980; 230: 9-13.

Liao, J., Keiser, J.A., Scales, W.E., Kunkel, S.L., Kluger, M.J. Role of corticosterone in TNF and IL-6 production in isolated perfused rat liver. *Am J Physiol.* 1995; 268: R699-R706.

Lippert, C., Seeger, H., wallwiener, D., Mueck, A.O. The effects of medroxyprogesterone acetate and norethisterone on the estradiol stimulated proliferation in MCF-7 cells: comparison of continuous combined versus sequential combined estradiol/progestin treatment. *Eur J Gynaecol Oncol.* 2001; 22 (5): 331-335.

Liuzzi, A., Chiodini, P.G., Botalla, L., Silvestrini, F. Role of medroxyprogesterone acetate (MPA) on the blood levels of STH in twelve patients with acromegaly. *Ann Endocrinol.* 1972; 33 (4): 426-430.

Maclaughlin, D.T. and Richardson, G.S. Specificity of medroxyprogesterone acetate binding in human endometrium: interaction with testosterone and progesterone binding sites. *J Steroid Biochem.* 1979; 10: 371-377.

MacPhee, I.A.M., Antoni, F.A., Mason, D.W. Spontaneous recovery of rats from experimental allergic encephalomyelitis is dependent on regulation of the immune system by endogenous adrenal corticosteroids. *J Exp Med.* 1989; 169: 431-445.

Maghazachi, A.A., al Aoukaty, A., Schall, T.J. C-C chemokines induce the chemotaxis of NK and IL-2-activated NK cells. Role for G proteins. *J Immunol.* 1994; 153: 4969-4977.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schütz, G. The nuclear receptor superfamily: the second decade. *Cell.* 1995; 83: 835-8839.

Mantovani, A. The interplay between primary and secondary cytokines. *Drugs.* 1997; 54 (Suppl 1): 15-23.

Margulies, R. and Miller, L. Increased medroxyprogesterone acetate use increase family planning program pharmaceutical supply costs. *Contraception.* 2001; 63 (3): 147-149.

Markiewicz, L. and Gurpide, E. Estrogenic and progestagenic activities coexisting in steroidal drugs: quantitative evaluation by in vitro bioassays with human cells. *J Steroid Biochem Mol Biol.* 1994; 48(1): 89-94.

Martin, H.L., Nyange, P.M., Richardson, B.A., Lavreys, L., Mandaliya, K., Jackson, D.J., Ndinya-Achola, J.O., Kreiss, J. Hormonal contraception, sexually transmitted diseases, and risk of heterosexual transmission of human immunodeficiency virus type 1. *JID.* 1998; 178: 1053-1059.

Marx, P.A., Spira, A.I., Gettie, A., Dailey, P.J., Veazey, R.S., Lackner, A.A., Mahoney, J., Miller, C.J., Claypool, L.E., Ho, D.D., Alexander, N.J. Progesterone implants enhance SIV vaginal transmission and early virus load. *Nat Med.* 1996; 2 (10): 1084-1089.

Mati, J.K.G., Hunter, D.J., Maggwa, B.N., Tukei, P.M. Contraceptive use and the risk of HIV infection in Nairobi, Kenya. *Int J Gynecol Obstet.* 1995; 48: 61-67.

May, L.T., Ghayeb, T., Santhanum, U., Tatter, S.B., Stoeber, Z., Helfgott, D.C., Chiorazzi, N., Griener, G., Seghal, P.B. Synthesis and secretion of multiple forms of beta 2-interferon/B cell differentiation factor 2/ hepatocyte-stimulating factor by human fibroblasts and monocytes. *J Biol Chem.* 1988; 263 (16): 7760-7766.

McEwan, J.A., Griffin, M., Fotherby, K., Trayner, I. Long-term use of depot-norethisterone enanthate: effect on blood lipid fractions. *Contraception.* 1992; 46: 49-60.

Mendoza-Rodriguez, C.A., Camacho-Arroyo, I., Garcia, G.A., Cerbon, M.A. Variations of progesterone receptor and c-fos gene expression in the rat uterus after treatment with norethisterone and its A-ring reduced metabolites. *Contraception.* 1999; 59(5): 339-343.

Meng, Y.X., Jiang, H.Y., Chen, A.J., Lu, F.Y., Yang, H., Zhang, M.Y., Shen, K.Y., Sun, D.L., Shao, Q.X., Fotherby, K. Hemostatic changes in women using a monthly injectable contraceptive for one year. *Contraception*. 1990; 42: 455-466.

Meng, Y-X., Jiang, H-Y., Lu, F-Y. The effects of long-term use of once-a-month injectable contraceptive norethisterone enanthate/estradiol valerate on coagulation in the Chinese. *Thromb Haemost*. 1990; 64: 548-549.

Mercurio, F. and Manning, A.M. Multiple signals converging on NF- κ B. *Curr Opin Cell Biol*. 1999; 11: 226-232.

Miesfeld, R.L. Molecular genetics of corticosteroid action. *Am Rev Respir Dis*. 1990; 141: S11-S17.

Mildvan, D., Yarrish, R., Marshak, A., Hutman, H.W., McDonough, M., Lamson, M., Robinson, P. Pharmacokinetic interaction between nevirapine and ethinyl estradiol/norethindrone when administered concurrently to HIV-infected women. *J Acquir Immune Defic Syndr*. 2002; 29: 471-477.

Million women study collaborators. *Lancet*. 2003; 362: 419-427.

Mishell, D.R. Pharmacokinetics of depot medroxyprogesterone acetate contraception. *J Reprod Med*. 1996; 41 (5): 381-390.

Mishell, D.R., el-Habashy, M.A., Good, R.G., Moyer, D.L. Contraception with a injectable progestin: A study of its use in postpartum women. *Am J Obstet Gynecol*. 1968; 101: 1046-1053.

Moguilewsky, M. and Deraedt, R. Interrelations between glucocorticoid and protein receptors. *J Steroid Biochem*. 1981; 15: 329-335.

Monier, M. and Laird, M. Contraceptives: a look at the future. *Amer J Nurs.* 1989; 89: 496-499.

Moore, L., Valuck, R., McDougall, C., Fink, W. A comparative study of one-year weight gain among users of medroxyprogesterone acetate, levonorgestrel implants, and oral contraceptives. *Contraception.* 1995; 52: 215-219.

Morale, M.C., Batticane, N., Gallo, F., Barden, N., Marchetti, B. Disruption of hypothalamic-pituitary-adrenocortical system in transgenic mice expressing type II glucocorticoid receptor antisense ribonucleic acid permanently impairs T cell function: Effects on T cell trafficking and T cell responsiveness during postnatal development. *Endocrinol.* 1995; 136: 3949-3960.

Morales, A., Kuruluta, G., Lotts, S. Hormones in the treatment of metastatic cancer. *J Urol.* 1975; 114: 692-693.

Mordacq, J.C. and Linzer, D.I. Co-localization of elements required for phorbol ester stimulation and glucocorticoid repression of proliferin gene expression. *Genes Dev.* 1989; 3(6): 760-769.



Mori, A., Kaminuma, O., Suko, M., Inoue, S., Ohmura, T., Hoshino, A., Asakura, Y., Miyazawa, K., Yokoto, T., Okumura, Y., Ito, K., Okudaira, H. Two distinct pathways of interleukin-5 synthesis in allergen-specific human T-cell clones are suppressed by glucocorticoids. *Blood.* 1997; 89 (8): 2891-2900.

Mori, T., Kobayashi, H., Nishimoto, H., Suzuki, A., Nishimura, T., Mori, T. Inhibitory effect of progesterone and 20 α -hydroxypregn-4-en-3-one on the phytohemagglutinin-induced transformation of human lymphocytes. *Am J Obstet Gynecol.* 1977; 15: 151-157.

Mosmann, T.R. and Sad, S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today.* 1996; 17(3): 138-146.

Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., Coffman, R.L. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol.* 1986; 136: 2348-2357.

Mostad, S.B., Overbaugh, J., DeVange, D.M., Welch, M.J., Chohan, B., Mandaliya, K., Nyange, P., Martin, H.L., Ndinya-Achola, J., Bwayo, J.J., Kreiss, J.K. Hormonal contraception, vitamin A deficiency, and other risk factors for shedding of HIV-1 infected cells from the cervix and vagina. *The Lancet.* 1997; 350: 922-927.

Mueck, A.O., Seeger, H., Wallwiener, D. Medroxyprogesterone acetate versus norethisterone enanthate: effect on estradiol-induced changes of markers for endothelial function and atherosclerotic plaque characteristics in human female coronary endothelial cell cultures. *Menopause.* 2002; 9: 273-281.

Munck, A. and Naray-Fejes-Toth, A. Glucocorticoids and stress: permissive and suppressive actions. *Ann N Y Acad Sci.* 1994; 746:115-130.

Nairn, R. and Helbert, M. *Immunology for medical students.* 1st. edition. Mosby. 2002.

Nakagomi, A., Freedman, S.B., Geczy, C.L. Interferon- γ and lipopolysaccharide potentiate monocyte tissue factor induction by C-reactive protein: relationship with age, sex, and hormone replacement treatment. *Circulation.* 2000; 101: 1785-1791.

Nash, H. Depo-provera: a review. *Contraception.* 1975; 12: 377-393.

Nelson, P.J., Kim, H.T., Manning, W.C. *et al.* Genomic organization and transcriptional regulation of the RANTES chemokine gene. *J Immunol.* 1993; 151: 2601-2612.

Neote, K., Darbonne, W., Ogez, J. *et al.* Identification of a promiscuous inflammatory peptide receptor on the surface of red blood cells. *J Biol Chem.* 1993a; 268: 12247-12249.

Neote, K., DiGregorio, D., Mak, J.Y. *et al.* Molecular cloning, functional expression, and signalling characteristics of a C-C chemokine receptor. *Cell*. 1993b; 72: 415-425.

Neote, K., Mak, J.Y., Kolakowski, L.J. *et al.* Functional and biochemical analysis of the cloned Duffy antigen: identity with the red blood cell chemokine receptor. *Blood*. 1994; 84: 44-52.

Neumann, F., Von Berswordt-Wallrabe, R., Elger, W. *et al.* Special problems of toxicity of long acting depot contraceptives. *Acta Endocrinol Suppl*. 1974; 185: 315-354.

Nishimura, R., Nagao, K., Matsuda, M. *et al.* Predictive value of serum medroxyprogesterone acetate concentration for response in advanced recurrent breast cancer. *Eur J Cancer*. 1997; 33: 1407-1412.

Northrop, J.P., Crabtree, G.R., Mattila, P.S. Negative regulation of interleukin 2 transcription by the glucocorticoid receptor. *J Exp Med*. 1992; 175(5): 1235-1245.

Okada, N., Kobayashi, M., Mugikura, K., Okamatsu, Y., Hanazawa, S., Kitano, S., Hasegawa, K. Interleukin-6 production in human fibroblasts derived from periodontal tissues is differentially regulated by cytokines and a glucocorticoid. *J Periodontal Res*. 1997; 32 (7): 559-569.

Ortiz, A., Horio, M., Stanczyk, F.Z., Goebelsmann, U., Mishell, D.R. Serum medroxyprogesterone acetate (MPA) concentrations and ovarian function following intramuscular injection of depo-MPA. *J Clin Endocrinol Metab*. 1977; 44: 32-38.

Otsuki, M., Saito, H., Xu, X. *et al.* Progesterone, but not medroxyprogesterone, inhibits vascular cell adhesion molecule-1 expression in human vascular endothelial cells. *Arterioscler Thromb Vasc Biol*. 2001; 21: 243-248.

Paine, C.H., Wright, F.W., Ellis, F. The use of progestogen in the treatment of metastatic carcinoma of the kidney and uterine body. *Brit J Cancer*. 1970; 24: 277-282.

Pasceri, V., Willerson, J.T., Yeh, E.T. Direct proinflammatory effect of C-reactive protein on human endothelial cells. *Circulation*. 2000; 102: 2165-2168.

Paul, C., Skegg, D.C., Spears, G.F. Depot medroxyprogesterone (Depo-Provera) and the risk of breast cancer. *Brit Med J*. 1989; 299: 759-762.

Peakman, M. and Vergani, D. Basic and clinical immunology. Int edition. Churchill Livingstone. 1997.

Pelner, L. and Rhoades, M.G. Host-tumor antagonism. 34. The use of progestational agents to retard homograft rejection in rabbits: A pilot study. *J Am Geriatr Soc*. 1965; 13: 765-771.

Perry, W. and Wiseman, R.A. Combined oral estradiol valerate-norethisterone treatment over 3 years in postmenopausal women: effect on lipids, coagulation factors, haematology and biochemistry. *Maturitas*. 2002; 42: 157-167.

Pfeiffer, A. and Barden, N. Estrogen-induced decrease of glucocorticoid receptor messenger ribonucleic acid concentration in rat anterior pituitary gland. *Mol Endocrinol*. 1987; 1: 435-440.

Pfeiffer, A., Lapointe, B., Barden, N. Hormonal regulation of type II glucocorticoid receptor messenger ribonucleic acid in rat brain. *Endocrinol*. 1991; 129: 2166-2174.

Pfeiffer, A., Morale, M.C., Barden, N., Marchetti, B. Modulation of glucocorticoid receptor gene expression in the thymus by the sex steroid hormone milieu and correlation with sexual dimorphism of immune response. *Endocr J*. 1994; 2:181-192.

Piccinni, M-P., Giudizi, M-G., Biagiotti, R., Beloni, L., Giannarini, L., Sampognaro, S., Manetti, R., Annunziato, F. Livi, C., Romagnani, S., Maggi, E. Progesterone favors the development of human T helper cells producing Th2-type cytokines and promotes both IL-4 production and membrane CD30 expression in established Th1 cell clones. *J Immunol.* 1995; 155: 128-133.

Powell, L.C. and Seymour, R.J. Effects of depot medroxyprogesterone acetate as a contraceptive agent. *Amer J Obstet Gynec.* 1971; 110: 36-41.

Pratt, W.B. Transformation of glucocorticoid and progesterone receptors to the DNA-binding state. *J Cell Biochem.* 1987; 35 (1): 51-68.

Primiero, F.M. and Benagiano, G. Unpublished data submitted to WHO. 1993 (as cited by Giwa-Osagie *et al.* 1994).

Ramierz, F., Fowell, D.J., Puklavec, M., Simmonds, S., Mason, D. Glucocorticoids promote a TH2 cytokine response by CD4+ T cells in vitro. *J Immunol.* 1996; 156(7): 2406-2412.

Ray, A. and Sehgal, P.B. Cytokines and their receptors: molecular mechanism of interleukin-6 gene repression by glucocorticoids. *J Am Soc Nephrol.* 1992; 2(12 Suppl): S214-S221.

Ray, K.P., Farrow, S., Daly, M., Talabot, F., Searle, N. Induction of the E-selectin promoter by interleukin 1 and tumor necrosis factor alpha, and inhibition by glucocorticoids. *Biochem J.* 1997; 328: 707-715.

Rehle, T., Brinkmann, U.K., Siraprasiri, T., Coplan, P., Aiemsukawat, C., Ungchusak, K. Risk factors of HIV-1 infection amongst female prostitutes in Khon Kaen, northeast Thailand. *Infection.* 1992; 20: 328-331.

Roberts, D.K., Morbelt, D.V., Powell, L.C. The ultrastructural response of human endometrium to medroxyprogesterone acetate. *Amer J Obstet Gynec.* 1975; 123: 811-818.

Rocken, M., Racke, M., Shevach, E.M. IL-4-induced immune deviation as antigen-specific therapy for inflammatory autoimmune disease. *Immunol Today.* 1996; 17(5): 225-231.

Romagnani, S. Lymphokine production by human T cells in disease states. *Annu Rev Immunol.* 1994; 12: 227-257.

Rome, E.S., Pokorny, S.F., Cromer, B.A. Management of clinical side effects of DMPA. *J Pediatr Adolesc Gynecol.* 2000; 13: 147-149.

Romero, R., Yoon, B.H., Mazor, M., Gomez, R., Gonzalez, R., Diamond, M.P., Baumann, P., Araneda, H., Kenney, J.S., Cotton, D.B. *et al.* A comparative study of the diagnostic performance of amniotic fluid glucose, white blood cell count, interleukin-6, and gram stain in the detection of microbial invasion in patients with preterm premature rupture of membranes. *Am J Obstet Gynecol.* 1993; 169 (4): 839-851.

Rook, G.A.W., Hernandez-Pando, R., Lightman, S.L. Hormones, peripherally activated prohormones and regulation of the Th1/Th2 balance. *Immunol Today.* 1994; 13: 301-303.

Rossouw, J.E., Andersen, G.L., Prentice, R.L., LaCroix, A.Z., Kooperberg, C., Stefanick, M.L., Jackson, R.D., Beresford, S.A., Howard, B.V., Johnson, K.C., Kotchen, J.M., Ockene, J. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: Principal results from the Women's Health Initiative randomized controlled trial. *JAMA.* 2002; 288: 321-333.

Rot, A., Krieger, M., Brunner, T. *et al.* RANTES and macrophage inflammatory protein 1 alpha induce the migration and activation of normal human eosinophil granulocytes. *J Exp Med.* 1992; 176: 1489-1495.

Rugarn, O., Hammar, M., Theodorsson, E. Progesterone and norethisterone have different effects on tachykinin-like immunoreactivity in rat cortex and striatum. *Regul Pept.* 2001; 101 (1-3): 87-91.

Rusconi, S. and Yamamoto, K.R. Functional dissection of the hormone and DNA binding activities of the glucocorticoid receptor. *EMBO J.* 1987; 6: 1309-1315.

Ryan, J.J., McReynolds, L.J., Keegan, A., Wang, L.H., Garfein, E., Rothman, P., Nelms, K., Paul, W.E. Growth and gene expression are predominantly controlled by distinct regions of the human IL-4 receptor. *Immunity.* 1996; 4(2): 123-132.

Schall, T.J. Biology of the RANTES/SIS cytokine family. *Cytokine.* 1991; 3: 165-183.

Schall, T.J. and Bacon, K.B. Chemokines, leukocyte trafficking and inflammation. *Curr Opin Immunol.* 1994; 6: 865-873.

Schall, T.J., Bacon, K., Toy, K.J. *et al.* Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature.* 1990; 347: 669-671.

Schall, T.J., Behrens, B.A., Gorgone, G.A., Bacon, K.B. Cytomegalovirus encodes a functional receptor for C-C chemokines. In: Grant McFadden, ed. *Viroceptors, virokines and related immune modulators encoded by DNA viruses.* R.G. Landes. 1994: 201-210.

Schall, T.J., Jongstra, J., Dyer, B.J. *et al.* A human T cell-specific molecule is a member of a new gene family. *J Immunol.* 1988; 141: 1018-1025.

Scheinman, R.I., Cogswell, P.C., Lofquist, A.K., Baldwin, A.S. Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. *Science*. 1995; 270(5234): 283-286.

Schmidt, J., Fleissner, S., Heimann-Weitschat, I., Lindstaedt, R., Szelenyi, I. The effect of different corticosteroids and cyclosporin A on interleukin-4 and interleukin-5 release from murine TH2-type T cells. *Eur J Pharmacol*. 1994; 260 (2-3): 247-250.

Schmidt, J., Fleissner, S., Heimann-Weitschat, I., Lindstaedt, R., Pomberg, B., Werner, U., Szelenyi, I. Effect of corticosteroids, cyclosporin A, and methotrexate on cytokine release from monocytes and T-cell subsets. *Immunopharmacology*. 1994; 27(3): 173-179.

Schoonen, W.G.E.J., Deckers, G.H., de Gooijer, M.E., de Ries, R., Kloosterboer, H.J. Hormonal properties of norethisterone, 7[alpha]-methyl-norethisterone and their derivatives. *J Steroid Biochem Mol Biol*. 2000; 74: 213-222.

Schust, D.J., Anderson, D.J., Hill, J.A. Progesterone-induced immunosuppression is not mediated through the progesterone receptor. *Hum Reprod*. 1996; 11: 980-985.

Schwallie, P.C. and Assenzo, J.R. The effect of depo-medroxyprogesterone acetate on pituitary and ovarian function, and the return of fertility following its discontinuation: A review. *Contraception*. 1974; 10: 181-202.

Shapiro, S.S., Dyer, R.D., Coles, A.E. Synthetic progestins: In-vitro potency on human endometrium and specific binding to cytosol receptor. *Amer J Obstet Gynec*. 1978; 132: 549-554.

Sharts-Hopko, N.C. Depo-provera. *Maternal Child Nursing*. 1993; 18: 128.

Sher, A. and Coffman, R.L. Regulation of immunity to parasites by T cells and T cell-derived cytokines. *Annu Rev Immunol*. 1992; 10: 385-409.

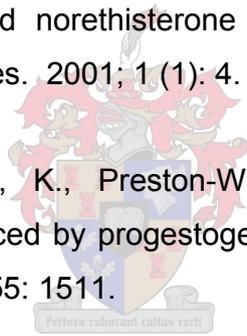
Sherman, M.R., Pickering, L.A., Rollwagen, F.M., Miller, L.K. Mero-receptors: proteolytic fragments of receptors containing the steroid-binding site. *Fed Proc.* 1978; 37: 167-173.

Skegg, D.C., Noonan, E.A., Paul, C., Spears, G.F., Meirik, O., Thomas, D.B. Depot medroxyprogesterone acetate and breast cancer: a pooled analysis of the World Health Organisation and New Zealand studies. *J Amer Med Ass.* 1995; 273: 799-804.

Smit, J., Botha, J., McFayden, L., Beksinska, M. Serum medroxyprogesterone acetate levels in new and repeat users of depot medroxyprogesterone acetate at the end of the dosing interval. *Contraception.* 2004; 69: 3-7.

Smit, J., Gray, A., McFayden, L., Zuma, K. Counting the costs: Comparing depot medroxyprogesterone acetate and norethisterone oenanthate utilization patterns in South Africa. *BMC Health Serv Res.* 2001; 1 (1): 4.

Smit, J., McFayden, L., Zuma, K., Preston-Whyte, E. Vaginal wetness: an underestimated problem experienced by progestogen injectable contraceptive users in South Africa. *Soc Sci Med.* 2002; 55: 1511.



Smith, R., Cromer, B., Hayes, J., Brown, R. Medroxyprogesterone acetate (Depo-Provera) use in adolescents: uterine bleeding and blood pressure patterns, patient satisfaction, and continuation rates. *Adolesc Pediatr Gynecol.* 1995; 8: 24.

Smith, S.M., Baskin, G.B., Marx, P.A. Estrogen protects against vaginal transmission of simian immunodeficiency virus. *J Infect Dis.* 2000; 182: 708-715.

Soderqvist, G. Effects of sex steroids on proliferation in normal mammary tissue. *Ann Med.* 1998; 30: 511-524.

Sornasse, T., Larenas, P.V., Davis, K.A., de Vries, J.E., Yssel, H. Differentiation and stability of T helper 1 and 2 cells derived from naive human neonatal CD4+ T cells, analyzed at the single-cell level. *J Exp Med.* 1996; 184(2): 473-483.

Sotaniemi, E.A., Hynnynen, T., Ahlqvist, *et al.* Effects of medroxyprogesterone on the liver function and drug metabolism of patients with primary biliary cirrhosis and chronic active hepatitis. *J Med.* 1978; 9: 117-128.

Special Programme of Research, Development and Research Training in Human Reproduction. Study 87901: Comparative study of two once-a-month injectable steroidal contraceptives (HRP 112 and HRP 102) and lipid, lipoprotein, and apolipoprotein metabolism. Report of analysis. May 1993.

Special Programme of Research, Development and Research Training in Human Reproduction. Study 87907: Comparative study of the effects of two once-a-month injectable steroidal contraceptives (Cyclofem and Mesigyna) and one combined oral contraceptive on coagulation and fibrinolysis. Draft report of final analysis. April 1993.

Spellacy, W.N., McLeod, A.G.W., Buhi, W.C., Birk, S.A. The effects of medroxyprogesterone acetate on carbohydrate metabolism: measurement of glucose, insulin and growth hormones after 12 months of use. *Fertil Steril.* 1972; 23: 239-244.

Stahlberg, C., Pedersen, A.T., Lynge, E., Andersen, Z.J., Keiding, N., Hundrup, Y.A., Obel, E.B., Ottesen, E. Increased risk of breast cancer following different regimens of hormone replacement therapy frequently used in Europe. *Int J Cancer.* 2004; 109: 721-727.

Stanczyk, F.Z. All progestins are not created equal. *Steroids.* 2003; 68: 879-890.

Stanczyk, F.Z. and Roy, S. Metabolism of levonorgestrel, norethindrone, and structurally related contraceptive steroids. *Contraception.* 1990; 42: 67-96.

Sternberg, E.M., Hill, J.M., Chrousos, G.P., Kamilaris, T., Listwak, S.J., Gold, P.W., Wilder, R.L. Inflammatory mediator-induced hypothalamic-pituitary-adrenal axis activation is defective in streptococcal cell wall arthritis-susceptible Lewis rats. *Proc N Ac Sc USA*. 1989; 86: 2374-2378.

Street, N.E., Schumacher, J.H., Fong, T.A.T., Bass, H., Fiorentino, D.F., Leverah, J.A., Mosmann, T.R. Heterogeneity of mouse helper T cells: evidence from bulk cultures and limiting dilution cloning for precursors of Th1 and Th2 cells. *J Immunol*. 1990; 144: 1629-1639.

Suni, M.A., Picker, L.J., Maino, V.C. Detection of antigen-specific T cell cytokine expression in whole blood by flow cytometry. *J Immunol Methods*. 1998; 212: 89-98.

Suthers, M.B., Pressley, L.A., Funder, J.W. Glucocorticoid receptors: evidence for a second, non-glucocorticoid binding site. *Endocrin*. 1976; 99 (1): 260-269.

Svec, F. and Rudis, M. Progesterin-induced enhancement of dexamethasone dissociation from glucocorticoid hormone receptors. *Arch Biochem Biophys*. 1981; 212: 417-423.



Svec, F., Teubner, V., Tate, D. Location of the second steroid-binding site on the glucocorticoid receptor. *Endo*. 1989; 125 (6): 3103-3108.

Svec, F., Yeakley, J., Harrison, R.W. Progesterone enhances glucocorticoid dissociation from the AtT-20 cell glucocorticoid receptor. *Endo*. 1980; 107: 566-572.

Swantek, J.L., Cobb, M.H., Geppert, T.D. Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor α (TNF- α) translation: glucocorticoids inhibit TNF- α translation by blocking JNK/SAPK. *Mol Cell Biol*. 1997; 17: 6274-6282.

Swenson, I., Khan, A.R., Jahan, F.A. A randomized single blind comparative trail of norethindrone enanthate and depot-medroxyprogesterone acetate in Bangladesh. *Contraception*. 1980; 21: 207-215.

Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Yasukawa, K., Matsuda, T., Hirano, T., Kishimoto, T. Interleukin 6 (IL-6) triggers the association of its receptor (IL-6R) with a possible signal transducer, gp130. *Cell*. 1989; 58 (3): 573-581.

Takusa, N., Komyia, I., Nagasawa, Y., Asawa, T., Yamada, T. Exacerbation of autoimmune thyroid dysfunction after unilateral adrenalectomy in patients with Cushing's syndrome due to adrenocortical adenoma. *New Eng J Med*. 1990; 322: 1708-1712.

Tankeyoon, M., Dusitsin, N., Poschyachinda, V., Larsson-Cohn, U. A study of glucose tolerance, serum transaminase and lipids in women using depot medroxyprogesterone acetate and a combined-type oral contraceptive. *Contraception*. 1976; 14: 199-214.

Telimaa, S., Penttila, I., Puolakka, J., Ronnberg, L., Kauppila, A. Circulating lipid and lipoprotein concentrations during danazol and high-dose medroxyprogesterone acetate therapy of endometriosis. *Fertil Steril*. 1989; 52: 31-35.

Terenius, L. Affinities of progestogen and oestrogen receptors in rabbit uterus for synthetic progestogens. *Steroids*. 1974; 23: 909-19.

Teubner, V.A., Tate, D.J., Svec, F. Antigluocorticoid interaction with the AtT-20 cell cytosolic glucocorticoid receptor. Annual Meeting of the Southern Section of the American Federation of Clinical Research. New Orleans. 1988 (Abstract 12A).

Teulings, F.A., van Gilse, H.A., Henkelman, M.S., Portengen, H., Alexieva-Figusch, J. Estrogen, androgen, glucocorticoid, and progesterone receptors in progestin-induced regression of human breast cancer. *Cancer Res*. 1980; 40(7): 2557-2561.

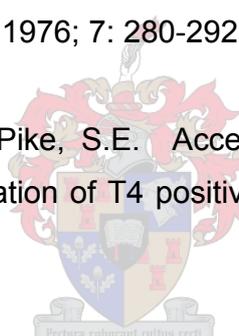
Thuneke, I., Schulte, H.M., Bamberger, A.M. Biphasic effects of medroxyprogesterone-acetate (MPA) treatment on proliferation and cyclin D1 gene transcription in T47D breast cancer cells. *Breast Cancer Res Treat.* 2000; 63: 243-248.

Tobena, R., Horikawa, S., Calvo, V., Alemany, S. Interleukin-2 induces gamma-S-adenosyl-L-methionine synthetase gene expression during T-lymphocyte activation. *Biochem J.* 1996; 319: 929-933.

Tobler, A., Meier, R., Seitz, M., Dewald, B., Baggiolini, M., Fey, M.F. Glucocorticoids downregulate gene expression of GM-CSF, NAP-1/IL-8, and IL-6, but not of M-CSF in human fibroblasts. *Blood.* 1992; 79(1): 45-51.

Tomoda, Y., Fuma, M., Miwa, T., Saiki, N., Ishizuka, N. Cell-mediated immunity in pregnant women. *Gynecol Invest.* 1976; 7: 280-292.

Tosato, G., Miller, J., Marti, G., Pike, S.E. Accessory function of interleukin-1 and interleukin-6: preferential costimulation of T4 positive lymphocytes. *Blood.* 1990; 75(4): 922-930.



Trifilieff, A., El-Hashim, A., Bertrand, C. Time course of inflammatory and remodeling events in a murine model of asthma: effect of steroid treatment. *Am J Physiol Lung Cell Mol Physiol.* 2000; 279(6): L1120-L1128.

Trinchieri, G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol.* 1995; 13: 251-276.

Truss, M. and Beato, M. Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. *Endocr Rev.* 1993; 14(4): 459-479.

Tsai, M-J and O'Malley, B.W. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Ann Rev Biochem.* 1994; 63: 451-486.

Tseng, L. and Zhu, H.H. Regulation of progesterone receptor messenger ribonucleic acid by progestin in human endometrial stromal cells. *Biol Reprod.* 1997; 57: 1360-1366.

Tuckermann, J.P., Reichardt, H.M., Arribas, R., Richter, K.H., Schutz, G., Angel, P. The DNA binding-independent function of the glucocorticoid receptor mediates repression of AP-1-dependent genes in skin. *J Cell Biol.* 1999; 147(7): 1365-1370.

Turner, B.B. and Weaver, D.A. Sexual dimorphism of glucocorticoid in rat brain. *Brain Res.* 1985; 343: 16-23.

Ungchusak, K., Rehle, T., Thammapornpilap, P., Spiegelman, D., Brinkmann, U., Siraprapasiri, T. Determinants of HIV infection amongst female commercial sex workers in northeastern Thailand: results from a longitudinal study. *J Acquir Immune Defic Syndr Hum Retrovirol.* 1996; 12: 500-507.

United Nations Population Division Department for Economic and Social Information and Policy Analysis. Levels and trends of contraceptive use as assessed in 1994. New York: United Nations; 1996: 61.



Upjohn. Contraceptive confidence 3 months at a time. The Upjohn Company. Kalamazoo, MI. 1993

Van Damme, J., Scaafsma, M.R., Fibbe, W.E., Falkenburg, J.H., Opdenakker, G., Billiau, A. Simultaneous production of interleukin-6, interferon-beta and colony stimulating activity by fibroblasts after viral and bacterial infection. *Eur J Immunol.* 1989; 19 (1): 163-168.

Van Veelen, H., Willemse, P.H., Sleijfer, D.T., Pratt, J.J., van der Ploeg, E., Sluiter, W.J., Doornbos, H. Adrenal suppression by oral high-dose medroxyprogesterone acetate in breast cancer patients. *Cancer Chemother Pharmacol.* 1984; 12: 83-86.

Van Veelen, H., Willemse, P.H., Sleijfer, D.T., van der Ploeg, E., Sluiter, W.J., Doornbos, H. Mechanisms of adrenal suppression by high-dose medroxyprogesterone acetate in breast cancer patients. *Cancer Chemother Pharmacol.* 1985; 15: 167-170.

Van Veelen, H., Willemse, P.H., Tjabbas, T., Schweitzer, M.J., Sleijfer, D.T. Oral high-dose medroxyprogesterone acetate versus tamoxifen. *Cancer.* 1986; 58: 7-13.

Vanden Berghe, W., Francesconi, E., De Bosscher, K., Resche-Rigon, M., Haegeman, G. Dissociated glucocorticoids with anti-inflammatory potential repress interleukin-6 gene expression by a nuclear factor- κ B-dependent mechanism. *Molec Pharmacol.* 1999; 56: 797-806.

Vassiliadou, N., Tucker, L., Anderson, D.J. Progesterone-induced inhibition of chemokine receptor expression on peripheral blood mononuclear cells correlates with reduced HIV-1 infectability in vitro. *J Immunol.* 1999; 162: 7510-7518.

Vayssiere, B.M., Dupont, S., Choquart, A., Petit, F., Garcia, T., Marchandeu, C., Gronemeyer, H., Resche-Rigon, M. Synthetic glucocorticoids that dissociate transactivation and AP-1 transrepression exhibit anti-inflammatory activity in vivo. *Mol Endocrinol.* 1997; 11 (9): 1254-1255.

Vedeckis, W.V. Limited proteolysis of the mouse liver glucocorticoid receptor. *Biochem.* 1983; 22: 1975-1983.

Vermeulen, M., Pazos, P., Lanari, C., Molinolo, A., Gamberale, R., Geffner, J.R., Giordano, M. Medroxyprogesterone acetate enhances *in vivo* and *in vitro* antibody production. *Immunol.* 2001; 104: 80-86.

Vermuelen, A. and Thiery, M. Hormonal contraceptives and carbohydrate tolerance: II. Influence of medroxyprogesterone acetate and chronic oral contraception. *Diabetologia.* 1974; 10: 253-259.

Wakatsuki, A., Okatani, Y., Ikenoue, N., Fukaya, T. Effect of medroxyprogesterone acetate on vascular inflammatory markers in postmenopausal women receiving estrogen. *Circulation*. 2002; 105: 1436-1439.

Wambach, G., Higgins, J.R., Kem, D.C., Kaufmann, W. Interaction of synthetic progestagens with renal mineralocorticoid receptors. *Acta Endocrinol (Copenh)*. 1979; 92: 560-567.

Wang, C.C., McClland, R.S., Overbaugh, J., Reilly, M., Panteleeff, D.D., Mandaliya, K., Chohan, B., Lavreys, L., Ndinya-Achola, J., Kreiss, J.K. The effect of hormonal contraception on genital tract shedding of HIV-1. *AIDS*. 2004; 18: 205-209.

Wang, Y., Campbell, H.D., Young, I.G. Sex hormones and dexamethasone modulate interleukin-5 gene expression in T lymphocytes. *J Steroid Biochem Molec Biol*. 1993; 44: 203-210.

Ward, S.G. and Westwick, J. Chemokines: understanding their role in T lymphocyte biology. *Biochem J*. 1998; 333: 457-470.

Wardlaw, A.J., Moqbel, R., Kay, A.B. Eosinophils: biology and role in disease. *Adv Immunol*. 1995; 60: 151-266.

Welnicka-Jaskiewicz, M. and Jassem, J. The risks and benefits of hormonal replacement therapy in healthy women and in breast cancer survivors. *Cancer Treat Rev*. 2003; 29: 355-361.

Wick, G., Hu, Y., Schwarz, S., Kroemer, G. Immunoendocrine communication via the hypothalamo-pituitary-adrenal axis in autoimmune disease. *Endocrinol Rev*. 1993; 14(5): 539-563.

Wierenga, E.A., Snoek, M., de Groot, C., Chretien, I., Bos, J.D., Jansen, H.M., Kapsenberg, M.L. Evidence for compartmentalization of functional subsets of CD4⁺ T lymphocytes in atopic patients. *J Immunol.* 1990; 144: 4651-4656.

Wilckens, T. Glucocorticoids and immune function: physiological relevance and pathogenic potential of hormonal dysfunction. *Trends Pharmacol Sci.* 1995; 16: 193-197.

Wilckens, T. and De Rijk, R. Glucocorticoids and immune function: unknown dimensions and new frontiers. *Immunol Today.* 1997; 18 (9): 418-424.

Wonodirekso, S., Affandi, B., Siregar, B., Barasila, A.C., Damayanti, L., Rogers, P.A. Endometrial endothelial integrity and subepithelial reticular fibre expression in progestin contraceptive acceptors. *Hum Reprod.* 2000; 15 (Suppl 3): 189-196.

World Health Organisation Collaborative study of Neoplasm and Steroid Contraceptives. Breast cancer and depot-medroxyprogesterone acetate: a multinational study. *Lancet.* 1991; 338: 833-838.

World Health Organisation Expanded Programme of Research, Development and Research Training in Human Reproduction: Task Force on Long Acting Systemic Agents for the Regulation of Fertility, Multinational comparative clinical evaluation of two long-acting injectable contraceptive steroids: Norethisterone oenanthate and medroxyprogesterone acetate: II. Bleeding patterns and side effects. *Contraception.* 1978; 17: 395-406.

World Health Organisation Expanded Programme of Research, Development and Research Training in Human Reproduction: Task Force on Long Acting Systemic Agents for the Regulation of Fertility, Multinational comparative clinical evaluation of two long-acting injectable contraceptive steroids: Norethisterone oenanthate and medroxyprogesterone acetate: 1. Use effectiveness. *Contraception.* 1977; 15: 513-533.

WHO Special programme of Research, Development and Research Training in Human Reproduction: Multinational comparative clinical trial of long-acting injectable contraceptives: Norethisterone enanthate given in two dosage regimens and depo-medroxyprogesterone acetate: Final Report. *Contraception*. 1983; 44: 1-20.

Wu, Z-Y., Shao, Q-X., Sang, G-W. The change of serum prolactin level after multiple injection of norethisterone enanthate and estradiol valerate. *Reprod Contracep*. 1991; 2: 44-48.

Wynn, T.A. and Cheever, A.W. Cytokine regulation of granuloma formation in schistosomiasis. *Curr Opin Immunol*. 1995; 7(4): 505-511.

Yamashita, J., Hideshima, T., Shrakusa, T., Ogawa, M. Medroxyprogesterone acetate treatment reduces serum interleukin-6 levels in patients with metastatic breast carcinoma. *Cancer*. 1996; 78: 2346-2352.

Yang, H., Zhang, M-Y., Lu, F-Y., Jiang, H-Y. The effect of a monthly injectable contraceptive on serum lipids in Chinese women. *Reprod Contraception*. 1993.

Yildirim, A., Aybar, F., Tokgozoglul, L., Yarali, H., Kabakci, G., Bukumez, O., Sinici, I., Oto, A. Effects of hormone replacement therapy on plasma homocysteine and C-reactive protein levels. *Gynecol Obstet Invest*. 2002; 53: 54-58.

Yoon, B.H., Romero, R., Kim, C.J., Jun, J.K., Choi, J.H., Syn, H.C. Amniotic fluid interleukin-6: a sensitive test for antenatal diagnosis of acute inflammatory lesions of preterm placenta and prediction of perinatal morbidity. *Am J Obstet Gynecol*. 1995; 172 (3): 960-970.

Yssel, H., Schanafelt, M.C., Soderberg, C., Schneider, P.V., Anzola, J., Peltz, G. *Borrelia burgdorferi* activates a T helper 1-like T cell subset in Lyme arthritis. *J Exp Med*. 1991; 174: 593-601.

Zalanyi, S., Landgren, B.M., Johannisson, E. Pharmacokinetics, pharmacodynamic and endometrial effects of a single dose of 200 mg norethisterone enanthate. *Contraception*. 1984; 30: 225-237.

Zanartu, J. and Navarro, C. Fertility inhibition by an injectable progestogen acting for 3 months – a clinical survey of 130 fertile women treated with norethisterone oenanthate. *Obstet Gynec*. 1968; 31: 627-633.

Zanartu, J. and Onetto, E. Long-acting injectable progestagens in fertility control. *Proceedings of the sixth Pan-American congress of endocrinology, Mexico City*. 1965; Inter Con Ser No. 112: 134.

Zhang, Y., Broser, M., Rom, W.N. Activation of the interleukin-6 gene by *Mycobacterium tuberculosis* or lipopolysaccharide is mediated by the nuclear factors NF-IL6 and NF-kappaB. *Proc Natl Acad Sci USA*. 1994; 91 (6): 2225-2229.

Zhao, D., Lebovic, D.I., Taylor, R.N. Long-term progestin treatment inhibits RANTES (regulated on activation, normal T cell expressed and secreted) gene expression in human endometrial stromal cells. *J Clin Endocrinol Metab*. 2002; 87 (6): 2514-2519.

Zhao, Q., Pang, J., Favata, M.F., Trzaskos, J.M. Receptor density dictates the behaviour of a subset of steroid ligands in glucocorticoid receptor-mediated transrepression. *Int Immunopharmacol*. 2003; 3: 1803-1817.

Zhu, Z., Tang, W., Ray, A., Wu, Y., Einarsson, O., Landry, M.L., Gwaltney, J., Elias, J.A. Rhinovirus stimulation of interleukin-6 in vivo and in vitro. Evidence for nuclear factor kappa B-dependent transcriptional activity. *J Clin Invest*. 1996; 97 (2): 421-430.