Investigating biocharacter and mechanisms of progestins used in contraception via the glucocorticoid receptor; Implications for choice of contraception and HIV-1 infection

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Abstract

Contraceptive use in sub-Saharan Africa has rapidly increased, with discreet injectable and implantable progestin-only methods being among the most preferred. However, concerns have emerged regarding the potential increased risk of HIV-1 acquisition associated with the progestin-only injectable depo-medroxyprogesterone acetate (DMPA-IM) but not norethisterone enanthate (NET-EN) use in this region with an already high HIV-1 prevalence. Substantial clinical, animal, and *in vitro* data suggest that medroxyprogesterone acetate (MPA), the synthetic progestin in DMPA-IM, may be associated with an increased risk of HIV-1 acquisition. In contrast, fewer studies have investigated norethisterone (NET), the synthetic progestin in NET-EN, and its potential risk of HIV-1 acquisition; however, the limited data available indicate that NET-use is not associated with an increased risk of HIV-1 acquisition. A plausible reason for this is that MPA, but not NET, exhibits glucocorticoid (GC)-like transcriptional activity with a relatively high affinity for the glucocorticoid receptor (GR). Whether other synthetic progestins used in contraception, such as levonorgestrel (LNG), etonogestrel (ETG) or nestorone (NES), exhibit GC-like activity and increase the risk of HIV-1 acquisition remains unclear.

This study investigated the GC actions of LNG, ETG and NES, compared to MPA and NET *in vitro* and *ex vivo*. For the first time, LNG, ETG and NES binding affinities for the GR were characterised by performing whole-cell binding assays in COS-1 cells exogenously expressing the GR. Progestin activity via the GR was assessed by performing dose-response analyses using promoter-reporter assays and

quantitative real-time PCR (qRT-PCR) in COS-1 and peripheral blood mononuclear cells (PBMCs). Immunomodulatory effects were investigated in HeLa cells and PBMCs using qRT-PCR, and soluble immune mediator levels were quantified in PBMCs using the Multiplex Luminex assay. Infection assays were conducted in PBMCs using HIV-$1_{BaL\_Renilla}$ to investigate progestin-induced regulation of HIV-1 infection. The GR and progesterone receptor (PR) antagonist, RU486, was employed to determine the role of the GR or PR in mediating progestin-induced responses. The GR and PR expression profiles were analysed using flow cytometry in PBMC immune cell subtypes ($CD3^+$, $CD4^+$, $CD8^+$ T cells and $CD14^+$ monocytes) and immunofluorescent staining in ectocervical tissue compartments. Progestin metabolism was quantified in COS-1 and HeLa cells and PBMCs using UHPSFC-MS/MS to provide insights into whether changes in progestin concentrations impact steroid receptor activity.

MPA and ETG exhibited higher relative binding affinities (RBAs) for the GR compared to NET, LNG, and NES in COS-1 cells. Like MPA, NES displayed full-to-partial agonist activity for transactivation and transrepression via the GR in COS-1 and HeLa cell lines and PBMCs. Gene-specific differences in the progestin efficacy and potency were observed in PBMCs. MPA and NES distinctly regulated the expression of select immune genes and soluble immune mediators and increased HIV-1 infection *ex vivo* through a GR-dependent mechanism in PBMCs. Notably, GR was predominantly expressed in systemic $CD4^+$ T cells and co-localised with CD4 in epithelial cells of ectocervical tissue explants. While progestins were metabolised to varying degrees across and within the model

systems, no significant correlation was found between progestin metabolism and GR binding affinity or activity via the GR.

This study characterised GR binding properties, activity via the GR, and metabolism profiles for LNG, ETG and NES in parallel with MPA and NET across distinct model systems. These results indicate that MPA and NES, and to a lesser extent ETG, may regulate select immune responses and increase HIV-1 infection *ex vivo* through a GR-dependent mechanism; whereas, NET and LNG displayed minimal GR activity. This study presents novel insights, suggesting a potential role for the GR in MPA- and NES-induced immune regulation and increased HIV-1 infection, particularly in systemic $CD4^+$ T cells and ectocervical epithelial cells co-expressing CD4 and GR. Overall, these findings provide valuable insights into the relevance of both SR expression and progestin actions when evaluating the safety and efficacy of contraceptives used in high-risk populations.

Declaration

I, **Maleshigo Komane**, declare that the work presented in this thesis is my original work unless otherwise acknowledged. Furthermore, I declare that neither this thesis nor any part of it has been submitted for another degree at any university. I authorise the University to reproduce all contents or any portion thereof for research purposes in any manner whatsoever.

Signature: Date:

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List of Abbreviations

AF	activation factor
AP-1	activator protein 1
AR	androgen receptor
ATCC	American type culture collection
bp	base pair
CCR5	C-C chemokine receptor 5
cDNA	complementary DNA
CoA	coactivator
CO_2	Carbon dioxide
COC	combined oral contraceptive
cs-FCS	charcoal-stripped foetal calf serum
Ct	cycle threshold
ctrl	control
CVL	cervicovaginal lavages
DBD	DNA binding domain
DEPC	diethyl pyrocarbonate
DEX	dexamethasone
DMEM	Dulbecco's modified Eagle's medium
DMPA	Depo-medroxyprogesterone acetate
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
EC50	effective concentration required for 50% of maximal response (potency)
ECHO	Evidence for Contraceptive Options and HIV Outcomes
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetra-acetic acid
ER	estrogen receptor
EtBr	ethidium bromide
ETG	etonogestrel
EtOH	absolute ethanol

FACS	fluorescence activated cell sorting
FCS	foetal calf serum
FGT	female genital tract
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	glucocorticoid
G-CSF	granulocyte-colony stimulating factor
GILZ	glucocorticoid induced leucine zipper
GMCSF	granulocyte-macrophage colony-stimulating factor
GR	glucocorticoid receptor
GREs	glucocorticoid response elements
HAT	histone acetyltransferase
HC	hormonal contraception
HDAC	histone deacetylase
H/SRE	hormone/steroid response element
HIV-1	human immunodeficiency virus
HSP90	heat shock protein 90
HREC	human research ethics committee
IC50	half maximal inhibitory concentration (inhibition constant)
IFN	interferon
IL	interleukin
IP	interferon γ-induced protein
IP3	inositol triphosphate
IU	infectious units
IUD	intrauterine device
K_d	equilibrium dissociation constant
kDa	kiloDalton
K_i	dissociation constant
LBD	ligand binding domain
LLOQ	lower limit of quantification
LNG	levonorgestrel
LOD	limit of detection
LUC	luciferase

M	Molar
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
MFI	mean fluorescence intensity
MPA	medroxyprogesterone acetate
mL	millilitre
mM	milliMolar
MOPS	4-morpholine-propanesulfonic acid
MPA	medroxyprogesterone acetate
MR	mineralocorticoid receptor
mRNA	messenger RNA
MRM	multiple reaction monitoring
MTBE	methyl-tert-butyl-ether
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
ND	not determined
NES	nestorone
NET	norethisterone
NET-EN	norethisterone enanthate
NFκB	nuclear factor kappa B
nGRE	negative GRE
nM	nanoMolar
NSB	nonspecific binding
NTD	amino-terminal domain
P4	progesterone
PAGE	polyacrylamide gel electrophoresis
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PI3K/AKT	phosphatidyl inositol 3-kinase/protein kinase B
PR	progesterone receptor
qRT-PCR	quantitative real-time-PCR
RBA	relative binding affinity

RLU	relative light units
RNA	ribonucleic acid
RPMI	Rosewell Park Memorial Institute
SB	specific binding
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SF	serum-free
SHIV	simian-human immunodeficiency virus
SR	steroid receptor
STAT6	signal transducer and activator of transcription 6
TA	transactivation
TAT	tyrosine aminotransferase
TBS	TRIS-buffered saline
TBST	TRIS-buffered saline tween
TF	transcription factor
TLR4	toll like receptor 4
TNFα	tumour necrosis factor alpha
TR	transrepression
UHPSFC-	ultra-high performance supercritical fluid chromatography–
MS/MS	tandem mass spectrometry
μL	microlitre
UN	United Nations
v/v	volume per unit volume
WHO	World Health Organization
w/v	weight per unit volume

Thesis Outline

This thesis comprises seven chapters and four appendices. There are three results chapters, and supplementary data are included in Appendix B.

Chapter 1 – Literature Review: This chapter outlines the available literature on SR mechanisms of action and methods used to study SR-mediated progestin activity, particularly the GR, with a focus on contraception methods containing progestins commonly used in sub-Saharan Africa. This chapter reviews current literature on how SR-mediated progestin activity impacts HIV-1 acquisition and immune activation through GR- or PR-mediated mechanisms. The role of progestin metabolism is also discussed. Furthermore, the chapter concludes with the research rationale, aims and hypotheses.

Chapter 2 – Materials and Methods: This chapter outlines the methods employed to obtain the results presented in the subsequent chapters.

Chapter 3 – Results: Progestins exhibit differential affinity for the GR and gene-specific transcriptional effects via the GR. This chapter investigates progestin binding affinities for the GR in a cell line devoid of competing SR, characterises progestin-induced activity on synthetic and endogenous GRE-promoters across distinct model systems and analyses the role of GR or PR in mediating these effects.

Chapter 4 – Results: MPA and NES differentially regulate IFNγ mRNA and protein levels and increase HIV-1 infection in PBMCs: Possible role of the GR or PR. This chapter investigates select markers relevant to HIV-1 infection, examines the progestin-induced effects on mRNA and protein levels and HIV-1 infection regulation, and explores the expression profiles of GR and PR in PBMCs and ectocervical tissue compartments.

Chapter 5 – Results: Progestins exhibit differential degrees of metabolism within and across different model systems. This chapter investigates progestin metabolism in model systems used to characterise progestin-induced activity via the GR.

Chapter 6 – Discussion: This chapter critically discusses the results presented in the previous chapters and contextualises the findings within the field of progestin-induced activity via the GR, as well as the potential biological mechanisms behind the immunomodulatory effects of progestins.

Chapter 7 – Conclusions and Future Perspectives: This chapter draws concluding remarks on the data presented in this thesis and outlines recommendations for future research.

References: This is an alphabetical list of all publications cited in this thesis.

Appendix A – Supplementary data for Chapter 2: This appendix includes the gating strategy used for flow cytometry analysis and UHPSFC-MS/MS method validation data.

Appendix B – Supplementary data for Chapters 3–5: This appendix includes supporting data referred to in Chapters 3–5.

Appendix C – Publications and statement of work conducted by the present author: This appendix includes statements of work done by the present author towards the listed publications and conference presentations.

Appendix D – Research articles: This appendix includes the PDF versions of the research articles co-authored by the present author.

Chapter 1

Literature review

1.1 Contraceptive use in sub-Saharan Africa

Unintended pregnancies account for 46% of births worldwide, with rates as high as 60.1% in Southern Africa (Ayalew *et al.*, 2022; Aragaw *et al.*, 2023). To address this issue, the United Nations (UN) developed the Sustainable Development Goal 3.7.1 on contraceptive use to ensure women of reproductive age (15-49 years) have access to modern family planning methods. Since 1994, contraceptive use has increased globally by 8.3% (**Figure 1.1.1**). Although Africa lags in overall contraceptive use, since 1994, the continent has recorded a 51.6% cumulative increase in contraceptive use (United Nations Department of Economic and Social Affairs, 2022) (**Figure 1.1.1**). As more women exercise their reproductive rights, it is essential to provide them with the option to choose a reliable and safe method of contraception that minimises potential side effects. A deeper understanding of the molecular mechanisms underlying these methods is critical for ensuring long-term safety and effectiveness.

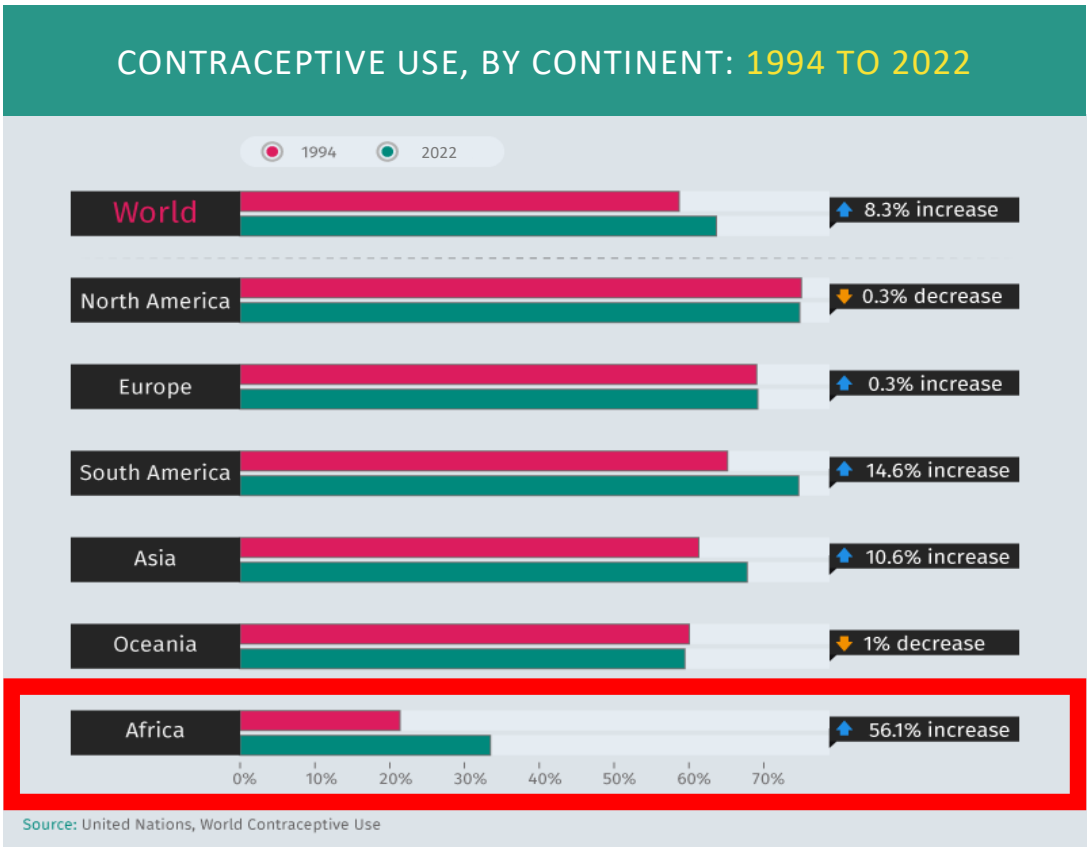


Figure 1.1.1: Contraceptive use by continent: 1994 to 2022. Figure adapted from ('Birth Control Around the World: Mapping Methods of Contraception,' 2022)

Since 1995, sub-Saharan Africa has documented a significant increase in contraception use, particularly the uptake of long-acting reversible contraceptive (LARC) methods (United Nations Department of Economic and Social Affairs, 2022). In 2020, fewer women chose to rely on the rhythmic method, female sterilisation and the pill, while injectable contraceptives remained the most commonly used method of contraception in sub-Saharan Africa (United Nations Department of Economic and Social Affairs, 2022) (**Figure 1.1.2**).

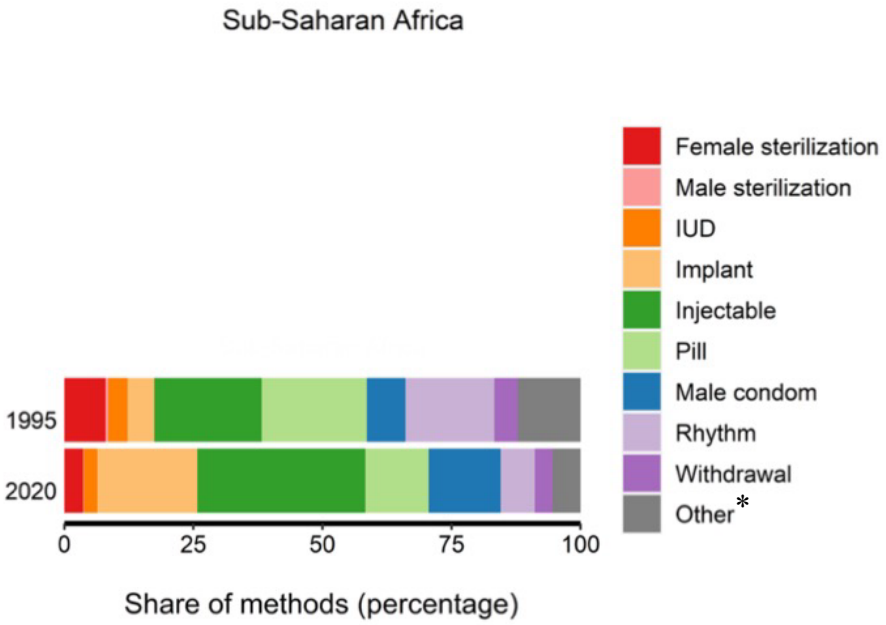


Figure 1.1.2: Contraceptive methods used in sub-Saharan Africa among women of reproductive age (15-49 years), 1995 and 2020. Figure adapted from Figure 11 in (United Nations Department of Economic and Social Affairs, 2022).“ *Other methods include modern methods such as female condoms, vaginal barrier methods (including diaphragms, cervical caps and spermicidal foams, jellies, creams and sponges), lactational amenorrhea method (LAM), emergency contraception and other modern and traditional methods not presented separately.”

1.2 Progestin-only contraceptives

Progesterone (P_4) is a crucial hormone in the female reproductive system, regulating the menstrual cycle and supporting pregnancy (Stanczyk, 2003; Schindler *et al.*, 2008). Synthetic progestins were developed to mirror the actions of P_4, but with improved pharmacological properties such as longer half-lives, higher potency and improved oral bioavailability (Kuhl, 2005; Africander, Verhoog, and Hapgood, 2011; Stanczyk *et al.*, 2013; Sitruk-Ware and El-Etr, 2013; Schindler, 2014). Synthetic progestins commonly used in contraceptives, including medroxyprogesterone acetate (MPA), norethisterone (NET), levonorgestrel (LNG), etonogestrel (ETG) and nestorone (NES), are synthesised as structural derivatives of either P_4 or Testosterone (T) (Schindler *et al.*, 2008; Kuhl, 2005; Sitruk-Ware, 2004) (**Figure 1.2.1**). Progestins are widely used in contraceptives, either alone or in combination with estrogen, to inhibit follicular maturation and ovulation, and are also employed in menopausal hormone therapy and the treatment of various gynaecological and endocrine disorders (Africander, Verhoog, and Hapgood, 2011; Stanczyk *et al.*, 2013; Schindler, 2014; Sitruk-Ware, 2006; Kuhl, 2005).

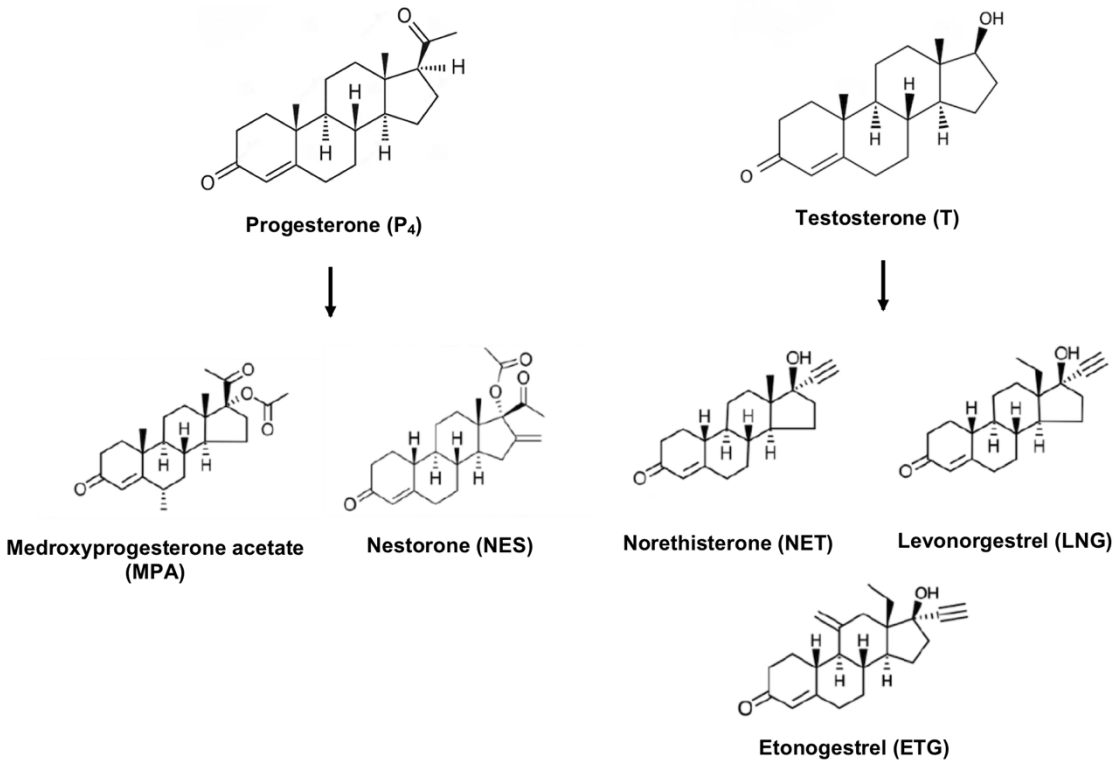


Figure 1.2.1: Synthetic progestins derived from progesterone or testosterone. Image adapted from (Stanczyk, 2003).

An overview of progestin-only contraceptives is provided below to highlight the relevance of these methods in sub-Saharan Africa and to contextualise the selection of progestins investigated in this thesis.

In sub-Saharan Africa, discreet, effective and reversible contraceptive methods are highly preferred. MPA is widely available as a three-monthly intramuscular injection (150 mg; Depo-Provera/DMPA-IM); whereas, NET (200 mg; Nuristerate/NET-EN) is administered intramuscularly every two months and has more limited distribution, largely being restricted to South Africa (Jacobstein and Polis, 2014; Hapgood, Kaushic, and Hel, 2018; Britton *et al.*, 2020).

While progestin-only injectables remain the most commonly used contraceptive method in sub-Saharan Africa, UN data indicate that in 2022, subdermal implants had become the preferred choice in five countries within sub-Saharan Africa (United Nations Department of Economic and Social Affairs, 2022). Long-acting reversible contraceptives (LARCs) such as implants and intrauterine devices (IUDs) releasing LNG are particularly effective in reducing unintended pregnancies (Croxatto, 2000; Sivin, 2003; Jacobstein and Polis, 2014; Rocca *et al.*, 2021; Brache and Faundes, 2010). Progestin-only contraceptives containing ETG are typically administered as a three-year slow-releasing subdermal implant or an intravaginal ring (IVR), while NES-releasing IVRs and implants are less widely used in the region (Bennink, 2000; Kumar *et al.*, 2000; Sitruk-Ware *et al.*, 2003; Sivin *et al.*, 2004; Funk *et al.*, 2005; Brache and Faundes, 2010; Jacobstein and Polis, 2014; Britton *et al.*, 2020; Renke *et al.*, 2023). **Table 1.2.1** summarises the effectiveness and mechanism of action of the most commonly used progestin-only methods in sub-Saharan Africa. Given the widespread use of progestins, their structural and pharmacokinetic differences underscore the importance of characterising progestin interactions with steroid receptors (SR), to advance understanding of the biological outcomes observed *in vivo*.

Table 1.2.1: Effectiveness and mechanism of action of widely used progestin-only contraceptive methods in sub-Saharan Africa*

Method	Effectiveness†	Mechanism of action
Progestin-only Injectables	0.2	• Inhibition of ovulation • Thickening of cervical mucus • Changes in endometrial lining
Implants	0.1	• Inhibition of ovulation • Thickening of cervical mucus
Intrauterine Devices (IUD)	0.5	• Endometrial suppression • Inhibit uterine implantation • Inhibit sperm migration
Intravaginal Rings (IVR)	0.3	• Ovulation suppression • Thickening of cervical mucus

*Table adapted from (World Health Organization, 2023)
†Pregnancies per 100 women per year with consistent and correct use.

1.3 Steroid Receptors

1.3.1 *Structure and mechanism of action*

1.3.1.1 *Structure*

Steroid receptors (SR) are ligand-activated transcription factors (TF) that belong to the nuclear receptor superfamily, which includes the androgen receptor (AR), estrogen receptor (ER), glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and progesterone receptor (PR) (Weikum, Liu, and Ortlund, 2018; Pecci *et al.*, 2022) (**Figure 1.3.1.1**). SRs mediate the biological actions of steroid hormones and share a conserved structural framework consisting of four distinct domains: a variable N-terminal domain (NTD), conserved DNA-binding domain (DBD), hinge region and a moderately conserved C-terminal ligand binding domain (LBD) (Beato, 2000; Huang, Chandra, and Rastinejad, 2010; Weikum, Liu, and Ortlund, 2018; Saha, Dey, and Nath, 2021; Kowalczyk *et al.*, 2021). The NTD and LBD contain activation function 1 (AF-1) and activation function 2 (AF-2), which are instrumental in modulating gene expression (Africander, Verhoog, and Hapgood, 2011; Weikum, Liu, and Ortlund, 2018).

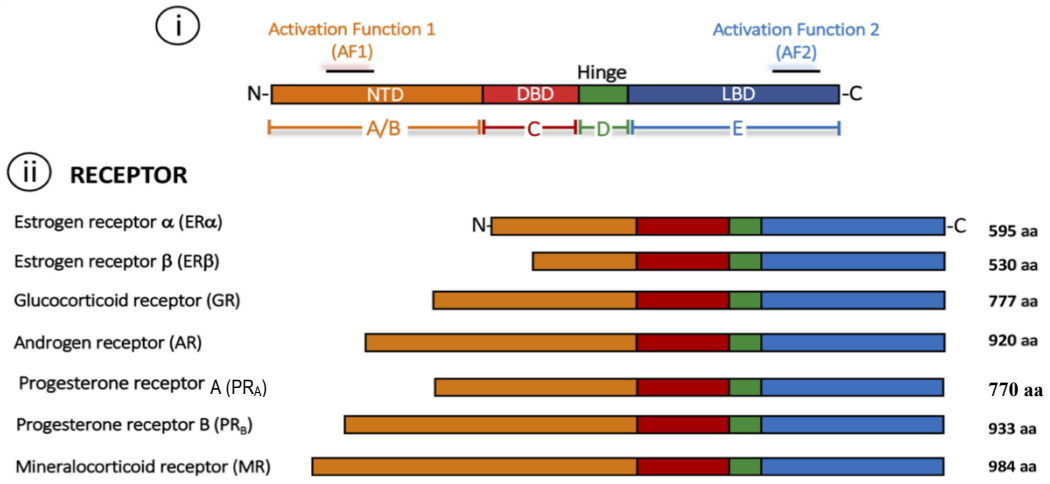


Figure 1.3.1.1 Domain structure of steroid receptors (SRs): i) Basic domain structure of SRs is composed of an N-terminal domain (NTD) that contains the activation function (AF-1) surface, a zinc finger DNA-binding domain (DBD), a flexible hinge region, and a ligand-binding domain (LBD) that binds to ligands and interacts with co-regulator proteins through the activation function (AF-2) surface. **ii)** Domain size and amino acid length of different isoform variants across members of the SR subgroup. The DBD and LBDs are the most conserved regions, whereas other domains are more variable in length and sequence composition. ERα and ERβ differ primarily in the N-terminal and ligand-binding domains. GR is represented by GRα, the transcriptionally active isoform, whereas GRβ carries a truncated LBD (not shown). PR-B includes an additional N-terminal activation function (AF-3) domain compared to PR-A. Figure adapted from (Pecci *et al.*, 2022).

1.3.1.2 *Mechanism of action*

SRs regulate gene expression through genomic and/or non-genomic mechanisms (Saha, Dey, and Nath, 2021; Wehling, 1997). In the classical genomic pathway, inactive SRs are bound to heat shock proteins (Hsp90/Hsp70 complexes) in the cytoplasm or nucleus (Smith and Toft, 1993; Beato, 2000; Kowalczyk *et al.*, 2021; Pecci *et al.*, 2022). Upon ligand binding, SRs undergo a conformational change, dissociate from the Hsp90/Hsp70 complexes, and translocate to the nucleus, dimerise and bind to steroid response elements (SREs) in target gene promoters (Trevino and Weigel, 2013; Weikum, Liu, and Ortlund, 2018). Transcription is either

activated through the recruitment of histone acetyltransferase (HAT) and coactivator (CoA) complexes, or repressed through histone deacetylase (HDAC) and corepressor (CoR) recruitment (Weikum, Liu, and Ortlund, 2018; Pecci *et al.*, 2022; Kowalczyk *et al.*, 2021; Saha, Dey, and Nath, 2021) (**Figure 1.3.1.2**). Alternatively, SR-ligand complexes can modulate transcription indirectly by tethering to other TFs, including AP-1, GATA-3, NFκB, and FOXA1, broadening the range of genes that SRs can influence without directly binding to DNA (Newton and Holden, 2007; Kowalczyk *et al.*, 2021; Pecci *et al.*, 2022) (**Figure 1.3.1.2**).

Non-genomic signalling occurs independently of the SR binding to DNA and involves rapid activation of secondary messenger pathways, such as inositol trisphosphate (IP3), or kinase cascades, including mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K)-Akt (Wehling, 1997; Schmidt *et al.*, 2000; Levin, 2008; Simoncini and Genazzani, 2003) (not shown). These mechanisms provide additional regulatory control and contribute to the tissue specificity of SR signalling.

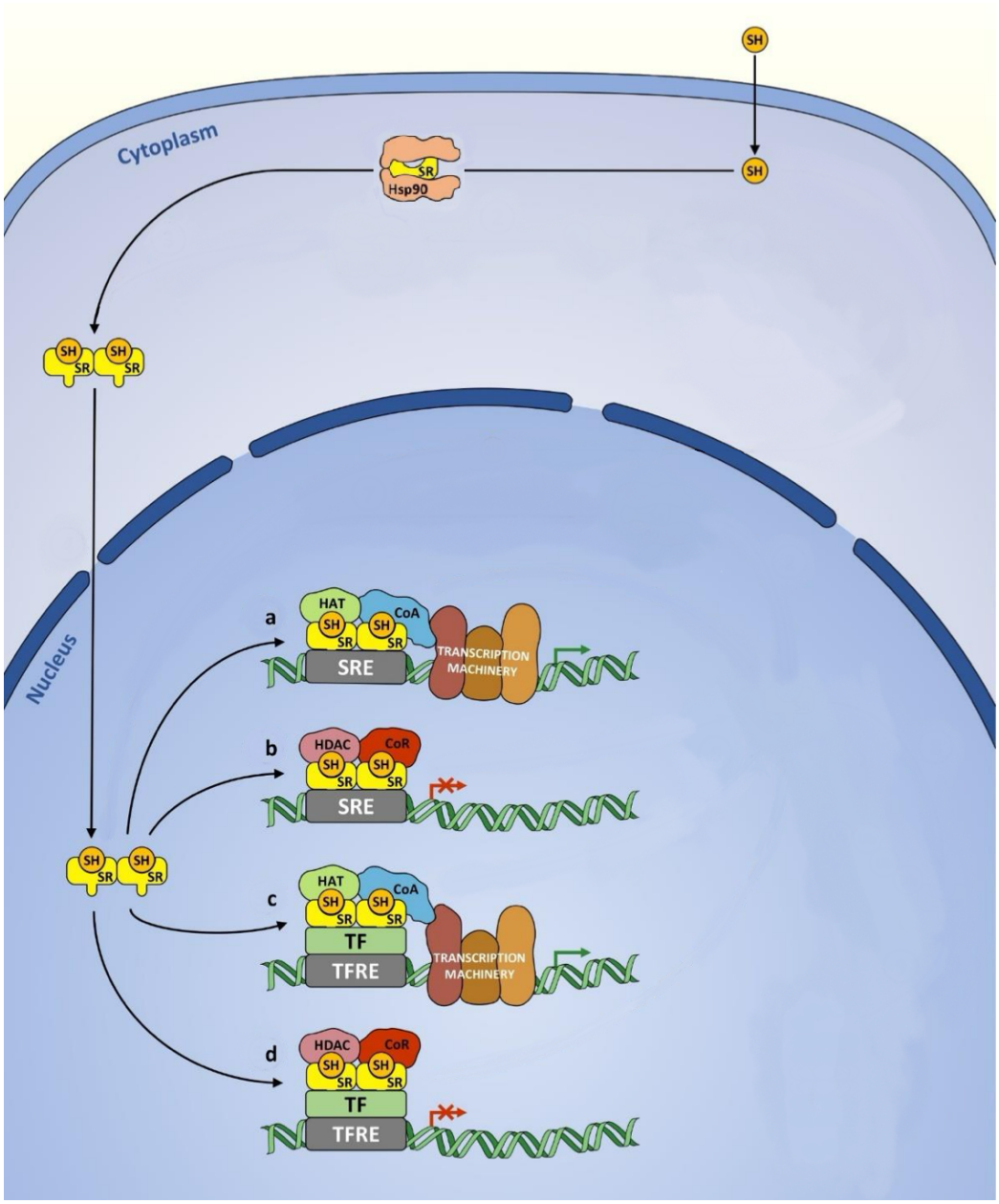


Figure 1.3.1.2 Genomic mechanism of steroid receptor (SR) action: Unliganded SRs are bound to heat shock protein (Hsp) complexes in the cytoplasm or nucleus. Upon steroid hormone (SH) binding, activated SRs dissociate from these complexes, dimerise, and may translocate to the nucleus. SRs bind steroid response elements (SREs) on target gene promoters and recruit histone acetyltransferase (HAT) and coactivator (CoA) complexes for upregulation (**a**) or histone deacetylase (HDAC) and corepressor (CoR) complexes for downregulation (**b**) of gene expression. Alternatively, SRs can bind to other transcription factors (TFs) on TF response elements (TFREs) and recruit CoA or CoR complexes to induce (**c**) or inhibit (**d**) gene expression. Figure adapted from (Kowalczyk *et al.*, 2021).

1.3.2 *Investigating progestin-induced SR activity*

A SRs mode of action can lead to distinct transcriptional responses with different ligands. SRs interact with structurally diverse ligands, forming SR-ligand complexes with different conformations (Katzenellenbogen, O'Malley, and Katzenellenbogen, 1996). The varied SR-ligand complexes selectively interact with transcriptional machinery to regulate gene expression in a tissue- and cell-specific manner (Hapgood, Africander*, et al.*, 2014; Katzenellenbogen, O'Malley, and Katzenellenbogen, 1996). Considering the structural diversity of progestins, it is essential to employ experimental approaches that evaluate binding affinity, progestin-induced conformational changes of the SR and transcriptional activity. Together, these assays provide a comprehensive framework for characterising SR-ligand interactions, thereby advancing understanding of the molecular mechanisms that may underpin differential biological effects.

Radiolabelled competitive binding assays determine the relative binding affinity (RBA) of a ligand for a specific SR *in vitro* by measuring the displacement of a constant, relatively low concentration of radiolabelled ligand of known affinity by varying concentrations of an unlabelled competing ligand (Caldwell *et al.*, 2012; Hapgood, Africander*, et al.*, 2014; Robertson *et al.*, 2013; Tomlinson and Hnatowich, 1988). The RBA is determined by measuring the concentration of competing ligand required to displace 50% of the radiolabelled ligand from the receptor (IC_{50}) (Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009; Hapgood, Africander*, et al.*, 2014) (**Figure 1.3.2.1**). However, the IC_{50} can vary depending on experimental

conditions, including the concentration of the radiolabelled ligand, receptor density, and the relative concentrations of other receptors and endogenous ligands (Caldwell *et al.*, 2012; Hapgood, Africander*, et al.*, 2014; Robertson *et al.*, 2013; Tomlinson and Hnatowich, 1988). In saturation binding assays, the concentration of radiolabelled ligand is varied to determine the equilibrium dissociation constant (K_d), which remains constant regardless of experimental conditions, thus providing a more accurate measure of affinity (Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009; Hapgood, Africander*, et al.*, 2014); however, K_d data remain limited for several progestins, including ETG and NES. Notably, binding assays only provide information on ligand affinity for SRs, and do not infer on the transcriptional activity of the SR-ligand complexes formed.

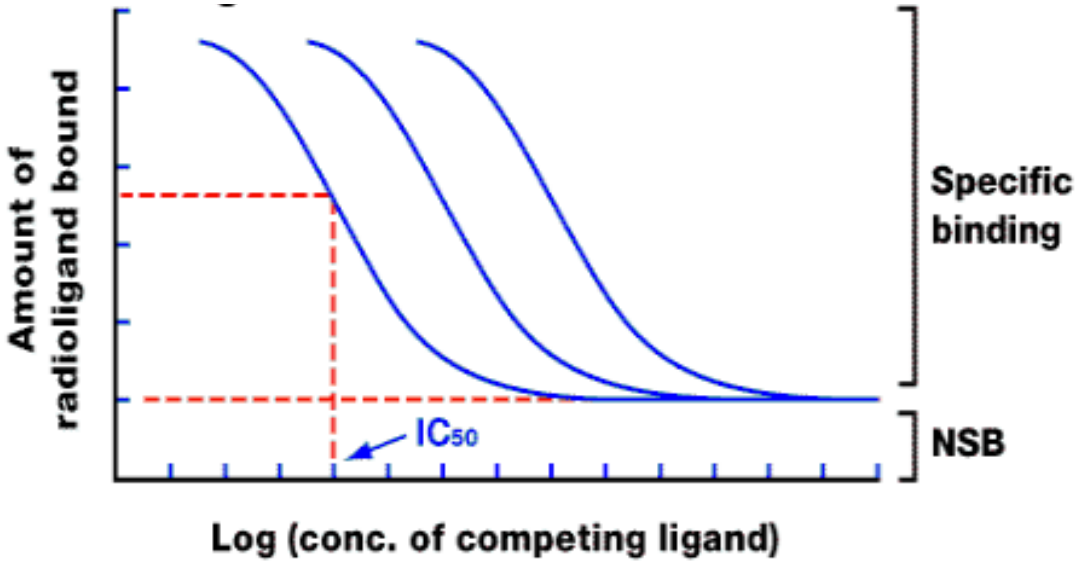


Figure 1.3.2.1. A schematic representation of competitive binding dose-response curves: Binding curves for multiple ligands in a competitive binding assay. IC_{50} denotes the competing ligand concentration displacing 50% of the radiolabelled ligand bound to the receptor. The fraction of radiolabelled ligand not displaced from the receptor by competing ligands is denoted as the non-specific binding (NSB). Figure adapted from (GlaxoWellcome, 2002).

The transcriptional activity of SR-ligand complexes can be determined *in vitro* using dose-response analyses in promoter-reporter or endogenous gene expression assays. While these assays may not entirely mimic physiological conditions *in vivo*, potentially eliciting cell- and promoter-specific transcriptional responses comparable to primary cells, these methods still offer valuable mechanistic insights into the potential biological effects of select SR-ligand complexes. Dose-response curves enable the precise determination of key pharmacological parameters for characterising SR-ligand transcriptional activities (Chow *et al.*, 2011; Hapgood, Africander*, et al.*, 2014; Hapgood, Kaushic, and Hel, 2018) (**Figure 1.3.2.2**). Potency (EC_{50}) indicates the ligand concentration required to elicit half the maximal transcriptional response; however, EC_{50} values can fluctuate between experiments in different cells depending on several factors, including SR availability, endogenous

gene or promoter-reporter construct concentration, and cell-specific variations in cofactor availability (Katzenellenbogen, O'Malley, and Katzenellenbogen, 1996; Africander, Verhoog, and Hapgood, 2011; Hapgood, Africander*, et al.*, 2014).

In contrast, efficacy reflects the maximal response elicited by a ligand when bound to a SR (Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009). The efficacy value classifies the biocharacter of ligand interactions with specific SRs. A ligand can be classified either as a full agonist (elicits maximal response), a partial agonist (elicits a response less than the maximal response) or an antagonist (reduces an agonist's efficacy) (Hapgood, Kaushic, and Hel, 2018; Africander, Verhoog, and Hapgood, 2011) (**Figure 1.3.2.2**). Furthermore, a ligand can be classified as a full, partial or neutral antagonist (binds to a receptor but does not elicit a response) (not shown) (Hapgood, Africander*, et al.*, 2014). Similar to potency, the efficacy of a ligand may fluctuate across cell systems due to differences in SR abundance, cell-specific coregulator expression, differential metabolism and uptake or efflux of the ligand (Katzenellenbogen, O'Malley, and Katzenellenbogen, 1996; Hapgood, Africander*, et al.*, 2014; Hapgood, Kaushic, and Hel, 2018). These factors do not directly alter SR-ligand binding conformations but influence the downstream transcriptional response by shaping SR-coregulator complex conformation and activity. As a result, SR-ligand interactions are not always directly comparable across different cell systems. Furthermore, SR expression levels influence the number of receptors occupied by ligand, which in turn can modulate both potency and efficacy (Katzenellenbogen, O'Malley, and Katzenellenbogen, 1996; Hapgood, Africander*, et al.*, 2014).

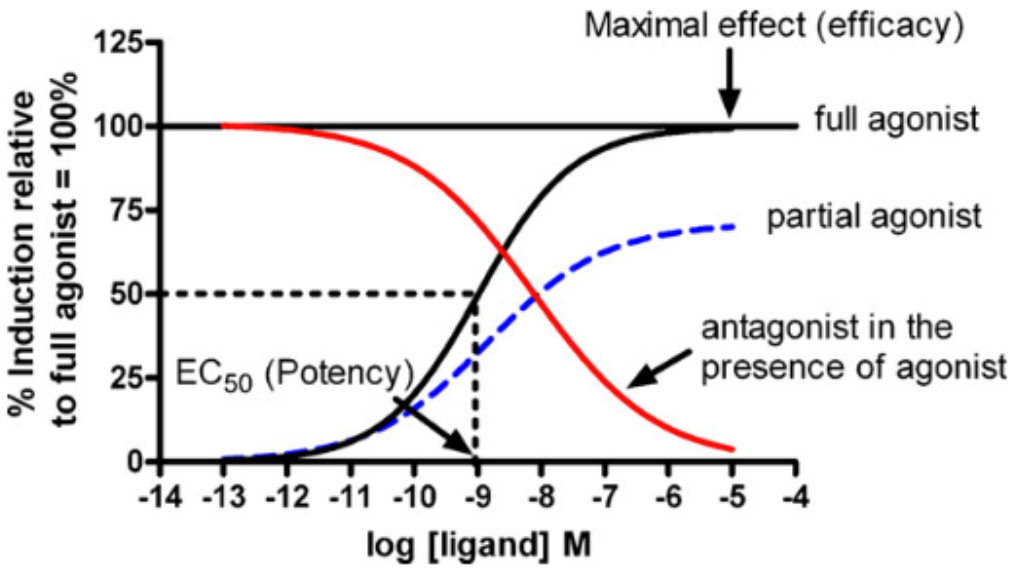


Figure 1.3.2.2 Schematic showing sigmoidal dose-response curves depicting the pharmacological parameters for classifying SR-ligand interactions: Full agonist ligand (solid black curve) exhibits maximal effect = 100% (efficacy). The full agonist potency (EC_{50}) is the ligand concentration required to elicit 50% of the maximal response (black dotted line). The blue dotted line represents a typical curve for a partial agonist, and the solid red curve depicts a full antagonist. Figure from (Africander, Verhoog, and Hapgood, 2011).

1.3.3 *SR-mediated progestin activity*

Progestins are designed to exhibit high affinity for the PR, however, select progestins bind to multiple SRs, including the AR, ER, GR, and MR, in addition to the PR, leading to potential off-target biological effects. (Hapgood, Kaushic, and Hel, 2018; Schindler *et al.*, 2008; Louw-du Toit *et al.*, 2017). Given the tissue-selective expression of SRs, off-target progestin activity may influence broader physiological pathways across multiple organ systems. For example, the PR is mainly expressed in the female genital tract (FGT), mammary glands and select immune cells (Medina-Laver *et al.*, 2021; Arruvito *et al.*, 2008); the AR and ER are highly

expressed across reproductive tissues, contribute to ovarian and breast development, and broader physiological functions (Wilson and McPhaul, 1996; Mertens *et al.*, 2001; Brosens *et al.*, 2004; Kuhl, 2005; Ildgruben *et al.*, 2005; Lee, Kim, and Choi, 2012); the GR is ubiquitously expressed and regulates key physiological functions, including development, immunity and metabolism (Timmermans, Souffriau, and Libert, 2019; Pooley *et al.*, 2020; Lockett, Inder, and Clifton, 2024); while the MR is widely expressed across multiple tissues, including kidney, heart and brain tissues (Funder, 2005; Stanczyk *et al.*, 2013; Africander, Louw, and Hapgood, 2013; Pooley *et al.*, 2020).

While the primary focus of this thesis is on the GR, it is also important to briefly examine published evidence of progestin interactions with AR, ER, and MR as possible off-target mechanisms.

Previous studies investigating off-target progestin affinity for the AR, ER, MR and GR report considerable variability in affinities across studies, primarily due to differences in experimental design, SR sources, model systems, and/or reference ligands (Africander, Verhoog, and Hapgood, 2011; Stanczyk *et al.*, 2013). *In vitro* studies investigating progestin interactions with the AR report variable findings. MPA, NET/NET-A and LNG consistently display relatively high AR affinity, comparable to dihydrotestosterone (DHT), and androgenic activity (Africander, Storbeck, and Hapgood, 2014; Stanczyk *et al.*, 2013; Phillips *et al.*, 1987; Ghatge *et al.*, 2005); whereas, ETG exhibits low AR affinity relative to DHT but still elicits AR-mediated transcriptional responses, consistent with partial agonist activity

(Kloosterboer, Vonk-Noordegraaf, and Turpijn, 1988; Kumar *et al.*, 2000; Phillips *et al.*, 1987). Published data on NES are inconsistent, with some studies reporting negligible AR affinity relative to T, while others describe relative AR affinity similar to LNG and P_4, comparable to DHT (Kumar *et al.*, 2000; Tuba *et al.*, 2000; Louw-du Toit *et al.*, 2017). These discrepancies likely reflect assay-specific variables, such as differences in the comparator ligand used across studies.

Although limited, published findings on ER-mediated progestin activity consistently report that, relative to estradiol (E_2), progestins display negligible ER affinity (Fuhrmann, Slater, and Fritzemeier, 1995; Philibert *et al.*, 1999; Kumar *et al.*, 2000; Kuhl, 2005; Africander, Verhoog, and Hapgood, 2011). One study examining isoform-specific interactions did not detect progestins affinity for ERβ, but found that compared to E_2, NET-A and LNG displayed ERα binding affinity and activation (Louw-du Toit *et al.*, 2017). The authors noted that since both NET-A and LNG are metabolised into by-products known to activate ERα, the apparent ERα affinity and estrogenic activity may be due to the progestin metabolites (Louw-du Toit *et al.*, 2017). This finding highlights the importance of isoform-specific analysis when evaluating potential off-target activity.

Regarding the MR, the available literature on MR-mediated interactions indicates that, relative to aldosterone (Ald), LNG exhibits the highest affinity for the MR compared to P_4, MPA, NET and ETG (Stanczyk *et al.*, 2013; Hapgood, Kaushic, and Hel, 2018; Philibert *et al.*, 1999; Africander, Louw, and Hapgood, 2013). Data for NES are limited and remain contradictory, with one study reporting negligible MR

activity (Stanczyk *et al.*, 2013; Micks and Jensen, 2020), and another suggesting NES, MPA, NET-A, LNG, and ETG display similar affinity for the MR (Louw-du Toit, Hapgood, and Africander, 2020).

Taken together, the literature consistently demonstrates that progestins exhibit diverse and often inconsistent off-target interactions with the AR, ER and MR to varying degrees. The variability observed in progestin activity across studies reflects not only inherent biological differences in SR-ligand interactions but also methodological heterogeneity. These methodological differences include differences in reference ligands (e.g., E_2, DEX, DHT, or T) and model systems used, which included non-human mammalian animal models and/or cell lines. These experimental factors likely account for the variable results, particularly NES and ETG, across AR, ER and MR studies. Despite these limitations, these data provide valuable mechanistic insights into the molecular actions of synthetic progestins. Furthermore, the data suggest that at physiologically relevant serum concentrations, off-target progestin interactions may modulate physiological functions in tissues expressing an array of SRs. To clarify progestin-specific off-target profiles and elucidate potential clinical effects, future studies should employ standardised experimental approaches.

1.3.4 *The glucocorticoid receptor (GR)*

Published data on off-target progestin activity via AR, ER and MR remain inconsistent, but the GR stands out as a biologically relevant off-target receptor due

to its ubiquitous expression and well-established role in immune regulation and hence possible role in HIV-1 susceptibility.

The GR is a nuclear TF that regulates between 1000 and 2000 genes, influencing key physiological processes such as immune function and metabolism (Heitzer *et al.*, 2007; Huang, Chandra, and Rastinejad, 2010; Timmermans, Souffriau, and Libert, 2019; Pooley *et al.*, 2020; Frank, Ortlund, and Liu, 2021; Lockett, Inder, and Clifton, 2024). Several isoforms of the GR have been identified, with GRα being the predominant transcriptionally active isoform; whereas, GRβ lacks ligand-binding capacity and can act as a dominant-negative regulator of the GRα, particularly under inflammatory conditions (Sanchez-Vega *et al.*, 2006; Kadmiel and Cidlowski, 2013; Liu *et al.*, 2019; Ramos-Ramirez and Tliba, 2021; Nicolaides, 2022).

The GR mechanism of action parallels that of other SRs (described in **Section 1.3.1.2**), with a few differences relevant for immune regulation (**Figure 1.3.4.1**). Within the nucleus, the GR regulates transcription through two principal genomic mechanisms. Firstly, GR-ligand complexes can bind directly to glucocorticoid response elements (GREs) to activate or repress transcription (Yudt and Cidlowski, 2002; Kassel and Herrlich, 2007; Newton and Holden, 2007; Nicolaides *et al.*, 2010; Timmermans, Souffriau, and Libert, 2019; Liu *et al.*, 2019; Praestholm, Correia, and Grontved, 2020; Kowalczyk *et al.*, 2021; Lockett, Inder, and Clifton, 2024). Secondly, the GR can modulate transcription by tethering to other TFs such as AP-1, NFκB, GATA-3, and STAT6 to repress pro-inflammatory genes. These pathways are central to the immunomodulatory effects of progestins (De Bosscher, Vanden Berghe, and Haegeman, 2003; Newton and Holden, 2007; Kassel and Herrlich,

2007; Nicolaides *et al.*, 2010; Ratman *et al.*, 2013; Timmermans, Souffriau, and Libert, 2019; Liu *et al.*, 2019; Praestholm, Correia, and Grontved, 2020; Zhang *et al.*, 2021; Liang *et al.*, 2021; Lockett, Inder, and Clifton, 2024).

Collectively, the GR signalling pathway encompasses a complex network of genomic regulatory mechanisms that modulate gene expression. Consequently, progestin-mediated off-target binding and activation of the GR is of particular relevance due to its potential to influence key immunomodulatory pathways. Elucidating the progestin-GR interactions is essential for understanding how off-target progestin effects via the GR influence immune regulation and HIV-1 susceptibility.

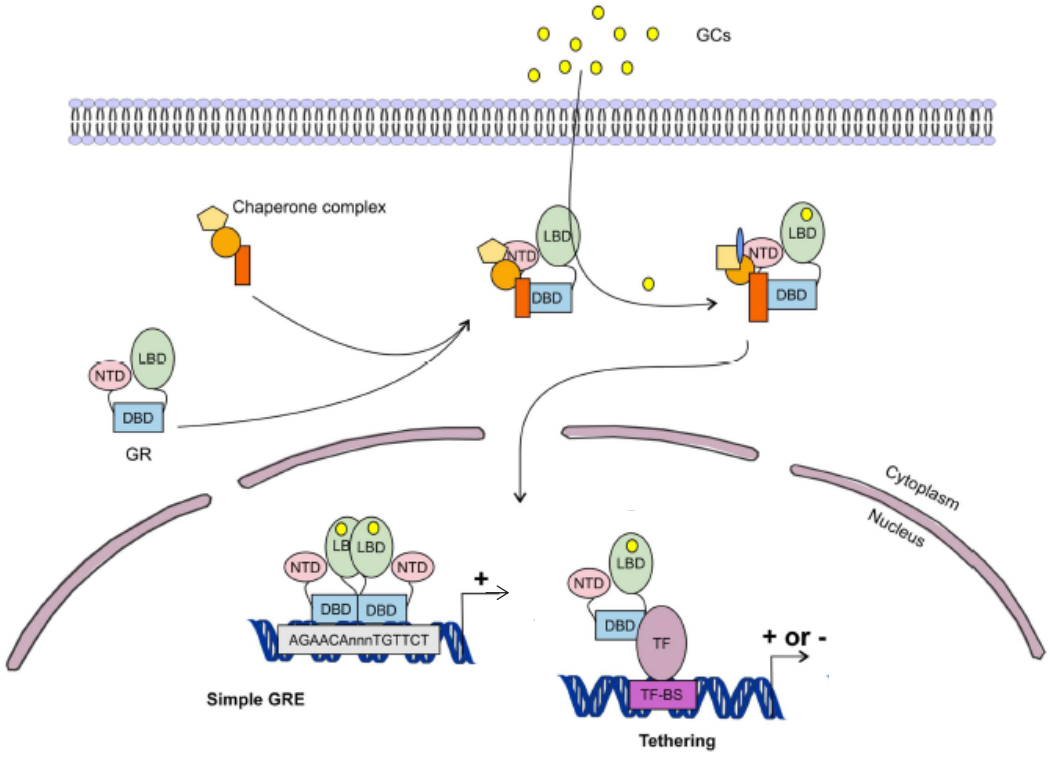


Figure 1.3.4.1. Simplistic model of genomic GR signalling pathways: Unliganded GR is associated with chaperone proteins in the cytoplasm. Upon GC binding, the GR-chaperone complex undergoes a conformational change, leading to activation. The GC-activated GR then translocates to the nucleus, regulating gene

transcription through direct and indirect mechanisms. The GR can directly transactivate genes by binding to GREs or indirectly regulate gene transcription by tethering to other TFs. Figure adapted from (Timmermans, Souffriau, and Libert, 2019)

1.4 Glucocorticoid (GC) actions of progestin-only contraceptives

As outlined above, progestins bind not only to PR but also to other SRs. Considering its ubiquitous expression and key role in immune regulation, investigating the off-target binding of progestins to the GR holds significant value, given the growing preference for progestin-only LARCs in developing countries with high HIV incidence.

Identifying both binding affinity and transcriptional activity is central to elucidating SR-ligand interactions (Hapgood, Africander*, et al.*, 2014; Katzenellenbogen, O'Malley, and Katzenellenbogen, 1996). **Section 1.4.1-2** provides an integrated overview of existing literature on the GC actions of select progestins.

1.4.1 *GR binding characteristics of synthetic progestins*

Determining GR binding affinity is essential for understanding the potential of progestins to elicit GR-mediated effects. Published findings from studies examining progestin affinity for the GR binding are summarised in **Table 1.4.1**. While competitive binding studies were widely employed to assess progestin affinities for the GR, the reported RBA, IC_{50}, or K_i values varied, indicating that the GR's affinity for progestins could differ depending on the method used. Despite methodological discrepancies between studies, some consistent patterns have emerged.

Table 1.4.1: Overview of published RBA, IC_{50} and K_i values of progestins via the GR

Ligand	RBA (%)*	IC_{50} (nM) ± SEM / ED_{50} (nM)	K_i (nM) ± SEM
MPA	42[a]; 26[c]; 58[c]; 79.1[f]	19 ± 3[g]	3.7± 1.8[b] 10.8 ± 1.1[f]
NET/-A	0.1[a]; 1.4[c]; 0.88[f]	1688 ± 300[g]	270 ± 1.3[f]
LNG	7.5[c]; 2.1[e]	-	-
ETG	40[d]; 10[e]	-	-
NES	-	56[h]	-

[a](Kontula *et al.*, 1983). [b](Selman, 1996). [c](Philibert *et al.*, 1999). [d](Fuhrmann, Slater, and Fritzemeier, 1995). [e](Pollow *et al.*, 1992). [f](Koubovec *et al.*, 2005). [g](Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009). [h](Kumar *et al.*, 2000).
*Relative to DEX=100%.

MPA consistently demonstrates relatively high GR affinity compared to DEX, with RBAs ranging from 26% to 79.1% (Kontula *et al.*, 1983; Selman, 1996; Philibert *et al.*, 1999; Koubovec *et al.*, 2005; Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009). Importantly, progestin binding appears stronger in human GR assays than in rodent models (Philibert *et al.*, 1999), indicating species-specific receptor differences. Given that receptor concentration may influence binding affinity, the differences between reported results may be due to varying GR concentration across model systems.

In contrast, NET and LNG consistently exhibit very low GR affinity compared to DEX (Kontula *et al.*, 1983; Philibert *et al.*, 1999; Pollow *et al.*, 1992; Kumar *et al.*, 2000; Koubovec *et al.*, 2005; Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009). However, the reproducibility of these results is uncertain as some studies do not report the number of biological repeats conducted or account for possible interference from competing SRs in the chosen model. Notably, some studies

implemented controls: One study confirmed that GR binding was not confounded by PR in human mononuclear leukocytes (Kontula *et al.*, 1983); another study saturated the MR with excess spironolactone to exclude MR interference (Selman, 1996). In another study, AR activity in A459 cells was diminished with excess amounts of hydroxyflutamide, an AR antagonist, and no differences were found in the reported GR affinities for the progestins (Koubovec *et al.*, 2005).

ETG and NES show intermediate or uncertain GR affinity; however, available studies are limited, often under-reported and inconsistent (Fuhrmann, Slater, and Fritzemeier, 1995; Kumar *et al.*, 2000).

Taken together, the inconsistencies in published GR-binding data likely stem from multiple sources, including the use of varied model systems (ranging from cell lines and animal tissues to primary human cells) with distinct SR expression profiles, heterogeneous experimental designs where not all progestins were tested in parallel, and incomplete methodological reporting, such as omission of replicate numbers or concentration ranges. An additional limitation is the inconsistent control for confounding activity via other SRs, such as the AR and MR, which may obscure actual GR-specific effects. While some studies applied appropriate controls (Selman, 1996; Koubovec *et al.*, 2005), this was not the case uniformly. Importantly, data for ETG and NES remain especially limited, precluding firm conclusions about their GR binding properties.

As mentioned above, previous studies reporting RBAs of MPA, NET/NET-A, LNG and ETG for the GR show considerable variations between the different progestins, while all progestins bind to the PR with higher affinity than P_4 (Africander, Verhoog, and Hapgood, 2011; Hapgood, Kaushic, and Hel, 2018). To accurately compare progestin affinities for the PR and the GR, it is essential to have precise K_d or K_i values for each progestin for these receptors, determined in model systems without competing SRs (Africander, Verhoog, and Hapgood, 2011; Hapgood, Africander*, et al.*, 2014); however, the available K_d or K_i data for these progestins are limited. For GR binding, specific K_d and K_i values are available only for MPA and NET (**Table 1.4.1**). One study demonstrated that MPA displayed high GR affinity compared to DEX, with a K_i of 3.7 nM (Selman, 1996). Another study reported that MPA has a K_d of approximately 4.2 nM and a K_i of 10.8 nM for the GR; whereas NET exhibits much weaker binding with a K_i around 270 nM (Koubovec *et al.*, 2005). Regarding PR binding, studies reporting K_d or K_i values are limited. One study reported a K_d of approximately 1.3 nM and a K_i of 6.8 nM for NET binding to the PR (Kasid *et al.*, 1978). No data are available on the K_d or K_i values for MPA, LNG, ETG or NES for the PR.

Nevertheless, it is noteworthy that studies evaluating MPA in comparison to one or more progestins consistently showed that relative to DEX, MPA exhibits a relatively higher affinity for the GR than the other progestins; whereas, NET exhibits the lowest relative affinity for the GR (Kontula *et al.*, 1983; Fuhrmann, Slater, and Fritzemeier, 1995; Philibert *et al.*, 1999; Koubovec *et al.*, 2005; Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009).

Future GR binding studies should be accurately designed to mitigate the limitations associated with current published studies, thereby providing more reliable and comparable data.

1.4.2 *Progestin-induced activation of the GR*

Several studies have examined progestin-induced GR activation to determine the functional outcomes of progestin activity via the GR, using various assays. Assays measuring glycogen storage, tyrosine aminotransferase (TAT) activity and the induction of synthetic GRE-driven promoters and GR target genes such as glucocorticoid induced leucine zipper (GILZ) and interleukin (IL)-6 are widely used because they represent well-established GC responses. Glycogen accumulation and TAT activity reflect classic metabolic regulation, while GILZ induction and IL-6 repression are directly linked to immunomodulation (Koubovec *et al.*, 2004; Koubovec *et al.*, 2005; Ronacher, Hadley, Avenant, Stubsrud, Simons Jr.*, et al.*, 2009; Kumar *et al.*, 2000).

While data on the activity of MPA via the GR are available, there is a scarcity of studies exploring the GC actions of NET, and even fewer studies are examining LNG, ETG, and NES.

Table 1.4.2 summarises reported efficacy and EC_{50} values of the progestins via the GR relative to DEX. Relative to DEX, MPA consistently behaves as a partial agonist for transactivation of GR-driven promoters, and as a potent full-to-partial agonist for

transrepression of AP-1 and NFκB signaling (Koubovec *et al.*, 2004; Koubovec *et al.*, 2005; Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009). Consistent with these findings, studies using RT-qPCR to measure gene expression have shown that relative to DEX, MPA upregulates GILZ mRNA levels while repressing the pro-inflammatory IL-6 and IL-8 mRNA levels (Govender *et al.*, 2014; Hapgood, Ray*, et al.*, 2014). A study by Bamberger *et al.,* extended these findings, reporting strong repression of IL-2 promoter activity; however, interpretation is limited by a lack of normalisation to DEX and the use of a single concentration of MPA. These findings demonstrate that MPA can regulate key immune-related genes across multiple cell models. Furthermore, diminished MPA responses in the presence of RU486, as well as GR localisation assays indicated potential GR involvement (Koubovec *et al.*, 2004; Koubovec *et al.*, 2005; Ronacher, Hadley, Avenant, Stubsrud, Simons Jr.*, et al.*, 2009; Bamberger *et al.*, 1999).

Table 1.4.2: Overview of published efficacy and EC_{50} values of progestins via the GR on GRE, AP-1, NFκB, and IL-8 *cis*-elements

	Transactivation		Transrepression					
	GRE		AP-1		NFκB		IL-8	
	Max (%) ± SEM	**EC_{50} (nM) ± SEM**	**Max (%) ± SEM**	**EC_{50} (nM) ± SEM**	**Max (%) ± SEM**	**EC_{50} (nM) ± SEM**	**Max (%) ± SEM**	**EC_{50} (nM) ± SEM**
MPA	5.7 ± 0.2[a] 72.2 ± 0.85[b,*] 73 ± 5[c,*]	92.0 ± 1.3[a] 15.5 ± 1.73[b,*] 2 ± 0.4[c]	98 ± 7[c,*]	0.0005 ± 0.0002[c]	87 ± 19[c,*]	0.7 ± 0.1[c,*]	40.6 ± 1.5[a] 95.4 ± 1.16[b,*]	90.0 ± 1.4[a] 7.90 ± 1.29[b*]
NET-A	0.40 ± 0.01[b,*] N/A[c,*]	N/A[b,c,*]	N/A[c,*]	N/A[c,*]	N/A[c,*]	N/A[c,*]	23.0 ± 3.44[b,*]	0.21[b, †]

[a](Koubovec *et al.*, 2004). [b](Koubovec *et al.*, 2005).[c](Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009).
*Relative to DEX=100%.
†EC_{50} to be interpreted with caution due to shallow slope.
N/A, no activity.

In contrast, limited studies found that NET-A consistently displays negligible GR activity, failing to induce a transactivation response on GRE-promoters (Koubovec *et al.*, 2005; Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009; Govender *et al.*, 2014; Hapgood, Ray*, et al.*, 2014) (**Table 1.4.2**). Interestingly, Govender *et al.*, found that NET-A may upregulate IL-6 and IL-8 mRNA levels at high concentrations after 24 and 4 hours, respectively (Govender *et al.*, 2014). The findings from the Govender *et al.,* study suggest that the transactivation response of NET-A via the GR may be dose and time dependent.

The available literature on LNG, ETG and NES is limited, making it difficult to draw firm conclusions from the reported findings. One study reported that ETG elicited a weak GR-mediated response in CV-1 cells exogenously expressing the GR (Fuhrmann, Slater, and Fritzemeier, 1995). Another study demonstrated that LNG and NES had no detectable effect on glycogen content and TAT activity in rats compared to DEX (Kumar *et al.*, 2000). However, the reproducibility of these studies is difficult to assess due to significant assay-dependent factors and inadequate replication (Fuhrmann, Slater, and Fritzemeier, 1995; Kumar *et al.*, 2000).

Taken together, the current *in vitro* and *ex vivo* literature consistently indicates that MPA is a potent partial to full agonist for the transactivation and transrepression of GR-regulated genes. Furthermore, the limited studies available on the GC actions of NET-A consistently indicated that NET-A does not exhibit substantial GC activity. The available data on LNG, ETG and NES are limited and cannot be reliably compared due to several limitations associated with experimental design. A

significant gap in the current literature is the absence of molecular studies investigating the potential off-target activation of the GR by LNG, ETG, and NES in parallel with MPA and NET in the same model system.

While GR binding and transcriptional activity provide mechanistic insight into how progestins engage receptor signalling, the functional consequences for immune regulation remain less clearly defined. **Section 1.5** reviews prior studies examining progestin effects on immune mediators and cell populations, critically evaluating the strength of this evidence and the extent to which it supports a role for GR-mediated immunomodulation.

1.5 HIV-1 acquisition and immune activation

1.5.1 *The female genital tract (FGT) and HIV-1 acquisition*

The HIV-1 epidemic continues to affect women disproportionately in sub-Saharan Africa, where girls and young women (15-24 years) remain particularly high risk of new HIV-1 infections compared to men (Ramjee and Daniels, 2013; Magadi, 2011). In 2021, women and girls accounted for 63% of all new HIV-1 infections (Cassim *et al.*, 2023). This gender disparity reflects intersecting socioeconomic vulnerabilities and physiological factors that together could enhance susceptibility to HIV-1infection (Magadi, 2011; Rodriguez-Garcia, Connors, and Ghosh, 2021; Ramjee and Daniels, 2013). Understanding the physiological factors that likely modify women's susceptibility to HIV-1 infection is crucial for understanding this gender disparity.

Vaginal transmission is considered the leading mechanism by which women engaging in heterosexual intercourse most likely acquire HIV-1 (Rodriguez-Garcia, Connors, and Ghosh, 2021). The rate of vaginal transmission is low in comparison to other routes of transmission, however, a greater proportion of new HIV-1 diagnoses are reported to result from vaginal transmission (Hladik and Hope, 2009). The FGT provides both a barrier and a portal of entry for HIV-1 (Wira *et al.*, 2005; Gholiof, Adamson-De Luca, and Wessels, 2022). The lower FGT, comprising the vagina and ectocervix, is lined with stratified squamous epithelium, offering greater protection; whereas, the cervical transformation zone and the upper FGT (uterus and endocervix) are lined by a single layer of columnar epithelium enriched with

activated immune cells (Wira *et al.*, 2005; Pudney, Quayle, and Anderson, 2005; Lee *et al.*, 2015; Kaushic, 2011; Gholiof, Adamson-De Luca, and Wessels, 2022).

To establish infection, HIV-1 targets immune cells, including dendritic cells, macrophages and T cells in the FGT (Greenhead *et al.*, 2000; Gholiof, Adamson-De Luca, and Wessels, 2022; Hapgood, Kaushic, and Hel, 2018). T cells are a diverse group of lymphocytes, including $CD3^+$ T cells, which are essential for T cell activation and function, and $CD4^+$ T cells, which support the activity of $CD8^+$ T cells and other immune components in identifying and destroying infected target cells; whereas, CD14, found on monocytes and macrophages, plays a key role in recognising pathogens and initiating the immune response by releasing inflammatory cytokines (Chandra *et al.*, 2013; Hapgood, Kaushic, and Hel, 2018; Rodriguez-Garcia, Connors, and Ghosh, 2021).

HIV-1 primarily targets $CD4^+$ T cells and replicates within these cells (Swanstrom and Coffin, 2012). To gain cellular entry and initiate infection, HIV-1 binds to the CD4 receptor and an co-receptor, either chemokine receptor 5 (CCR5) or chemokine receptor 4 (CXCR4) (Rottman *et al.*, 1997; Li *et al.*, 2009; Swanstrom and Coffin, 2012; Rodriguez-Garcia, Connors, and Ghosh, 2021). HIV-1 can alternate between CCR5 and CXCR4 tropism, however, a recent study indicates that only dual-tropic or CCR5-tropic viruses mediate mucosal HIV-1 transmission (Swanstrom and Coffin, 2012; Rodriguez-Garcia, Connors, and Ghosh, 2021).

1.5.2 *FGT and systemic inflammation*

Considering that innate immune responses are the first line of defence against viral infection, understanding how progestins-induced GR binding and activation influence downstream inflammatory responses within this compartment is essential for linking receptor activation to potential alterations in HIV-1 susceptibility.

Inflammation plays a dual role in host defence and susceptibility to infection. While transient production of cytokines and chemokines is essential for recruiting immune cells and containing pathogens, sustained or dysregulated inflammation disrupts mucosal homeostasis and may increase vulnerability to HIV-1 infection (Ahmed, 2011; Gholiof, Adamson-De Luca, and Wessels, 2022).

Inflammation of the FGT is considered to play a role in HIV-1 acquisition (Masson *et al.*, 2015; Hapgood, Kaushic, and Hel, 2018). Clinical studies have consistently shown an association between HIV-1 seroconversion and elevated levels of proinflammatory chemokines and cytokines in the FGT (Li *et al.*, 2009; Masson *et al.*, 2015; Passmore, Jaspan, and Masson, 2016). For example, studies found that HIV-1 seroconversion correlated with elevated levels of chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β and interferon γ-induced protein (IP)-10, IL-8 in the FGT (Li *et al.*, 2009; Masson *et al.*, 2015; Passmore, Jaspan, and Masson, 2016) (Summarised in **Table 1.5.2**). These findings suggest that elevated inflammatory markers in the FGT facilitate the recruitment of HIV-1 target cells like $CD4^+$ T cells, thereby increasing the risk of HIV-1 acquisition.

In contrast to reported findings on genital cytokines, there are mixed reports on the correlation between systemic cytokine levels and risk of HIV-1 acquisition. Some studies, such as one by Lehman *et al.* did not find a significant correlation between plasma cytokine levels (IL-6, IL-10 and IL-7) in HIV-1 acquisition in seroconverted women (Lehman *et al.*, 2014); whereas, another study among HIV-1 serodiscordant couples detected higher plasma levels of IL-10 and IP-10 for couples in which HIV-1 was transmitted, suggesting an association between elevated plasma cytokines and HIV-1 acquisition (Kahle *et al.*, 2015). The precise mechanisms by which systemic inflammation increases HIV-1 acquisition in the FGT are not fully understood; however, enhanced immune activation due to systemic infections may be an indirect mechanism for increased susceptibility to HIV-1 (Kahle *et al.*, 2015).

Taken together, these studies indicate that elevated levels of select FGT and systemic inflammatory biomarkers are most likely associated with HIV-1 acquisition, although the data regarding systemic involvement remains unclear.

Table 1.5.2: Inflammatory biomarkers for HIV-1 risk.

Immune Marker*	
Proinflammatory chemokines	MIP-1α
	MIP-1β
	IP-10
	IL-8
Proinflammatory cytokines	IL-1α
	IL-1β
	IL-6
	TNFα
	MCP-1
Anti-inflammatory cytokine	IL-10
Hematopoietic cytokines	G-CSF
	IL-7

*G-CSF, granulocyte-colony stimulating factor; IL, interleukin ; IP, interferon γ-induced protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; TNF, tumour necrosis factor.

1.6 Progestin-only contraceptives and the risk of HIV-1 acquisition

While several observational and epidemiological studies have investigated the association between DMPA-IM use and an increased risk of HIV-1 acquisition, fewer studies have examined this relationship for NET-EN.

Meta-analyses of high-quality observational studies detected a significant 40%–50% increased risk of HIV-1 acquisition associated with DMPA-IM use compared to women not on hormonal contraception (Heffron *et al.*, 2012; Polis *et al.*, 2014; Morrison *et al.*, 2015; Ralph, Gollub, and Jones, 2015; Polis *et al.*, 2016). In light of this, the Evidence for Contraceptive Options and HIV Outcomes (ECHO) trial was conducted to compare the differences in HIV-1 risk between DMPA-IM, LNG implant and Cu-IUD (Evidence for Contraceptive and Consortium, 2019). The trial aimed to detect a 50% or more increase in HIV-1 incidence when comparing each of the contraceptive methods to one other. Given this threshold, the authors reported that no substantial differences were detected in HIV-1 risk between DMPA-IM, LNG implant, and CU-IUD (Evidence for Contraceptive and Consortium, 2019). The results of this trial influenced the revision of the WHO Medical Eligibility Criteria for DMPA-IM use from risk category 2 to category 1, which stipulates no restriction on the use of DMPA-IM for women at high risk of HIV-1 (World Health Organization, 2019). However, the study's 50% threshold limited the detection of substantial increases, such as a potential 45%. Furthermore, the absence of a control group of women not using contraception made it impossible to determine the individual risk associated with each contraceptive method. As ongoing research suggests, the

debate on this issue remains unresolved, requiring a deeper understanding of the biological mechanisms at play (Steyn *et al.*, 2024; Gollub *et al.*, 2019; Hapgood, 2020).

In contrast to the controversial findings for DMPA-IM, observational data for NET-EN, LNG-implant and LNG-IUD do not show a significant association with an increased risk of HIV-1 acquisition (Lavreys *et al.*, 2004; Ralph, Gollub, and Jones, 2015; Noguchi *et al.*, 2015; Morrison *et al.*, 2015; Wall *et al.*, 2015; Curtis *et al.*, 2020). Currently, there are no clinical studies assessing the potential increased risk of HIV-1 acquisition associated with ETG- and NES-implant and -IVR use, leaving significant gaps in safety profiles for these methods.

Collectively, available *ex vivo* and *in vitro* studies on the effect of MPA on HIV-1 infection are limited but report consistent findings in agreement with clinical observations. Results from *ex vivo* studies showed that exogenous MPA significantly increases HIV-1 infection in activated CD8-depleted PBMCs (Huijbregts *et al.*, 2013), non-activated total PBMCs (Sampah *et al.*, 2015; Maritz *et al.*, 2018; Bick *et al.*, 2022) and cervical tissue explants (Ray *et al.*, 2019). Furthermore, a significant increase in HIV-1 infection was observed in TZM-bL cells stimulated with exogenous MPA in an *in vitro* study (Maritz *et al.*, 2018).

Similarly, limited *ex vivo* and *in vitro* studies investigating the effect of NET on HIV-1 infection consistently showed no detectable effect of NET on HIV-1 infection when compared to MPA in both PBMCs, TZM-bL cells (Maritz *et al.*, 2018), and cervical

tissue explants (Ray *et al.*, 2019). Currently, the regulatory effects of LNG, ETG, or NES on HIV-1 infection remain unknown.

While the association between DMPA-IM use and an increased risk of HIV-1 acquisition remains controversial, reported findings across clinical, *ex vivo* and *in vitro* studies, albeit limited, consistently support NET-EN as a safer alternative for women in high HIV-1 risk areas.

To elucidate the mechanisms contributing to the increased risk of HIV-1 acquisition associated with the use of DMPA-IM, several biological mechanisms in the FGT have been put forward as potential pathways through which MPA may facilitate an increase in HIV-1 acquisition (Blish and Baeten, 2011; Murphy, Irvin, and Herold, 2014; Hapgood, Kaushic, and Hel, 2018). The mechanisms are summarised in **Figure 1.6.1.1** (Hapgood, Kaushic, and Hel, 2018). The mechanisms relevant to the present study will be discussed in more detail **(Section 1.6.1-1.6.2)**.

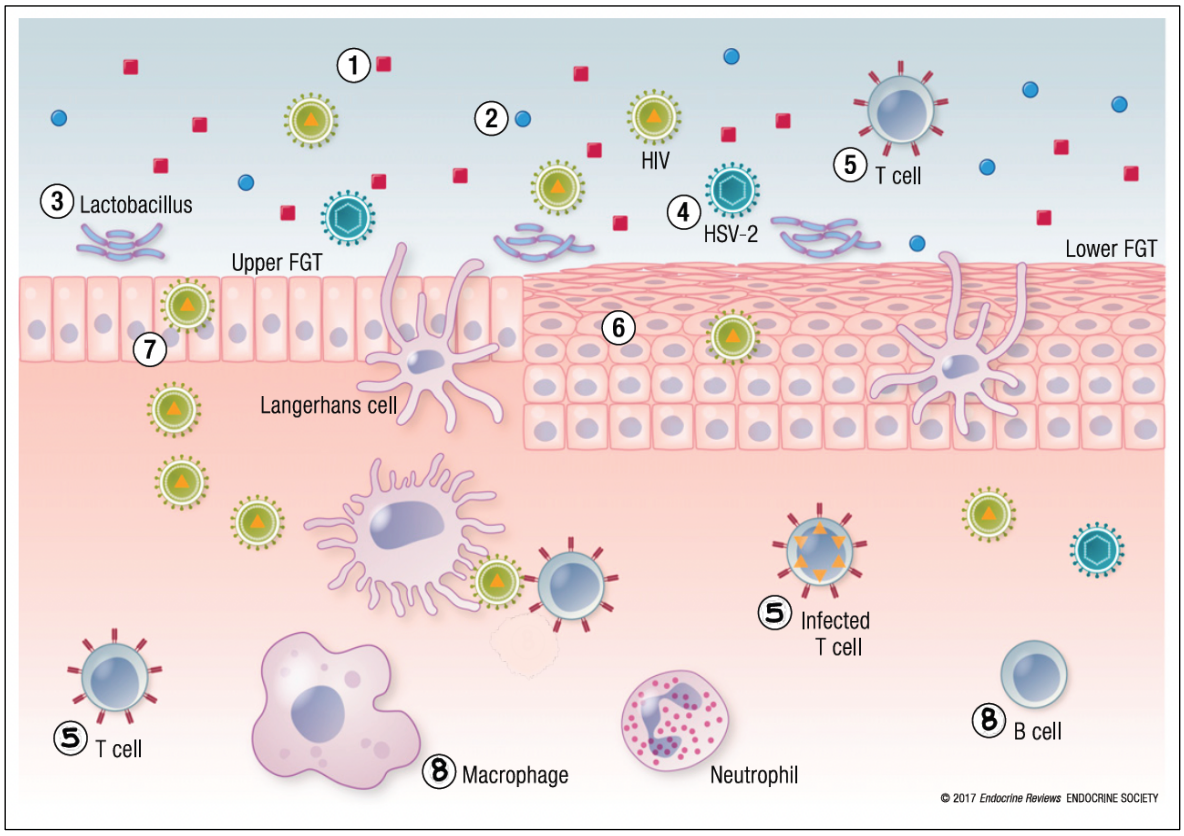


Figure 1.6.1.1 Potential biological mechanisms whereby MPA can facilitate increased HIV-1 acquisition: Experimental evidence suggests that MPA can increase HIV-1 acquisition in the FGT through several mechanisms, including: **1.** Decreasing the levels of soluble mucosal protective mediators. **2.** Changing the levels of secreted soluble immune mediators. **3.** Altering the composition of the vaginal microbiome. **4.** Increasing HSV-2 infection. **5.** Increasing the frequency and infectivity of HIV-1 target T cells, including $CCR5^{+}$ $CD4^{+}$ T cells. **6.** Increasing barrier permeability by decreasing the expression of proteins involved in barrier integrity. **7.** Increasing HIV-1 transcytosis through the columnar epithelial monolayer in the upper FGT. **8.** Modifying the function of innate and adaptive immune cells. Figure is adapted from (Hapgood, Kaushic, and Hel, 2018)

1.6.1 *Progestin-induced regulation of soluble immune mediators*

While some studies investigating the effects of progestins on immune mediator levels have shown consistent results, others have presented contrasting findings. The effects of progestins on systemic immune mediator levels are not well understood. Currently, available data on the effects of progestins on systemic immune mediator levels are limited, as most studies have primarily focused on FGT immune mediator levels.

The available literature reports contrasting findings across various studies investigating the immunomodulatory effects of DMPA-IM use (Hapgood, Kaushic, and Hel, 2018). Some studies observed enhanced pro-inflammatory immune mediators in FGT samples from DMPA-IM users (Morrison *et al.*, 2014; Deese *et al.*, 2015; Francis *et al.*, 2016), while others detected immunosuppressive effects of DMPA-IM use on select FGT immune mediators (Michel *et al.*, 2015; Ngcapu *et al.*, 2015; Molatlhegi *et al.*, 2020; Achilles *et al.*, 2020) (**Table 1.5.2**).

Consistent with the study by Michel *et al.,* which found DMPA-IM use increases select pro-inflammatory immune mediator levels in the FGT while lowering plasma immune mediator levels (Michel *et al.*, 2015), an animal study in DMPA-IM-treated rhesus macaques similarly detected significantly higher levels of IFNγ in FGT samples and lower levels in plasma samples (Goode *et al.*, 2014). This observation contrasts with the well-established association of IFNγ suppression with GC activity (Goode *et al.*, 2014).

Clinical findings on the immunomodulatory effects of NET-EN are limited and present contrasting results. One study found that NET-EN use increases the levels of pro-inflammatory immune mediators in FGT samples (Deese *et al.*, 2015), and another study did not detect significant changes in FGT immune mediator levels among women using NET-EN (Achilles *et al.*, 2020). Similarly, limited clinical studies examining the effects of LNG-implant and -IUD use on immune response report contrasting findings. Some clinical studies found that LNG-implant and -IUD use increases the levels of select inflammatory immune mediator levels in FGT samples (Shanmugasundaram *et al.*, 2016; Radzey *et al.*, 2022); whereas, other studies reported that LNG-implant use did not significantly alter the levels of inflammatory immune mediators in the FGT (Achilles *et al.*, 2020; Haddad *et al.*, 2023). Similarly, studies comparing FGT and systemic immune responses did not observe detectable immunomodulatory effects with ETG-implant use (Achilles *et al.*, 2020; Haddad *et al.*, 2020).

Data from limited *ex vivo* and *in vitro* studies investigating the regulatory effects of progestins on immune function appear consistent with reported clinical findings. For example, studies in PBMCs and cervical cell lines showed that exogenous MPA may suppress pro-inflammatory, and increase anti-inflammatory immune gene expression (Kleynhans *et al.*, 2011; Huijbregts *et al.*, 2013; Huijbregts, Michel, and Hel, 2014; Hapgood, Ray*, et al.*, 2014; Louw-du Toit, Hapgood, and Africander, 2014; Govender *et al.*, 2014; Piccinni *et al.*, 2019; Matubu *et al.*, 2021); whereas, studies in PBMCs, cervical tissue explants, and cell lines did not detect significant regulation of select immune genes or secreted soluble immune mediators following

stimulation with exogenous NET (Africander, Verhoog, and Hapgood, 2011; Govender *et al.*, 2014; Hapgood, Ray*, et al.*, 2014; Huijbregts, Michel, and Hel, 2014; Ray *et al.*, 2019; Matubu *et al.*, 2021), LNG and ETG (Huijbregts, Michel, and Hel, 2014).

Whether NES-implant and -IVR use exert immunomodulatory effects has not been investigated in clinical, *ex vivo*, or *in vitro* studies, and thus remains unknown.

Collectively, these results suggest that MPA may induce immunosuppressive or distinct immunomodulatory effects on FGT and systemic immune responses in DMPA-IM users. This implies that DMPA-IM use most likely increases HIV-1 susceptibility by either suppressing both FGT and systemic immunity or inducing inflammation in select compartments.

Variability in available clinical findings may be attributed to differences across clinical studies. Reported findings suggest that NET-EN, LNG-implant and -IUD use potentially induce inflammation in select cell and tissue compartments; whereas, available ETG-implant studies consistently report undetectable changes in FGT and systemic immunity. Alternatively, variability in clinical findings most likely indicate that NET-EN, LNG-implant and -IUD, as well as ETG-implant use have no significant immunomodulatory effects.

The available data on progestin-induced immunomodulatory effects remain limited, and variable findings have been reported across studies. Differences between

clinical and in vitro results can be attributed to variations in experimental conditions, such as the duration of progestin exposure, the concentration at the target site, immune responses, and hormonal regulation. In clinical settings, women using contraceptives undergo prolonged exposure to progestins over extended periods, during which interactions with multiple physiological signals likely influence the observed biological effects. In contrast, *in vitro* studies often involve shorter exposure periods under controlled conditions, which may not fully mimic the complex signalling environment *in vivo*, potentially underestimating immunomodulatory effects observed in clinical studies. Additional *in vitro* studies with refined experimental conditions and different model systems may offer new insights into the differences between *in vitro* and clinical immunomodulatory effects of progestins and potential association with increased HIV-1 susceptibility.

1.6.2 *Progestin-induced regulation of HIV-1 target cells and co-receptors*

The available clinical and *ex vivo* studies investigating whether progestins alter FGT and systemic immunity report variable findings on progestin-induced regulation of the frequency of HIV-1 target cells in both FGT and systemic immune cells. Multiple factors, including differences in study design, patient populations, and methods of immune cell detection, most likely contribute to the contrasting results reported across studies. Notably, FGT samples collected for analyses differed across studies, ranging from cervical or vaginal tissues to CVLs or vaginal swabs. Given the inherent differences in immune cell composition across FGT samples and donor heterogeneity, these differences most likely influence the variability observed in

clinical outcomes. Furthermore, differences in progestin concentration between systemic models and FGT tissue compartments most likely contribute to the differences in clinical findings.

Considering that MPA potentially increases HIV-1 acquisition by regulating HIV-1 target cell populations, more information is available on the effects of DMPA-IM use on FGT and systemic immunity than NET-EN, LNG-implant and -IUD, as well as ETG-implant and -IVR use (**Table 1.6.2**). Whether NES-implant and -IVR use alter HIV-1 target cell populations has not been investigated in clinical, *ex vivo*, or *in vitro* studies, and thus remains unknown.

Overall, there is a significant disparity in the available data, with more information on the effects of progestin on FGT immunity compared to systemic immunity. Available clinical studies report considerably variable findings regarding cell number, frequency (% of cells expressing a particular marker compared to the total cell population) and/or CCR5 expression (measured by mean fluorescence intensity [MFI]) between studies.

Reported clinical findings on progestin effects on HIV-1 target cell populations in FGT and PBMCs are summarised in **Table 1.6.2**. Briefly, clinical findings on the effect of DMPA-IM use on the frequency of HIV-1 target cell populations varied between some studies, with consistencies in others. Several studies have shown that DMPA-IM use increases the frequency of $CD3^+$, $CD4^+$, $CD8^+$, $CD4^+CCR5^+$ and $CD8^+CCR5^+$ T cells in the FGT (Ildgruben, Sjoberg, and Hammarstrom, 2003;

Chandra *et al.*, 2013; Byrne *et al.*, 2016; Thurman *et al.*, 2019; Tasker *et al.*, 2020; Lajoie *et al.*, 2019; Li *et al.*, 2019); whereas, other studies do not detect significant changes in select HIV-1 target cell populations in the FGT (Mitchell *et al.*, 2014; Achilles *et al.*, 2020; Michel *et al.*, 2015; Lajoie *et al.*, 2019; Omollo *et al.*, 2020; Bunjun *et al.*, 2022; Smith-McCune *et al.*, 2017). While studies found that DMPA-IM users express increased cervical $CCR5^{+}CD4^{+}$ T cells (Byrne *et al.*, 2016; Tasker *et al.*, 2020), other studies did not detect significant changes in CCR5 expression in the cervix (Bunjun *et al.*, 2022; Omollo *et al.*, 2020).

In contrast, clinical studies in PBMCs have consistently observed no detectable effects of DMPA-IM use on the frequency of systemic $CD4^{+}$, $CD8^{+}CCR5^{+}$ T cells (Byrne *et al.*, 2016; Achilles *et al.*, 2020; Omollo *et al.*, 2020; Sciaranghella *et al.*, 2015; Li *et al.*, 2019; Tasker *et al.*, 2017). One study showed that DMPA-IM use increased systemic $CCR5^{+}CD4^{+}$ T cells (Sciaranghella *et al.*, 2015), other studies detected decreased CCR5 expression in systemic $CD4^{+}$ T cells (Mitchell *et al.*, 2014; Lajoie *et al.*, 2019), and others did not detect significant changes in CCR5 expression in PBMCs from DMPA-IM users (Byrne *et al.*, 2016; Omollo *et al.*, 2020).

Clinical studies investigating the effects of NET-EN, LNG-implant and -IUD, as well as ETG-implant and -IVR use on FGT, and systemic immunity are limited and reported findings show minimal variability (**Table 1.6.2**). One study demonstrated that NET-EN use decreased $CD4^{+}$ and $CD8^{+}$ T cell numbers in FGT samples, despite showing no overall detectable effect on the FGT immune cell numbers (Achilles *et al.*, 2020). Some clinical studies detected increases in the frequency of

$CD4^+$ and $CD8^+$ T cells with LNG-implant and -IUD use (Ildgruben, Sjoberg, and Hammarstrom, 2003; Shanmugasundaram *et al.*, 2016), whereas other studies did not observe detectable effects with LNG-implant and -IUD use on the frequency of HIV-1 target cell populations in the cervix (Li *et al.*, 2019; Achilles *et al.*, 2020; Bunjun *et al.*, 2022). One study found that ETG-implant and -IVR use increased the frequency of cervical $CD4^+CCR5^+$ and $CD8^+$ T cells (Haddad *et al.*, 2020); whereas, other studies did not detect significant changes in the frequency of select cervical HIV-1 target cell populations associated with ETG-implant and -IVR use in the cervix (Li *et al.*, 2019; Haddad *et al.*, 2020; Achilles *et al.*, 2020).

Overall, studies using PBMCs from NET-EN, LNG-implant and -IUD, as well as ETG-implant and -IVR users did not detect significant changes in select HIV-1 target cell populations compared to those using non-hormonal contraceptives (Li *et al.*, 2019; Haddad *et al.*, 2020; Achilles *et al.*, 2020). In contrast, one study found that LNG-implant and -IUD use increases the frequency of $CD3^+CCR5^+$, $CD4^+CCR5^+$ and $CD8^+CCR5^+$ T cells in PBMCs (Sciaranghella *et al.*, 2015).

Currently, *ex vivo* studies investigating the effects of MPA on HIV-1 target cell populations are limited and report contrasting findings (**Table 1.6.2**). Briefly, one study using cervical tissue explants showed that exogenous MPA increases the frequency of $CD4^+$ T cells but has no detectable effect on the frequency of $CD3^+CCR5^+$, $CD4^+CCR5^+$ and $CD8^+CCR5^+$ T cell populations (Ray *et al.*, 2019). In PBMCs, the effects of exogenous MPA on HIV-1 target cell populations are consistent in some studies, but not others. Some studies found that MPA increases

CCR5 expression and the frequency of $CD4^+$ and $CD8^+$ T cells, while having no detectable effect on other HIV-1 target cell populations. (Sampah *et al.*, 2015; Maritz *et al.*, 2018; Bick *et al.*, 2022).

Overall, the data available on the regulation of HIV-1 target cell populations induced by select progestins remain limited, and variable findings are reported across studies. Currently, the literature suggests that MPA most likely induces compartment-specific immune regulation, resulting in distinct FGT and systemic immune responses in DMPA-IM users. Alternatively, the varying clinical findings most likely suggest that DMPA-IM has no significant effect on HIV-1 target cell populations, as indicated by the limited literature on the effects observed in NET-EN, LNG-implant and -IUD, as well as ETG-implant and -IVR users.

Further research is required to understand the complex relationship between contraceptive methods and HIV-1 susceptibility to safeguard the health and well-being of young women at high risk of HIV. The discrepancies observed in clinical findings highlight the need for standardised methodologies to improve the comparability and interpretation of results across different study designs.

Table 1.6.2: Overview of progestin effects on HIV-1 target cell populations in FGT and PBMCs: Clinical and *ex vivo* studies (↑/⇑, increase; ↓/⇓ , decrease; ↔/⇔, no change)

	FGT samples*		PBMCs	
	Frequency (%)†	Density (cells/mm²)	Frequency (%)†	
CD3⁺ T cells	↑[2,4] ↓[6,10]	↔[8]	⇑[17] ⇔[18] ⇔[18]	**CCR5 MFI** ⇑[17,18] ⇔[18]
CD3⁺CCR5⁺	↔[6] ⇔[13]	↓[6]	⇔[17,19] ⇑[18] ⇓[18] ↑[14]	
CD4⁺ T cells	↑[4,5] ↔[9–12] ⇑[13] ↓[7] ⇔[13] ↔[7,11] ↑[20] ↔[7,21]	↔[8] ↑[1] ↔[8]	↔[7] ↑[9] ⇔[18,19] ↔[7] ⇔[18] ↔[7] ↔[7,21]	**CCR5 MFI** ↔[3,10] ↓[9,16] ↑[14] ⇑[17,18] ⇔[18] ↑[14]
CD4⁺CCR5⁺	↑[9,15] ↔[7,10,12] ⇔[13] ↔[7] ⇔[13] ↓[20] ↔[7,15] ↑[21] ↔[7,15]	**CCR5 MFI** ↑[3,5] ↔[1] [0,11] ↔[11]	↓[9] ↔[7,10,14–16] ⇔[17–19] ↔[7] ↔[7,15] ↑[14] ↔[7,15,21]	
CD8⁺ T cells	↑[1,2,4] ↔[7,12] ↓[7] ↔[7] ↑[20] ↔[7] ↑[21]	↔[8]	⇑[17] ⇔[18] ↔[7] ⇔[18] ↔[7] ↔[7]	**CCR5 MFI** ⇔[17] ⇑[18] ⇔[18] ↑[14]
CD8⁺CCR5⁺	↑[15] ↔[7,12] ⇔[13] ↔[7] ↓[20] ↔[7,15] ↔[7,15]	—	↔[7,14,15] ⇔[17] ⇑[18] ↑[7] ⇓[18] ↔[7,15] ↑[14] ↔[7,15]	

Legend	↑ DMPA-IM	↑ NET-EN	↑ LNG-implant/IUD	↑ ETG-implant/IVR
	⇑ MPA	⇑ NET	⇑ LNG	⇑ ETG

*FGT samples may include vaginal and cervical tissue and/or cells.
†Includes data reported as changes in cell numbers from (Achilles *et al.*, 2020).
[1](Ildgruben, Sjoberg, and Hammarstrom, 2003). [2](Chandra *et al.*, 2013). [3](Byrne *et al.*, 2016). [4](Thurman *et al.*, 2019). [5](Tasker *et al.*, 2020). [6](Mitchell *et al.*, 2014). [7](Achilles *et al.*, 2020). [8](Michel *et al.*, 2015). [9](Lajoie *et al.*, 2019). [10](Omollo *et al.*, 2020). [11](Bunjun *et al.*, 2022). [12](Smith-McCune *et al.*, 2017). [13](Ray *et al.*, 2019). [14](Sciaranghella *et al.*, 2015). [15](Li *et al.*, 2019). [16](Tasker *et al.*, 2017). [17](Bick *et al.*, 2022). [18](Maritz *et al.*, 2018). [19](Sampah *et al.*, 2015). [20](Shanmugasundaram *et al.*, 2016). [21](Haddad *et al.*, 2020).

1.6.3 *MPA-induced regulation of systemic immunity and HIV-1 infection: potential GR/PR-mediated mechanisms*

Multiple studies have demonstrated that MPA activates both the GR (reviewed in **Section 1.4.2**) and PR (Hapgood, Africander*, et al.*, 2014; Enfield *et al.*, 2020). It is not well understood whether progestins cross-react with the GR, potentially exerting regulatory effects on systemic immune function and HIV-1 infection. Previous *ex vivo* studies that used the GR/PR antagonist RU486 to investigate the role of the GR reported that MPA most likely exerts immunosuppressive effects and increases HIV-1 infection through mechanisms most likely involving the GR in PBMCs (Huijbregts *et al.*, 2013; Hapgood, Ray*, et al.*, 2014; Bick *et al.*, 2022). However, it is challenging to draw conclusions from these results as RU486 is not a GR-specific antagonist and can reduce the effects of both the GR and PR. Furthermore, considering that PBMCs consist of a mixed population of cell types expressing varying levels of several SRs, including the AR, ER, and MR, as well as both the GR and PR, it remains unclear whether the GR-mediated effects observed *ex vivo* occur *in vivo* (Hapgood, Ray*, et al.*, 2014; Bick *et al.*, 2022).

Currently, studies examining SR expression in PBMCs report contrasting findings. The variability in the available literature reflects experimental differences, including the use of whole versus sorted PBMCs, as well as inter-individual variations, which encompass relative differences in PBMC cell subtypes. Furthermore, few studies compare SR mRNA and protein expression within the same study, with only one

published study on SR expression conducting parallel assessments that include AR, ER, GR, MR, and PR in PBMCs (Tomasicchio *et al.*, 2013).

Overall, some studies have shown that whole PBMCs express detectable mRNA levels of ER, GR, MR (Tomasicchio *et al.*, 2013) and PR (Polikarpova *et al.*, 2019). In contrast, some studies reported that PBMCs only express detectable levels of GR, but not AR, ER, MR or PR protein (Tomasicchio *et al.*, 2013); whereas, others found detectable levels of ER and PR protein (Asin *et al.*, 2008; Cabrera-Munoz *et al.*, 2012). The literature on SR expression levels within PBMC cell subtypes is limited. The available data suggest that $CD4^+$ and $CD8^+$ T cells, as well as $CD14^+$ monocytes, only express detectable levels of ER and GR mRNA (Brundin *et al.*, 2021); however, low levels of PR protein were detected in $CD4^+$ and $CD8^+$ T cells (Arruvito *et al.*, 2008). Considering the differences in SR expression between whole PBMCs and PBMC immune cell populations, it is likely that SR-mediated responses in whole PBMCs and select PBMC cell subtypes will differ and potentially involve various SRs. Despite the detection of ER mRNA and protein, it is unlikely that the ER plays a role in mediating progestin activity (as reviewed in **Section 1.3.3**). Considering the absence of detectable MR protein, these findings collectively suggest that whole PBMCs express GR, low levels of PR, and negligible amounts of ER protein.

Taken together, the available data, albeit limited, suggest that GR, and to a lesser extent PR, likely mediate progestin-induced regulation of immune function and HIV-1 infection in PBMCs. However, further research is necessary to confirm these

findings. Notably, the expression of GR across PBMC cell subtypes remains undetermined. To better understand the SRs most likely involved in regulating systemic immune function and HIV-1 infection induced by select progestins, additional studies characterising the expression profiles of GR and PR in PBMC cell subtypes are required.

1.7 Progestin metabolism

Progestins used in contraceptives are subject to various metabolic processes influencing the levels of circulating progestins that can elicit biological effects (Bick *et al.*, 2021b). Progestins can be metabolised into numerous metabolites by enzymes in different metabolic pathways (Schindler *et al.*, 2008; Skosana *et al.*, 2019; Bick *et al.*, 2021b). Beginning with the route of administration, the liver is the primary location for the metabolism of parenterally administered progestins. Still, progestin metabolism most likely occurs in other tissues or even commences at the administration site (Stanczyk, 2003; Africander, Verhoog, and Hapgood, 2011; Bick *et al.*, 2021b). The pharmacokinetics of progestins have been described in a limited number of clinical and animal studies (Edelman, Cherala, and Stanczyk, 2010; Prasad *et al.*, 2010; Ravinder *et al.*, 1997; Hümpel *et al.*, 1978; Lørber *et al.*, 1981). However, these studies do not provide insight into whether progestin metabolism is cell- or tissue-specific, nor do they elucidate the implications thereof.

SR activation is known to be influenced by the concentration of circulating progestin (Africander, Verhoog, and Hapgood, 2011); however, the effect of progestin metabolism on SR activity is poorly understood. While multiple *in vitro* studies have investigated SR transcriptional activity through concentration-dependent experiments across various cell models, most studies do not account for potential changes in progestin levels due to metabolism. A key consideration in such studies is whether commonly used cell lines actively metabolise progestins. Metabolic activity can alter the dose-response relationship, affect RBAs, and shift EC_{50} values

to the right, leading to an apparent reduction in reported relative EC_{50} and/or efficacy. Without evaluating metabolism within the specific cell models, it is difficult to determine whether an observed loss of SR activity is due to rapid metabolic activity or an inherent absence of receptor activation.

Overall, progestin metabolism is an underexplored but potentially critical determinant of SR activation and biological outcomes. The literature suggests that metabolism varies across tissues and model systems, likely altering both the magnitude and nature of progestin signalling. Further research is needed to determine if progestin metabolism occurs in standard model systems that characterise progestin-induced SR activity and assess its potential impact on receptor activation outcomes.

1.8 Thesis rationale and aims

The recent ECHO trial findings did not detect substantial differences in HIV-1 risk between DMPA-IM, LNG implant, and CU-IUD (Evidence for Contraceptive and Consortium, 2019), however, evidence from several clinical studies suggests that MPA, administered as DMPA-IM, may be associated with an increased risk of HIV-1 acquisition (Heffron *et al.*, 2012; Polis *et al.*, 2014; Morrison *et al.*, 2015; Ralph, Gollub, and Jones, 2015; Polis *et al.*, 2016). Fewer studies have been conducted to assess the potential risk of HIV-1 acquisition associated with NET, administered as NET-EN; however, limited clinical findings indicate that there is no association between NET-EN use and an increased risk of HIV-1 acquisition (Ralph, Gollub, and Jones, 2015; Noguchi *et al.*, 2015; Morrison *et al.*, 2015). It is hypothesised that the differences in the risk of HIV-1 acquisition most likely result from DMPA-IM, but not NET-EN, exhibiting off-target GC-like activity. Literature on the GC-like effects or potential risk of HIV-1 acquisition associated with LNG-implant or -IUD use is limited (Curtis *et al.*, 2020; Wall *et al.*, 2015; Lavreys *et al.*, 2004), while no clinical findings are available on ETG- and NES-implant and -IVR use.

Several *ex vivo*, and *in vitro* studies have examined the GR-mediated effects of MPA, with limited information available on NET (Koubovec *et al.*, 2004; Koubovec *et al.*, 2005; Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009; Govender *et al.*, 2014; Hapgood, Ray*, et al.*, 2014; Bamberger *et al.*, 1999; Maritz *et al.*, 2018; Ray *et al.*, 2019; Bick *et al.*, 2022), and even fewer studies on the potential GC-like

actions of LNG, ETG and NES (Fuhrmann, Slater, and Fritzemeier, 1995; Kumar *et al.*, 2000).

The available data on the GR-mediated actions of progestins present contrasting findings and cannot be reliably compared. Some *in vitro* studies are conducted across various model systems or investigate only one or two progestins in parallel. Studies designed to examine the GR-mediated effects of progestins in parallel and within the same model system are necessary to characterise the potential GC actions of progestins accurately. The first central hypothesis of this study addresses some of these deficiencies.

First hypothesis: **Progestins display varying binding affinities for the GR and induce gene-specific transcriptional effects via the GR.**

Aim 1: To investigate the RBAs of the selected progestins for the GR compared to dexamethasone (DEX), a synthetic GR agonist. Competitive binding assays were performed in COS-1 cells exogenously expressing the GR. The cells were incubated with [^{3}H] DEX in the presence of increasing MPA, NET, LNG, ETG and NES concentrations.

Aim 2: To investigate progestin-induced activity via the GR, dose-response analyses were performed to determine the relative efficacy and EC_{50} values for LNG, ETG, and NES in parallel with MPA and NET, on synthetic and endogenous GRE-promoters. Promoter-reporter assays were conducted in COS-1 cells exogenously

expressing the GR to characterise the progestin activity for transactivating a synthetic GRE-promoter construct relative to DEX. The progestin-induced responses on GILZ and IL-6 mRNA levels were examined using qRT-PCR in COS-1 cells, HeLa cells and PBMCs.

Aim 3: To determine GR or PR involvement in the potential progestin-induced effects on GR target gene mRNA levels in PBMCs. Progestin-induced effects on GILZ and IL-6 mRNA levels were investigated using qRT-PCR in PBMCs stimulated with MPA, NET, LNG, and NES, in the absence and presence of RU486, a GR/PR antagonist.

Aim 4: To determine whether a relationship exists between progestin activity and affinity for the GR. Pearson correlation analyses assessed the relationship between progestin RBA for the GR and the progestin-induced GR EC_{50}, and efficacies on synthetic and endogenous GRE-promoters in COS-1 cells and PBMCs.

Several clinical studies suggest that DMPA-IM use is likely to be associated with a 40%–50% increased risk of HIV-1 acquisition (Heffron *et al.*, 2012; Polis *et al.*, 2014; Morrison *et al.*, 2015; Ralph, Gollub, and Jones, 2015; Polis *et al.*, 2016), a finding supported by *ex vivo* and *in vitro* studies demonstrating that MPA significantly enhances HIV-1 infection (Huijbregts *et al.*, 2013; Sampah *et al.*, 2015; Maritz *et al.*, 2018; Ray *et al.*, 2019; Bick *et al.*, 2022). Based on this evidence, some studies propose that MPA may facilitate HIV-1 acquisition by altering immune mediator

levels and increasing the frequency of HIV-1 target cell populations (Blish and Baeten, 2011; Murphy, Irvin, and Herold, 2014; Hapgood, Kaushic, and Hel, 2018).

Currently, limited data are available on the regulatory effects of progestins on systemic immune function (Huijbregts *et al.*, 2013; Huijbregts, Michel, and Hel, 2014; Hapgood, Ray*, et al.*, 2014; Piccinni *et al.*, 2019; Haddad *et al.*, 2020; Achilles *et al.*, 2020). The gap in the available data is the absence of studies that accurately evaluate the regulatory effects of MPA and NET in parallel with LNG, ETG, and NES on immune function genes and soluble immune mediators. Furthermore, there are no *ex vivo* data available comparing the MPA-induced increase in HIV-1 infection with that of LNG, ETG, and NES. Furthermore, the current study investigates the potential involvement of the GR in the progestin-induced effects on HIV-1 infection. However, the antagonist employed in this study diminishes both GR and PR effects.

Considering that the GR and PR also recognise similar DNA target sequences, and some progestins bind to and activate both steroid receptors, therefore, it is crucial to examine the expression profiles of both the GR and PR in model systems that may express both SRs. The second central hypothesis of this study addresses some of these deficiencies.

Second hypothesis: **MPA, ETG, and NES differentially regulate select immune gene mRNA and soluble immune mediator levels, as well as HIV-1 infection in PBMCs *ex vivo*, potentially through GR-dependent mechanisms.**

Aim 1: To investigate whether LNG, ETG and NES, in parallel with MPA and NET, regulate immune gene mRNA, and secreted immune mediator protein levels in PBMCs. PBMCs isolated from female donors were incubated with 100 nM of the panel of progestins in parallel with DEX for 48 hours. Following RNA isolation, IFNγ, CD4 and CCR5 mRNA levels were measured and quantified using qRT-PCR.

IFNγ was selected because it plays a critical role in innate and adaptive immunity against viral infections, including HIV-1, by inhibiting viral replication and enhancing immune cell activation (Dighe, Farrar, and Schreiber, 1993; Haase, 2005). CD4 and CCR5 expression were assessed because $CD4^+$ T cells serve as primary HIV-1 targets, while CCR5 functions as a co-receptor that R5-tropic HIV-1 strains utilise for cellular entry (Swanstrom and Coffin, 2012; Rottman *et al.*, 1997).

The protein levels of secreted G-CSF, IFNγ, IL-10, IL-1β, IL-5, IL-13, TNFα, IL-1α, IL-6, and IL-17 were determined using a Multiplex Luminex assay. These cytokines were selected because some are crucial in regulating immune responses, while others are associated with increased susceptibility to HIV-1 acquisition (Akdis *et al.*, 2011; Arnold *et al.*, 2016; Ngcobo *et al.*, 2022).

Aim 2: To determine whether NET, LNG, ETG and NES, in parallel with MPA, regulate HIV-1 infection via GR- or PR-dependent mechanisms in PBMCs. PBMCs were treated with progestins and/or RU486, infected with R5-tropic $HIV\text{-}1_{BaL\_Renilla}$, then harvested to assess infection levels by measuring luciferase activity.

Aim 3: To determine relative GR and PR expression across immune cell types in PBMCs and ectocervical tissue explants. The relative expression of GR and PR was investigated in PBMC cell subtypes ($CD3^+$, $CD4^+$, $CD8^+$ and $CD14^+$). The relative expression of CD4, GR and PR were measured in epithelial and stromal cells of ectocervical explants using immunofluorescence with confocal microscopy.

Progestins undergo various metabolic processes in the body. Understanding whether progestins are metabolised in a cell- or tissue-specific manner may provide insight into the effect of progestin metabolism on SR activity. Numerous studies have investigated the transcriptional effects of progestins via multiple SRs. Still, none have explored progestin metabolism in the model systems of choice or assessed the potential effects of metabolism on progestin-induced transcriptional responses. The third central hypothesis of this study addresses some of these deficiencies.

Third hypothesis: **Progestins are differentially metabolised within and across different model systems, and the extent of metabolism correlates with the biocharacter of the progestin.**

Aim 1: To determine whether progestins are metabolised in COS-1 cells, HeLa cells, and PBMCs. Progestin metabolism in these models was studied using a validated UHPSFC-MS/MS method, and progestin levels in the cells were compared to those in the absence of cells.

Aim 2: To determine the relationship between the extent of progestin metabolism, and progestin RBA for the GR, and progestin-induced GR efficacy or EC_{50} on select GR-regulated genes, Pearson correlation analyses were conducted to assess correlation.

Chapter 2

Materials and Methods

The present author conducted all experimental work for this thesis. Support was provided as follows: Dr Chanel Avenant (co-supervisor) provided intellectual, experimental and technical support when preparing cells for flow cytometry experiments and data analysis. Dr. Michele Tomasicchio at the Lung Infection and Immunity Unit (University of Cape Town) performed flow cytometry analysis on fixed cells prepared by the author. For immunofluorescence, tissue samples were processed by Gcina Dlamini (research assistant), and Assoc. Prof Dirk Lang at the Confocal and Light Microscope Unit (University of Cape Town) analysed immunofluorescence slides prepared by the present author. Luminex® samples were prepared by the author and Luminex assays were performed by Candice Snyders at the SUN Immunology Research Group, Stellenbosch University.

2.1 Biosafety and ethics statement

This project is part of a larger study investigating the mechanisms by which steroid receptors, hormones, and contraceptives modulate HIV-1 pathogenesis in cell lines, primary human cells or tissues, and human clinical samples. Ethics approval from the Human Research Ethics Committee at the University of Cape Town was obtained for both studies: HREC ref# 210/2011 and HREC ref# 682/20211. All blood and tissue sample donors provided written informed consent.

This study adhered to the biosafety procedures established by the Health and Safety Committee of the Faculty of Science at the University of Cape Town: BSC021_2022.

2.2 Inducing compounds

The following compounds were obtained from Sigma-Aldrich, South Africa: (11β,6α)-9-Fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione (dexamethasone; DEX, catalogue #D4902); 6α-methyl-17α- hydroxy-progesterone acetate (medroxyprogesterone acetate; MPA, catalogue #M0250000); 17α-Ethynyl-17β-hydroxy-19-nor-4-androsten-3-one (norethindrone; NET, catalogue #BP266); 13β-Ethyl-17α-ethynyl-17β-hydroxygon-4-en-3-one (levonorgestrel; LNG, catalogue #1362602); 1-methylene-17α-ethynyl-18-methyl-19-nortestosterone (etonogestrel; ETG, catalogue #SML0356); 17-acetyl-13-methyl-16-methylidene-3-oxo-2,6,7,8,9,10,11,12,14,15-decahydro-1H-cyclopenta[a]phenanthren-17-yl acetate (nestorone; NES, catalogue #SML0550); 11β-(4-Dimethylamino)-phenyl-17β-hydroxy-17-(1-propynyl)-estra-4,9-dien-3-one (mifepristone, RU486, catalogue #M8046); 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT, catalogue #M5655); Bovine serum albumin (BSA, R&D Systems, USA, catalogue #DY995). Interleukin 2 (IL2) was obtained from Gentaur, Belgium (catalogue #04RHIL2-08E02). Deuterated testosterone-16,16,17-D3 (T-d3) used as an internal standard for the ultra-high performance supercritical fluid chromatography–tandem mass spectrometry (UHPSFC-MS/MS) was purchased from Toronto Research Chemicals (North York, ON, Canada). [^{3}H]-Dexamethasone (78 Ci/mmol) was

obtained from AEC-Amersham, South Africa (catalogue #NET1192001MC) and was used as a final concentration of 20 nM diluted in serum-free, phenol-red free media. All ligands were prepared in absolute ethanol (EtOH) and added to the cells, with the final [EtOH] being 0.1% (v/v).

2.3 Cell culture

The African green monkey kidney fibroblast (COS-1, catalogue #CRL-1650) was purchased from the American Type Culture Collection (ATCC, USA). The human cervical carcinoma cell line HeLa was obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme Inc. (ARP, NIH, USA).

Cells were maintained at 37°C in a water jacket incubator (90% humidity and 5% CO2) at the Biosafety Level 2 (BSL2) Mammalian Tissue Culture Facility in the Department of Molecular & Cell Biology at the University of Cape Town. All cells were cultured in 75 cm^2 flasks (Greiner Bio-one International, Austria) in Dulbecco's modified Eagle's medium (catalogue #D0819, DMEM, Sigma Aldrich, South Africa) supplemented with 1 mM sodium pyruvate (catalogue #P5280, Sigma Aldrich, South Africa), 44 mM sodium bicarbonate (catalogue #144-55-8, Sigma Aldrich, South Africa), 10% (v/v) foetal calf serum (FCS, catalogue #26140, Thermo Scientific, South Africa) and 100 IU/mL penicillin and 100 mg/mL streptomycin (catalogue #15140122, Gibco, Invitrogen, UK) (from here on termed full DMEM). For sub-culturing, cells were washed with warm 1X phosphate-buffered saline (PBS,

catalogue #P4417, Sigma Aldrich, South Africa) and incubated at 37°C with 2 mL 0.25% trypsin/0.1% EDTA in PBS (catalogue #252000056, Highveld Biological, South Africa) for 5 minutes. Trypsin was neutralised by adding full DMEM containing 10% fetal FCS.

Cells were regularly tested for mycoplasma by Hoechst staining and fluorescence microscopy (Freshney, 1987) in the Department of Molecular and Cell Biology, University of Cape Town. Only Mycoplasma-negative cells were used in this study.

2.4 Plasmids

The luciferase reporter gene plasmid, pTAT-GRE-E1b-LUC (TAT-GRE-LUC), containing two glucocorticoid response elements (GREs) from the rat tyrosine amino transferase (TAT) gene under the control of the E1b promoter was a gift from G. Jenster (Erasmus University of Rotterdam, Rotterdam, Netherlands) (Jenster *et al.*, 1997). The empty vector, pcDNA3.1, containing a cytomegalovirus promoter and no downstream DNA sequence, was obtained from Invitrogen, UK (catalogue #V79020). The steroid receptor plasmid for the GR, pcDNA3-hGR, containing full-length untagged human GRα cloned into the pcDNA3 vector, was a gift from D. W. Ray (University of Manchester, UK) (Ray *et al.*, 1999) An R5 infectious molecular clone, HIV-1_{BaL}, with a luciferase gene inserted adjacent to the *env* gene in the HIV-1 NL4-3 backbone, known as NL–LucR.T2A–BaL.ecto, was kindly gifted by Dr. Christina Ochsenbauer (Edmonds *et al.*, 2010). For this study, this clone was named HIV-$1_{BaL\_Renilla}$.

2.4.1 *Plasmid transformation and purification*

Plasmids were transformed into *Escherichia coli* DH5α cells using heat shock as previously described (Sambrook and Russel, 2006). Competent DH5α cells (100 µL) were incubated with 10 ng plasmid DNA on ice for 30 minutes, at 42°C for 2 minutes and on ice for 2 minutes. Cells were subsequently mixed into 900 µL Luria broth (LB, 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl) and incubated at 37°C for 1 hour with shaking. Transformed cells were plated onto LB-agar plates (1% (w/v) tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar) containing 100 µg/mL final concentration of the antibiotic ampicillin (catalogue no. 10835242001; Sigma Aldrich, South Africa) and incubated overnight at 37°C. Day cultures of 5 mL LB containing 100 µg/mL ampicillin were inoculated into single colonies and incubated at 37°C for 8 hours with shaking. Plasmid glycerol stocks were prepared by mixing 500 µL of 70% (v/v) glycerol with 500 µL of the transformed cell suspension and stored at -80°C. Overnight cultures of 200 mL LB containing 100 µg/mL ampicillin were inoculated with 200 µL day culture cell suspension and incubated at 37°C for 16 hours with shaking. Plasmid DNA was purified using the Nucleobond Xtra Midi kit for transfection-grade plasmid DNA (catalogue no. NC0389369, Thermo Scientific, USA) according to the manufacturer's instructions. The purity and yield of the plasmid preparations were assessed using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies).

2.4.2 *Restriction enzyme digest*

The supercoiled conformation of plasmid DNA was assessed by agarose gel electrophoresis and compared with the linearised plasmid after restriction enzyme digestion. According to the manufacturer's instructions, reactions containing 300 ng DNA, 1 unit (U)/µL restriction enzyme (or equivalent volume of water for the undigested control), and 1X FastDigest universal buffer containing sample application dye (Fermentas, Thermo Scientific, USA) were incubated for 10 minutes at 37°C. Digested and undigested samples were separated by electrophoresis on a 0.8% (w/v) 1X Tris-Acetate-EDTA (TAE) agarose gel containing 10 µg/mL ethidium bromide (catalogue no. E1510, Sigma Aldrich, RSA). Samples were subsequently visualised under ultraviolet light on a Syngene G: Box (Vacutec, England), and images were acquired using GeneSnap version 7.08 (SynGene, England). The identity of the plasmids was assessed by restriction enzyme digestion pattern.

2.4.3 *Plasmid transfection*

For promoter-reporter assays, $1.5x10^6$ COS-1 cells were seeded into 10 cm dishes (Greiner Bio-One International, Austria) in full DMEM. After a 24-hour incubation, to ensure consistent transfection efficiency, cells were transiently bulk-transfected with 10 µg pcDNA3-hGR or the empty vector pcDNA3.1, as a negative control, and 3.75 µg pTAT-GRE-E1b-LUC using XtremeGENE9 (catalogue #06365787001; Roche, South Africa) according to the manufacturer's instructions. Where endogenous GR-regulated mRNA expression was determined, cells were transfected as described

above, excluding pTAT-GRE-E1b-LUC. For competitive binding assays, COS-1 cells were transfected with 5 µg pcDNA3-hGR or the empty vector pcDNA3.1.

2.5 Promoter-reporter assays

COS-1 cells were seeded as described previously (Komane *et al.*, 2022) at 1.5 x 10^6 cells in 10 cm dishes and allowed to adhere overnight. Cells were transiently bulk-transfected with the GR expression vector pcDNA3-hGR or pcDNA3.1 and luciferase reporter pTAT-GRE-E1b-LUC as specified above. After 24 hours, cells were incubated in trypsin and seeded into 96-well plates (Greiner Bio-One International, Austria) at a density of 1 x 10^4 cells per well in 200 µL full DMEM and allowed to adhere overnight. Following a 24-hour incubation with either DEX, MPA, NET, LNG, ETG, NES or 0.1% (v/v) EtOH (Control – Ctrl) in serum free (SF) media, cells were washed with warm 1X PBS. Thereafter, cells were washed with ice cold 1x PBS and harvested in 25 µL 1X luciferase reporter lysis buffer (catalogue #PRE1501, Promega, USA) by shaking on a rotating shaker followed by a freeze-thaw step at -20 ^oC. Luciferase activity was assessed using a Luciferase Assay System (catalogue #PRE1501, Promega, USA). Relative light units (RLU) in 10 µL cell lysate after the addition of 50 µL luciferin substrate in white 96-well plates (Greiner Bio-One International, Austria) were determined using a Modulus microplate luminometer (Turner Biosystems, USA). Luciferase activity was calculated as RLU relative to the total protein content per well, as determined spectrophotometrically using the Bradford assay (Bradford, 1976). Thereafter, all data were expressed relative to the DEX maximal response set as 100% for dose responses. Data were analysed using the nonlinear regression model with three-parameters [log (agonist) vs response] with the hill slope set to 1 using Prism version 9. When only one concentration was investigated, DEX was used as the reference

ligand for maximum activity, and all data were calculated relative to the reference ligand set as 100%.

2.6 Whole cell ligand binding

Whole cell binding assays were performed as previously described (Komane *et al.*, 2022). COS-1 cells were seeded at 1.5 x 10^6 cells in 10 cm dishes and allowed to adhere overnight. Cells were transiently bulk-transfected with 5 μg pcDNA3-hGR or pcDNA3.1, as specified above. After 24 hours, cells were incubated in trypsin and seeded into 24-well plates (Greiner Bio-One International, Austria) at a density of 5 x 10^4 cells per well in phenol red-free full DMEM. The following day, the cells were washed with PBS and incubated for 3 hours at 37°C with 20 nM [^{3}H]-Dexamethasone (78 Ci/mmol) in the presence of 0.1% EtOH or increasing concentrations of unlabelled competitor compound in antibiotic-free, phenol red-free and serum-free media. Working on ice at 4°C, cells were washed three times with ice-cold PBS containing 0.2% (w/v) bovine serum albumin (catalogue #A9418, Sigma Aldrich, RSA) for 15 minutes. Cells were lysed in 1X lysis buffer. The cell lysate was transferred to scintillation vials containing 1 mL scintillation fluid (Flo-ScintTM II, Perkin Elmer catalogue #6013529). Total binding ([^{3}H]-Dexamethasone only) was determined by scintillation counting using the Beckman Tri-Carb 2810 Beta-scintillation counter (Perkin Elmer machine purchased from Separations Scientific) which uses Quantasmart software (Perkin Elmer product purchased from Separations Scientific) and expressed as 100%. Specific bound [^{3}H] DEX was calculated as the difference between the total and nonspecific binding ([^{3}H] DEX

plus 10 μM unlabelled DEX). All values were normalised against total protein concentration as determined by the Bradford assay (Bradford, 1976). Relative binding affinities (RBAs) (%) were calculated as follows: [IC_{50} value of DEX (M) / IC_{50} value for each ligand (M)] x 100, with the DEX IC_{50} value set as 100%.

2.7 PBMC isolation and culture

All PBMCs were obtained from female donors only and were isolated from whole blood, as previously described (Tomasicchio *et al.*, 2013). Buffy packs from healthy female HIV-1-negative donors of reproductive age were obtained from Western Province Blood Transfusion Services (Pinelands, South Africa). PBMCs were isolated by Histopaque (catalogue #H1077, Sigma-Aldrich, South Africa) density centrifugation in Leucosep tubes (catalogue #P1TUB039Z-000050ST, Lasec, South Africa). Cell number and viability were determined by diluting the PBS-suspended PBMCs 1:10 with trypan blue (catalogue #T8154, Sigma-Aldrich, South Africa) and counting on a haemocytometer. PBMCs were added to a freezing media comprised of 20% dimethyl sulfoxide (DMSO, catalogue #D2438, Sigma-Aldrich, South Africa) (v/v) in cs-FCS and stored at -80°C until required.

PBMCs were maintained in high-glucose (4.5 g/mL) RPMI medium (catalogue #R8758, Sigma-Aldrich, South Africa) containing 10% (v/v) cs-FCS (Thermo Scientific, South Africa), 2 mM L-glutamine (catalogue #G7513, Sigma Aldrich, South Africa), 100 IU/mL penicillin and 100 mg/mL streptomycin (catalogue #P4333, Sigma-Aldrich, South Africa) and 30 U/mL final concentration IL-2 (catalogue

#04RHIL2-08E02, Gentaur, Belgium) (from here on termed full RPMI), at 37°C in a humidified incubator containing 5% CO2 at the BSL2+ Mammalian Tissue Culture Facility in the Department of Molecular & Cell Biology at the University of Cape Town.

For experiments, frozen PBMC stocks were thawed and seeded into sterile 50 mL V-bottomed tubes (Greiner Bio-One International, Austria), at a concentration of 1 million cells/mL in 45 mL full RPMI. Cells were incubated overnight, following which PBMCs were pelleted and washed twice by centrifugation at 1200 rpm in 1X PBS supplemented with 1% (v/v) cs-FCS. Cells were seeded into 12 mL round-bottomed tubes (catalogue #PGRE163160, Lasec, South Africa) at a density of 2 million cells in 2 mL full RPMI. Subsequently, PBMCs were stimulated with ligands for 48 hours as indicated in figure legends. Thereafter, PBMCs were pelleted by centrifugation at 1200 rpm for 5 minutes and harvested in 400 µL TriReagent® (catalogue #T9429, Sigma-Aldrich, South Africa) for RNA isolation.

2.8 Gene expression assays

2.8.1 *RNA isolation*

RNA was isolated from cell lines and PBMCs using Tri-Reagent® as described previously (Komane *et al.*, 2022). PBMCs pelleted by centrifugation or adherent cells seeded in 12-well plates were incubated in 400 µL Tri-Reagent® for 5 minutes at room temperature. Lysed cells were transferred to microfuge tubes. After adding 80

µL chloroform (catalogue #288306, Sigma Aldrich, South Africa), the tubes were vortexed vigorously for 15 seconds and centrifuged at 20 000 x g at 4°C for 15 minutes. The aqueous phase was carefully removed by pipetting and transferred into a new microfuge tube, into which 200 µL isopropanol (catalogue #I9516, Sigma Aldrich, South Africa) was added. Samples were mixed by gentle inversion and incubated for 10 minutes at room temperature. RNA was pelleted by centrifugation at 4°C for 10 minutes at 20 000 x g. The supernatants were discarded, and the RNA pellet was washed twice with 75 % (v/v) EtOH in diethyl pyrocarbonate (DEPC) catalogue #D4758, Sigma Aldrich, South Africa)-treated water (1:1000). Samples were air-dried, and RNA pellets were resuspended in 12 µL of DEPC-treated water. RNA was precipitated by adding 24 µL 100% (v/v) EtOH and 1.2 µL 3 M sodium acetate (pH 5.5), followed by storage at -80°C for 30 minutes. RNA was pelleted by centrifugation at 20 000 x g for 15 minutes at 4°C, washed in 75 % (v/v) EtOH (in DEPC-treated water), air-dried, and resuspended in 12 µL of DEPC-treated water. Following the clean-up procedure, RNA was quantified by spectrophotometry (NanoDrop Technologies), and integrity was measured by denaturing formaldehyde agarose gel electrophoresis (Sambrook and Russel, 2006). Briefly, 500 ng RNA was mixed with sample loading buffer [12% (v/v) DEPC-treated water, 5% (v/v) bromophenol blue, 7% (v/v) glycerol, 10% (v/v) 10X MOPS buffer (0.2 M MOPS in DEPC-treated water, 0.05 M sodium acetate, 0.01 M EDTA), 17% (v/v) 12.3 M formaldehyde and 49% (v/v) formamide] and 20 µg/mL ethidium bromide Samples were electrophoresed on a 1% formaldehyde agarose gel [70% (v/v) DEPC-treated water, 10% (v/v) 10X MOPS buffer, 20% (v/v) formaldehyde] in 1X MOPS buffer (40 mM MOPS; 10 mM sodium acetate; 1 mM EDTA, pH 8.00) at 65V for 40 minutes.

Samples were visualised under ultraviolet light on a Syngene G: Box (Vacutec, England), and images were acquired using GeneSnap version 7.08 (SynGene, England). RNA was stored at -80°C in 2 X volume EtOH + 1X volume sodium acetate (catalogue #S2889-250g, Thermo Fisher Scientific, RSA) as a precipitate until cDNA synthesis.

2.8.2 *cDNA synthesis*

According to the manufacturer's instructions, cDNA was synthesised from 250 ng of RNA using the Transcriptor First Strand Synthesis cDNA kit (catalogue #04379012001, Roche Applied Science, South Africa). The reverse transcription method is based on anchored oligo d(T) priming. Samples were stored at -20 °C.

2.8.3 *Quantitative real-time PCR (qRT-PCR)*

Relative gene expression was determined by qRT-PCR using 1X SensiMixTM SYBR® no ROX master mix (catalogue #QT650-05, Bioline, USA), forward and reverse primers (sequences and concentrations used are shown in **Table 2.8.3.1**) and 1 µL cDNA template in a 20 µL reaction. Samples were analysed on a RotorGene 3000 qRT-PCR machine (Qiagen, Netherlands). Cycling conditions included an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 8 seconds, annealing (primer-specific annealing temperatures are indicated in **Table 2.8.3.1**) for 10 seconds and elongation for 8 seconds at 72°C. Melting curve analysis and cycle threshold (Ct) determination were

performed using RotorGene software (version 1.7). Quantitative RT-PCR product samples were analysed by 2% agarose gel electrophoresis in 1X TAE at 70 V for 1 hour. mRNA transcript levels normalised to the housekeeping gene GAPDH were calculated using the Pfaffl method (Pfaffl, 2001). The fold-change was calculated relative to the EtOH control (Ctrl) which was set to 1. For dose responses, all data were calculated relative to the DEX maximal response as 100%.

Table 2.8.3.1: Primers used for qRT-PCR reactions, primer sequences, concentrations, annealing temperatures, and product sizes

Primer	Sequence	Final conc	Ta (°C)	Product size (bp)	Reference
GAPDH	F: 5'-TGAACGGGAAGCTCACTGG-3' R: 5'TGTCAGTTGATAAAACCGCTGCC-3'	200 nM	60	307	(Ishibashi et al. 2003)
GILZ	Quantitect Primer catalogue no. QT00091035	1X	60	69	Qiagen, Netherlands
IL-6	F: 5'-TCTCCACAAGCGCCTTCG-3' R: 5'-CTCAGGGCTGAGATGCCG-3'	250 nM	60	193	(Wolf et al. 2002)
IFN γ	F: 5' - AATGCAGGTCATTCAGATGTAGCGG-3' R: 5' - GGATGAGTTCATGTATTGCTTTGCG-3'	250 nM	60	298	(Scott et al. 1999)
CCR5	F: 5' TGGACCAAGCTATGCAGGTG 3' R: 5' CGTGTCACAAGCCCACAGAT 3'	500 nM	55	240	(Maritz et al, 2018)
CD4	F: 5'-GGGGATACAGTGGAACTGACC-3' R: 5'-TCCCAAAGGCTTCTTCTTGAG-3'	500 nM	55	164	(Eszterhas et al. 2011)

2.9 Protein Assays

2.9.1 *Western blotting*

For positive controls, COS-1 cells were seeded into 12-well plates at a density of 1 x 10^5 cells per well and incubated for 24 hours. Thereafter, cells were transiently transfected with 1 µg/well of the GR expression vectors using X-tremeGENE 9 and incubated for 24 hours. Cells were washed in ice-cold 1x PBS, lysed with 50 µL 2 x SDS sample buffer (5 x SDS sample buffer: 100 mM Tris pH 6.8, 5% v/v SDS, 20% v/v glycerol, 5% v/v β-mercaptoethanol, 0.1% w/v bromophenol blue), boiled at 100°C for 10 minutes and stored at -20°C until use. For experiments, following transfection of COS-1 cells with the GR expression vector, as described in **Section 2.4.3**, cells were incubated in trypsin and seeded into 12-well plates at a density of 1 x 10^5 cells per well. Cells were stimulated as indicated in figure legends for 24 hours, after which they were washed in ice-cold 1X PBS, lysed with 50 µL 2 x SDS sample, boiled at 100°C for 10 minutes and stored at -20°C until use. Western blotting was conducted as previously reported (Avenant, Kotitschke, and Hapgood, 2010). Equal volumes of the lysate were loaded onto a 10% SDS-polyacrylamide gel. Samples were separated by electrophoresis at 75 V for 25 minutes and 150 V for 1 hour in 1X running buffer (25 mM TRIS-HCl, 250 mM glycine and 0.1% (v/v) SDS pH 8.4) using a Bio-Rad Mini Protean II electrophoresis system (Bio-Rad, South Africa). Subsequently, the sample was transferred onto a Hybond-ECL nitrocellulose membrane (Amersham, South Africa) for 1 hour at 0.18 A in ice-cold 1X transfer buffer (25 mM TRIS, 200 mM glycine, 20% (v/v)

methanol). Subsequently, nitrocellulose membranes were blocked in 4% (w/v) ECL advance blocking reagent powder (catalogue #GERPN2232, AEC- Amersham, South Africa) in 1X TRIS-buffered saline (50 mM TRIS, 150 mM NaCl, pH 7.6; TBS) containing 0.1% (v/v) Tween (catalogue #P1379, Sigma Aldrich, South Africa) (TBS-Tween; TBST) for 1 hour. The nitrocellulose membranes were incubated in 10 mL of 4% ECL-TBST containing primary antibodies and shaken gently overnight at 4°C. The primary and secondary antibodies used are listed in **Table 2.9.1.1**. Following this, membranes were washed twice in 1X TBST for 5 minutes and once for 10 minutes. Membranes were incubated at room temperature with secondary antibodies diluted in 5% (w/v) skim milk powder in 1X TBST, on a rotating shaker for 1 hour. After three washes in 1X TBST as above, membranes were placed in 1X TBS followed by a 1-minute incubation with Pierce ECL-chemiluminescent western blotting substrate (catalogue #32106, Thermo Scientific, USA). Proteins were visualised by autoradiography using Amersham Hyperfilm™ MP high-performance autoradiography film (catalogue #28906842; AEC-Amersham, South Africa). Densitometric quantification of the film was performed using ImageJ software, version 1.8.0 (NIH, USA).

Table 2.9.1.1: Antibodies used in western blotting assay.

Primary Antibody	Primary Antibody Dilution	Secondary Antibody	Secondary Antibody Dilution	Detected Size (kDa)
GRα (G-5, sc-393232, Santa Cruz Biotechnology)	1:3000	Anti-mouse HRP linked antibody (sc-516102; Santa Cruz Biotechnology)	1:5000	95
GAPDH (0411, sc-47724, Santa Cruz Biotechnology)	1:2000	Anti-mouse HRP linked antibody (sc-516102; Santa Cruz Biotechnology)	1:5000	37

2.9.2 *Fluorescence activated cell sorting (FACS) by flow cytometry*

Fluorescence-activated cell sorting (FACS) was carried out as previously described (Tomasicchio *et al.*, 2013), with a few modifications. PBMCs were thawed and seeded at 3 X 10^6 in 5 mL Falcon tubes (Becton Dickson Scientific, South Africa). Cells were stained with the viability dye ZOMBIE Aqua BV510 (catalogue #423102, Biolegend, USA) for 15 minutes in the dark at room temperature. For extracellular staining, cells were stained with anti-CD3 AF700 (catalogue #300324, Biolegend, USA), anti-CD4 APC Fire 750 (catalogue #300560, Biolegend, USA), anti-CD8 PerCP (catalogue #300922, Biolegend, USA) and anti-CD14 PE-DAZZLE 594 (catalogue #325634, Biolegend, USA) for 30 minutes in the dark at room temperature. After surface staining, PBMCs were permeabilised in

1X permeabilisation solution (catalogue #554714 Becton-Dickinson, USA) for 45 minutes at room temperature in the dark and washed twice with 1X perm wash solution (catalogue #554714 Becton-Dickinson, USA). PBMCs were subsequently stained with either PR-AF488 (D8Q2J) (catalogue #35591, Cell Signalling, USA) or GR-PE (D8H2) (catalogue #19560, Cell Signalling, USA) and incubated for 45 minutes at room temperature in the dark. Cells were washed with 1X PBS and resuspended in 1X Cell Fix solution (catalogue#554714 Becton-Dickinson, USA). Fluorescence minus one (FMO) controls were prepared for all antibodies. Samples were acquired at the Lung Infection and Immunity Unit, Department of Medicine, University of Cape Town, using a BLSRII Becton-Dickinson flow cytometer (Becton-Dickinson, USA), and analysed using FlowJo software version 10.1 (Treestar Inc., Ashland, Oregon, USA). Gates were set using the FMO controls and the gating strategy is shown in **Appendix A, Figure A1**. Results were plotted as frequency (the percentage of total cells), or expression, as median fluorescence intensity (MFI) per number of double-positive cells.

2.9.3 *Luminex®*

PBMC's seeded and stimulated for gene expression assays (section 2.8), were pelleted by centrifugation 1200 rpm for 5 minutes and the supernatants were processed for secreted cytokine and chemokine levels using a Miliplex® Human Cytokine/Chemokine magnetic bead panel (catalogue #HCYTOMAG-60K, Merck, Germany), according to the manufacturer's instructions. Data was obtained on a Luminex 200 pro (Bio-Rad, Germany) at the SUN Immunology Research Group,

University of Stellenbosch (South Africa) that had been validated and calibrated before the analysis. Sample concentrations were determined using the equation generated for each cytokine or chemokine. Relative fold-change in expression was determined by setting the EtOH control (Ctrl) to 1. Analytes that were investigated and the lower level of detection (LOD) included: IL-6 (0.9 pg/mL), TNFα (0.7 pg/mL), IL-10 (1.1 pg/mL), IFNα2 (2.9 pg/mL), IL-1β (0.8 pg/mL), IL-1α (9.4 pg/mL), IL-12p40 (7.4 pg/mL), G-CSF (1.8 pg/mL), IFNγ (0.8 pg/mL), IL-13 (1.3 pg/mL), IL-4 (4.5 pg/mL), IL-5 (0.5 pg/mL) and IL-17 (0.7 pg/mL).

2.10 HIV-1 infection assays

2.10.1 *Preparation of virus and determination of viral titres*

Viral stocks were prepared by transfection of HEK293T cells, as previously described (Pear *et al.*, 1993), with a few modifications. HEK293T cells were seeded at 4 X 10^6 cells per 10 cm dish in full DMEM supplemented with 25 mM HEPES buffer (Lonza, Germany) for 24 h. Cells were transfected with 12 µg HIV-$1_{BaL\text{-}Renilla}$ or control (DMEM) as a negative no-virus control, for 48 hours, using XtremeGENE-9 transfection reagent. The media was collected and passed through a 0.22 µM filter, after which cs-FCS (Thermo Scientific, USA) was added to a final concentration of 12.5%. Aliquots of the viral stock and no-virus control were stored at -80°C.

The viral titres were determined in TZM-bl cells as previously described (Edmonds *et al.*, 2010). Briefly, TZM-bl cells were seeded at 2.5 X 10^4 cells/well in a 96-well plate in full DMEM for 24 hours. The virus was serially diluted 1:5 in full phenol red-free DMEM (Sigma Aldrich, South Africa) for 8 dilutions in a separate 96-well plate. The media was removed from the TZM-bl cells, and the diluted virus, or the no-virus control, was added to quadruplicate wells. After 72 hours the cells were harvested in 70 µL/well Bright-Glo luciferase lysis buffer (Promega, USA) in the dark for 5 minutes. Samples were transferred into a white 96-well plate (Greiner, Germany) and fluorescence in RLU was measured using a luminometer (Modulus microplate, Promega, USA). The viral titre was calculated as log infectious units

(IU)/mL, using the Reed and Muench method (Reed and Muench, 1938). The typical viral titre ranged from 1 X 10^4-4 X 10^5 IU/mL.

2.10.2 *PBMC infection assays*

Owing to the wide inter-individual variability in HIV-1 infection observed in PBMC donors, PBMCs were pre-screened for infectability before hormone (progestin) plus infection assays. After isolation (as described in Section 2.7), aliquots of PBMCs were frozen at -80°C in full RPMI containing 5% cs-FCS (until infectability was determined), while the remaining PBMCs were seeded at 2 X 10^5 cells/well in U-bottom 96-well plates. The following day, the virus was serially diluted as described in the TZM-bl assay above. The media was removed from pelleted PBMCs, and the diluted virus was added in quadruplicate wells. Control cells (no-virus control) underwent mock infection by incubation with diluted media collected from mock-transfected HEK293T cells. After infection for 2 hours at 37°C, the cells were washed three times in 1X PBS containing 1% cs-FCS, resuspended in full RPMI containing IL-2 and incubated for a further 5 days. PBMCs were pelleted by centrifugation at 1200 rpm for 5 minutes and harvested for Renilla luciferase expression (infection) using Renilla luciferin according to the manufacturer's specifications (Promega, USA), with a few modifications. Briefly, PBMCs were resuspended in 70 µL/well 1X reporter lysis buffer with Renilla luciferin after incubation for 10 minutes in the dark at room temperature. Lysed cells were transferred into a white 96-well plate (Greiner, Germany), and fluorescence in RLU was measured using a luminometer (Modulus microplate, Promega, USA).

Infectability was quantified using the Reed and Muench $TCID_{50}$ method (Reed and Muench, 1938), and donors were deemed acceptable if infection reached the 50% endpoint dilution with consistent relative light unit (RLU) signals at least 2.5-fold higher than no virus control across replicate wells. In parallel, cell viability was confirmed using the MTT assay. PBMCs (200 µL/well) were incubated with MTT at a final concentration of 0.5 mg/mL for 2 h at 37 °C, followed by solubilisation in 0.1 N HCl/isopropanol and absorbance reading at 520 nm with background subtraction at 695 nm. Infection values were normalised to MTT optical density to account for viability differences. Relative infection was then calculated by setting vehicle control (EtOH) to 100% infection.

PBMCs meeting these infectability and viability thresholds were then used for subsequent progestin plus infection experiments. This pre-screening ensured that variability in infection rates across donors was minimised.

For experiments, infectable PBMCs were thawed from frozen stock and seeded into sterile 50 mL V-bottomed tubes (Greiner Bio-One International, Austria) at a concentration of 1x 10^6 cells/mL in 45 mL of full RPMI. Cells were incubated overnight, seeded at 2 x 10^6 cells in 12 mL round-bottomed tubes, and stimulated with the indicated ligands for 2 days. PBMCs were infected with 10 IU/mL HIV-$1_{BaL\text{-}Renilla}$ or an equivalent volume of no-virus control for 2 hours at 37°C, followed by three washes in 1X PBS containing 1% cs-FCS. Cells were resuspended in full RPMI containing IL-2. Five days post-infection, cells were seeded into two U-bottom 96-well plates in quadruplicate wells. Cells on the first

plate were pelleted by centrifugation at 1200 rpm for 5 minutes and harvested for Renilla luciferase expression, as described above. Cells on the second plate were analysed for cell viability using the MTT assay. Briefly, cells were incubated with MTT solution (5 mg/mL MTT in PBS, filter-sterilized) to a final concentration of 0.5 mg/mL for 2 hours in an incubator at 37°C. Cells were pelleted by centrifugation at 1200 rpm for 5 minutes, resuspended in 70 µL/well solubilisation solution (0.1 N HCl in isopropanol), and transferred into a new 96-well plate. The absorbance was measured at 595 nm using a spectrophotometer (Thermo Scientific, USA). Infection was calculated as the RLU of each quadruplicate divided by the average absorbance at 595 nm of four quadruplicate wells (RLU/MTT). The relative infection was determined by setting the EtOH control (Ctrl) in the presence of the virus to 100% or as indicated in the figure legend.

2.11 Progestin metabolism

2.11.1 *Cell line and PBMC incubation with ligands*

COS-1 and HeLa cells were seeded at 1 × 10^5 cells in 24-well plates and cells were allowed to adhere for 24 hours. PBMCs were thawed from frozen stock and seeded into sterile 50 mL V-bottomed tubes (Greiner Bio-One International, Austria) at a concentration of 1x 10^6 cells/mL in 45 mL of full RPMI. Cells were incubated overnight and subsequently seeded at 2 x 10^6 cells in 12 mL round-bottomed tubes. For analysis of extent of metabolism, the cells and "no-cells" control (absence of cells/PBMCs) were washed with pre-warmed media and treated with 100 nM ligand or Ctrl (0.1% v/v EtOH). COS-1 and HeLa cells were treated for 24 hours, while PBMCs were treated for 48 hours. Upon treatment with ligand, 500 µL of the ligand- and EtOH-containing media were aliquoted into separate glass tubes and stored at –20°C; this served as the time zero (T0) control. After 24/48 hours, 500 µL aliquots of media were removed from the cells (and no-cell control), transferred into clean glass tubes, and stored at –20°C before extraction.

2.11.2 *Preparation of standards and samples*

An internal standard stock solution, containing 0.01 ng/µL deuterated testosterone-16,16,17-D3 (T-d3), as well as individual stock solutions of the six steroids (DEX, NET, ETG, LNG, MPA and NES) (1 mg/mL) were prepared in absolute EtOH and stored at –20°C until use. These individual steroid stock

solutions were later used to prepare two standard master mixes (1 000 ng/mL and 1 ng/mL) containing all the above-mentioned steroids (except for T-d3) in EtOH. These standard master mixes were subsequently used to prepare standards (1 mL, 0.01–100 ng/mL) by the addition of the appropriate volume of the standard master mix to the appropriate matrix (phenol-red containing DMEM without penicillin-streptomycin or unsupplemented RPMI-FCS).

2.11.3 *Steroid extractions*

Samples and standards were treated with 100 μl internal standard (1 ng final T-d3) and extracted using a 1:3 ratio of sample to methyl-tert-butyl-ether (MTBE) (v/v) (catalogue #20256-1L-F, Sigma Aldrich, RSA). The samples were shaken at 1 000 rpm for 15 minutes before being placed at –80°C for an hour to allow the aqueous phase to freeze. The MTBE layer containing steroids was transferred to a glass tube, and the MTBE was evaporated at room temperature in a fume hood overnight or under a stream of nitrogen gas. The samples were subsequently reconstituted in 150 μL of 50% methanol (v/v methanol and deionised water) and stored at –20°C before analysis.

2.11.4 *Ultra-high performance supercritical fluid chromatography–tandem mass spectrometry (UHPSFC-MS/MS)*

2.11.4.1 *UHPSFC-MS/MS method validation*

This method has been validated as previously described (Skosana *et al.*, 2019)(**Appendix A, Table A1**). Briefly, the limit of detection (LOD) for each steroid was defined as the lowest concentration at which the signal-to-noise (S/N) ratio was greater than 3 for the quantifier ion. The lower limit of quantification (LLOQ) was defined as the lowest concentration at which the S/N ratio was greater than 10 for the quantifier ion and greater than 3 for the qualifier ion. The upper limit of quantification (ULOQ) was defined as the highest concentration at which the % relative standard deviation (RSD) was no higher than 20. Accuracy was defined as % RSD from the analysis of independent replicate samples. Precision was defined as the %RSD of the average calculated concentrations following repeated injections of a single sample. The LOD, LLOQ, precision, accuracy, multiple reaction monitoring (MRM) mass transitions and retention time are summarised in **Appendix A, Table A1**.

2.11.4.2 *Instruments and chromatographic conditions for UHPSFC-MS/MS*

Steroids were separated using an Acquity Ultra High Performance Convergence Chromatography (UPC2) system (Waters Corporation, Milford, USA) with an Acquity UPC2 Ethylene Bridged Hybrid column (3 mm x 100 mm, 1.7 µm particle size) as

previously described (Skosana *et al.*, 2019). The mobile phase consisted of liquid CO2 (mobile phase A) and methanol (mobile phase B; MPB). A 2.5-minute gradient inlet method was used to separate the steroids using a constant flow rate of 1.9 mL/min according to the following protocol: 4% MPB from 0–1 min; 10% MPB from 1–1.5 min; 25% MPB from 1.5–2.5 min and back to 4% MPB at 2.5 min for re-equilibration. The column temperature and automated back-pressure regulator were set to 60°C and 1700 pounds-force per square inch (psi), respectively. The injection volume was 2.0 µL. Quantitative mass spectrometric detection was performed using a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, USA). A make-up pump fed 1% formic acid in methanol into the mixer preceding the MS line at a constant flow rate of 0.2 mL/min. All steroids were analysed in MRM mode using an electrospray probe in the positive ionisation mode (ESI+). The following settings were used: capillary voltage of 3.8 kV, desolvation temperature 350°C, desolvation gas 900 L/h and cone gas 150 L/h. MRM transitions are included in **Appendix A, Table A1**. Data collection and analysis were performed using MassLynx 4.1 (Waters Corporation). Using MassLynx, the area detected under the peak of each ligand was quantified and normalised to the internal standard peak area. Thereafter, the standard curve equation for each ligand (determined by MassLynx) was used to calculate the concentration of the respective ligand in each sample (ng/mL). The concentration of ligand detected in the no-cells control after the incubation period was set to 100% and the concentration of ligand detected in the cells was calculated as a percentage thereof. Thereafter, the % metabolism of all samples was expressed relative to the concentration of the respective no-cells control set to 0% metabolism.

2.12 Immunofluorescence

Ectocervical tissue explants were obtained after informed consent from HIV-1 negative pre-menopausal women undergoing hysterectomy for benign conditions. Explants were collected from Tygerberg Hospital in Cape Town and transported in full RPMI 1640 media containing 10 µg/mL fungizone, 10% charcoal stripped-FCS, 2 mM L-glutamine, 100 U penicillin, and 100ug/ml streptomycin (catalogue #85886 Sigma Aldrich, South Africa) and were processed within 4 hours after surgery. In brief, tissue was washed with 1X PBS, cut and placed into the embedding mould and submerged in tissue freezing media (Catalogue #14020108926 Leica Biosystems, Germany). Tissue was stored at -80°C until sectioning. Frozen tissue blocks were sectioned using a Leica CM1850 Cryotome cryostat at -20°C and 8 µm slices were mounted onto charged Menzel Superfrost slides (Catalogue # 631-9483 Thermo Scientific, South Africa). A research assistant carried out the aforementioned procedure.

Tissue sections on slides were fixed with 2% formaldehyde at room temperature for 15 minutes and washed twice with 1X PBS for 5 minutes. Antigen retrieval was performed by incubating sections with 5% H_2O_2 in methanol for 10 minutes and thereafter washed twice followed by permeabilisation with 0.25% Triton X-100 in PBS for 10 minutes. After three washes tissue sections were blocked with 1% BSA (w/v in PBS-Tween) for 30 minutes at room temperature and incubated with the following dilutions of monoclonal antibodies: 1:250 mouse anti-human GRα (G-5, sc-393232; Santa Cruz Biotechnology), 1:1000 mouse anti-human PR

(NCL-LPGR-312, Leica Biosystems) and 1:200 rabbit anti-human CD4 (AB133616, Abcam). Antibodies were diluted in 1% BSA (w/v in PBS) and incubated in a humidified chamber overnight at 4°C. Sections were washed three times and thereafter incubated with the following dilutions of secondary antibodies: 1:500 Alexa Fluor 488 (Alexa 488)-labelled anti-mouse and 1:1000 Cyanine 3 (Cy3)-labelled anti-rabbit antibodies in 1% BSA (w/v in PBS-Tween) in the dark for 1 hour at room temperature. After three washes, sections were counterstained with 1 µg/mL Hoechst in PBS for 5 minutes, and coverslips were subsequently mounted onto the sections using Mowiol containing N-propyl gallate antifade (catalogue #475904 Merck, South Africa). Slides were dried overnight and stored in the dark at 4°C prior to viewing on a Carl Zeiss LSM 880 confocal microscope. For each tissue donor, a research assistant performed an autofluorescence control (not stained with any antibodies) to determine minimal background fluorescence in the tissue before the above-mentioned experiments (data not shown). For the experiment performed in this study, a secondary antibody control containing both the Alexa 488 anti-mouse and the Cy3 anti-rabbit antibodies, but none of the primary antibodies were included. In addition, single stain controls containing one primary antibody, for example mouse anti-GR, and both secondary antibodies (Alexa 488 anti-mouse and the Cy3 anti-rabbit) were performed. Three single-stain controls were performed, one for each primary antibody (mouse anti-GR, mouse anti-PR and rabbit anti-CD4). All imaging was performed by technicians not involved in the research.

2.13 Data analysis

Data were analysed using GraphPad Prism (version 9) software from GraphPad Software Inc. (La Jolla, California, USA). For binding data, dose-response curves were plotted using non-linear regression with one site – fit logIC_{50} options. Associations between the extent of progestin metabolism and biocharacter were examined using Pearson correlation analysis.

All data were tested for normal distribution and the specific tests used for normality and statistical significance are given in the individual figure legends. In general, for sample sets with an n-value less than 8, the Shapiro-Wilk test was used, and for sample sets with an n-value greater than 8, the D'Agostino-Pearson normality tests were used. Parametric data were analysed for statistical significance with one/two-way ANOVA with Tukey or Dunnett post-tests. Significant differences in non-parametric data were analysed using one-way ANOVA or non-parametric Friedman test with Dunns post-test for multiple comparisons. Mann-Whitney t-tests or two tailed paired t-tests were performed for comparisons. For the binding data, the Newman-Keuls post-test was used. Statistical differences between treatment groups are indicated by "a, b, c, d, e, or f" in multiple comparisons. Treatment groups with different letters indicates statistically significant differences between groups, while treatment groups with the same letter are not significantly different from one another. Significant differences comparing each sample to control are indicated by hashtags where #, ##, ### represent $p<0.05$, $p<0.01$, and $p<0.001$ respectively, while

asterisks where *, **, ***, **** represent $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively, were used to indicate significance between groups.

Chapter 3

Progestins exhibit differential affinity for the GR and gene-specific transcriptional effects via the GR

3.1 Background

Available literature on the GC actions of progestin-only contraceptives report results from several *in* vitro and *ex vivo* studies investigating MPA and/or NET binding affinity for the GR (Kontula *et al.*, 1983; Selman, 1996; Philibert *et al.*, 1999; Koubovec *et al.*, 2005; Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009) and transcriptional activity via the GR (Bamberger *et al.*, 1999; Koubovec *et al.*, 2004; Koubovec *et al.*, 2005; Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009; Hapgood, Ray*, et al.*, 2014; Govender *et al.*, 2014; Louw-du Toit, Hapgood, and Africander, 2014). The limited literature on the LNG and ETG affinity for the GR and transcriptional activity via the GR consists of contrasting findings (Pollow *et al.*, 1992; Fuhrmann, Slater, and Fritzemeier, 1995; Kumar *et al.*, 2000); whereas, only one study has investigated the GC actions of NES (Kumar *et al.*, 2000). Moreover, the available data may be confounded by multiple factors including the fact that not all the progestins were investigated in parallel and different model systems were utilised, which may not have accounted for the presence of competing SR. Given the discrepancies in the limited available data, conducting accurate comparative studies on the GR-mediated progestin effects, in parallel, within the same model

system, will provide more information on whether the progestin-induced off-target activation of the GR may exert significant biological effects.

This chapter will directly compare the RBAs and the relative efficacy and EC_{50} values of MPA, NET, LNG, ETG, and NES on synthetic and endogenous GRE-promoters in COS-1 cells, HeLa cells, and PBMCs using the synthetic GR agonist DEX as a reference ligand.

Considering the progestins demonstrate off-target binding with multiple steroid receptors, the COS-1 cell line was selected as it expresses negligible levels of endogenous steroid receptors including the GR (Koubovec *et al.*, 2005; Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009). HeLa cells are immortalised cervical epithelial cells that endogenously express the GR and are well-established for mechanistic studies (Fichorova, Rheinwald, and Anderson, 1997; Govender *et al.*, 2014). PBMCs were chosen as a more physiologically relevant model system expressing GR mRNA and protein (Tomasicchio *et al.*, 2013; Ray, 2015). PBMCs likely express low levels of PR mRNA and protein, though results vary across studies. While some studies found detectable levels of PR mRNA and protein (Asin *et al.*, 2008; Arruvito *et al.*, 2008; Cabrera-Munoz *et al.*, 2012; Polikarpova *et al.*, 2019), others have reported no detectable PR mRNA and protein expression (Bamberger *et al.*, 1999; Tomasicchio *et al.*, 2013; Ray, 2015; Brundin *et al.*, 2021).

GILZ and IL-6 target genes were selected for this study, as they have been previously investigated and are known to be regulated by GCs and MPA via the GR

(Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009; Govender *et al.*, 2014; Hapgood, Ray*, et al.*, 2014).

Progestin concentrations of 100 nM were employed to allow direct comparison of effects at the same molecular concentration. While this concentration may achieve near-saturating occupancy for high-affinity ligands such as MPA, it is unlikely to fully saturate the GR for progestins with relatively low affinity. Nonetheless, the use of a fixed concentration enables meaningful comparisons between the progestins under standardised conditions. Experimental endpoints were optimised in prior work by the present authors' research group and varied between assays to account for differences in sensitivity, detection methods, and technical constraints. This ensured that each assay was conducted under conditions that maximised the reliability and reproducibility of the measured outcomes.

The present author has published some data from this chapter (Komane *et al.*, 2022).

3.2 Synthetic progestins and the GR: affinity and activity

3.2.1 MPA and ETG exhibit higher affinity for the GR compared to NET, LNG and NES in COS-1 cells

The experimental conditions were first optimised to investigate the RBAs of the progestins, MPA, NET, LNG, ETG, and NES for the GR compared to the GC agonist DEX. The experiment first aimed to investigate the optimal concentration of the radiolabelled ligand and optimise previously determined incubation periods with competing ligands (Koubovec *et al*., 2005). COS-1 cells were transfected with pGR or pcDNA and incubated with 20 nM or 40 nM of [^{3}H]-DEX and 10 μM of the competing ligand for either 90 minutes or 3 hours. Specific binding was calculated as the difference between total binding ([^{3}H]-DEX only) and nonspecific binding ([^{3}H]-DEX + 10 μM unlabelled DEX). More specific binding was observed after a 3-hour incubation period (**Appendix B**, **Figure B1A** and **B**). Higher counts per minute (cpm) values were observed with 40 nM of [^{3}H]-DEX; however, the cpm values obtained with 20 nM [^{3}H]-DEX were adequate for this study. A notable increase in non-specific binding was observed with increasing concentrations of [^{3}H]-DEX, likely due to low-affinity interactions with off-target receptors and other cellular proteins. Under these experimental conditions, the observed binding most likely suggest an additive effect of these non-specific interactions rather than actual ligand displacement.

Next, the optimal transfection conditions and cell densities were established. COS-1 cells were seeded at 0.5 x10^{-5} or 1 x10^{-5} or 2 x10^{-5} cells/well and transfected with 2.5 μg, 5 μg or 10 μg of pGR. The cells were treated for 3 hours with 20 nM of [^{3}H]-DEX and 10 μM unlabelled DEX. More specific binding was observed when the cells were seeded at 2 x10^{-5} cells/well and transfected with 5 μg of pGR (**Appendix B, Figure B1C**). These results indicate that optimal binding conditions in this model system involve a 3-hour incubation using 20 nM [^{3}H]-DEX and transfecting 5 μg pGR in COS-1 cells seeded at 2x10^{-5} cell/well. Western blot analysis was conducted to confirm the expression of the varying concentrations of transfected pGR (**Appendix B**, **Figure B2**).

After establishing the optimal conditions for conducting the binding assays, the next step was to determine whether the progestins bind to the GR, using saturating concentrations of the progestins before proceeding with competitive binding assays. These experiments were conducted both in the absence and presence of exogenously expressed GR to confirm GR involvement. As shown in **Figure 3.2.1.1**, relative to DEX, MPA, NET, LNG, ETG, and NES bind to the GR at high concentrations (10 μM). No significant binding was detected in the absence of exogenous GR; however, the minimal binding observed with DEX in the absence of exogenous GR was considered negligible and may be attributed to low levels of endogenous GR in COS-1 cells.

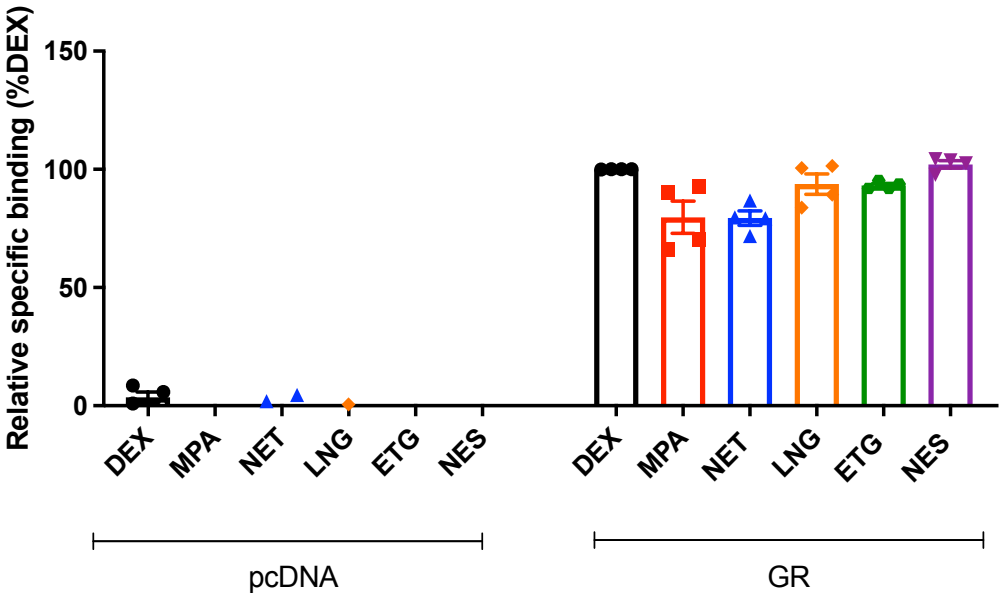


Figure 3.2.1.1: MPA, NET, LNG, ETG and NES bind to the GR. COS-1 cells were transfected with pGR or pcDNA and stimulated for 3 hours with 20 nM [^{3}H] DEX in the absence and presence of 10 μM of competing unlabelled ligands or 0.1% (v/v) EtOH (Ctrl). Counts per minute (cpm) were normalised to the protein concentration. Total specific binding of [^{3}H]-DEX only was expressed as 100% and the binding of the unlabelled competitors was expressed as a percentage relative to this. Relative specific binding is shown for four independent experiments where each condition was performed in triplicate and is shown as mean ± SEM.

To determine the RBA of the progestins for the GR, competitive binding assays were performed in COS-1 cells expressing pGR which were stimulated for 3 hours with 20 nM [3H]-DEX and increasing concentrations (1 x 10^{-10} M – 1 x 10^{-5} M) of unlabelled progestins to compete for binding to the GR (**Figure 3.2.1.2**). RBAs were expressed as a % DEX (reference agonist =100%) and indicated that the progestins exhibit significantly distinct binding affinities for the GR. The rank order from the highest to lowest affinity was DEX > MPA > ETG > NES > LNG > NET (**Table 3.2.1**). When compared with one another, NES, LNG and NET displayed significantly different affinities. Interestingly, no statistically significant differences were observed between the IC_{50} values for the synthetic GR agonist DEX compared to MPA and

ETG; whereas, NES, LNG and NET exhibited significantly lower IC_{50} values compared to DEX. Unlike NET, MPA and ETG displayed substantial % binding at concentrations higher than 1 nM; whereas, NES and LNG exhibit relative % binding at concentrations exceeding 10 nM. The RBA and IC_{50} values for the GR are summarised in **Table 3.2.1**.

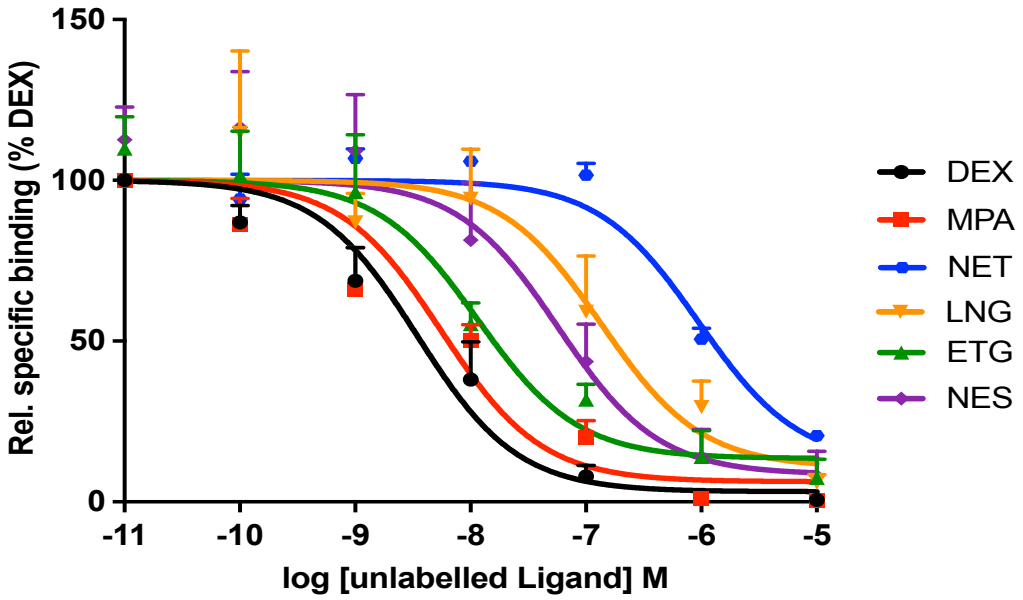


Figure 3.2.1.2: MPA and ETG display similar IC_{50} values to DEX, and higher affinity for the GR than NET, LNG and NES. COS-1 cells were transiently transfected with pGR and treated for 3 hours with 20 nM [^{3}H]-DEX in the absence or presence of increasing concentrations (1 x 10^{-10} M – 1 x 10^{-5} M) of competing unlabelled ligands or 0.1% (v/v) EtOH (Ctrl). Counts per minute (cpm) were normalised to the protein concentration (mg/mL). The data represent six independent experiments plotted as mean ± SEM, where each condition was performed in triplicate. Non-linear regression and one site – fit logIC_{50} options, and the Newman-Keuls post-test was used for statistical analysis. The relative specific binding of [^{3}H]-DEX only was expressed as 100% and the binding of the unlabelled competitors expressed as a percentage relative to DEX.

Table 3.2.1: Table summarising the RBA and IC_{50} values of the progestins via the GR*

Ligand	RBA (%) ± SEM	IC_{50} (M) ± SEM
DEX	100.0 ± 0.00[a]	3.46 ± 1.0 x 10^{-9a}
MPA	62.0 ± 0.50[b]	5.58 ± 2.0 x 10^{-9a}
NET	0.360 ± 0.30[c]	9.50 ± 2.9 x 10^{-7d}
LNG	2.56 ± 0.10[d]	1.35 ± 9.1 x 10^{-7c}
ETG	30.1 ± 0.20[e]	1.15 ± 5.2 x 10^{-8a}
NES	6.03 ± 0.30[f]	5.73 ± 3.6 x 10^{-8b}

*Data shown in Figure. 3.2.1.2, were analyzed to obtain the RBA ± SEM and IC_{50} ± SEM values. RBAs were calculated for each ligand for individual experiments from the IC_{50} (M) values (obtained using GraphPad Prism™ software version 9) and expressed as a percentage relative to DEX (100%) for that experiment. The mean RBA ± SEM values were calculated for each ligand.
[a,b,c,d,e,f] Statistical significance between treatment groups was determined using one-way ANOVAs with Newman-Keuls post-test.

3.2.2 MPA, ETG and NES display partial GR agonist activity for transactivation in COS-1 cells

To characterise progestin-induced transcriptional activity via the GR, relative agonist efficacy and EC_{50} values for transactivating a synthetic GRE-promoter construct (pGRE) were determined in COS-1 cells exogenously expressing the GR.

Initially, progestin activity on the synthetic GRE promoter was investigated using saturating progestin concentrations (10 μM) to ensure that maximal transcriptional activity of the GRE-promoter construct could be observed. To confirm GR involvement, experiments were conducted in the absence and presence of exogenously expressed pGR. Compared to DEX, MPA, ETG, and NES significantly activated the GR compared to DEX, while no significant transactivation of pGRE

was detected for LNG and NET (**Figure 3.2.2.1**). Consequently, LNG and NET were excluded from further promoter-reporter dose response analysis.

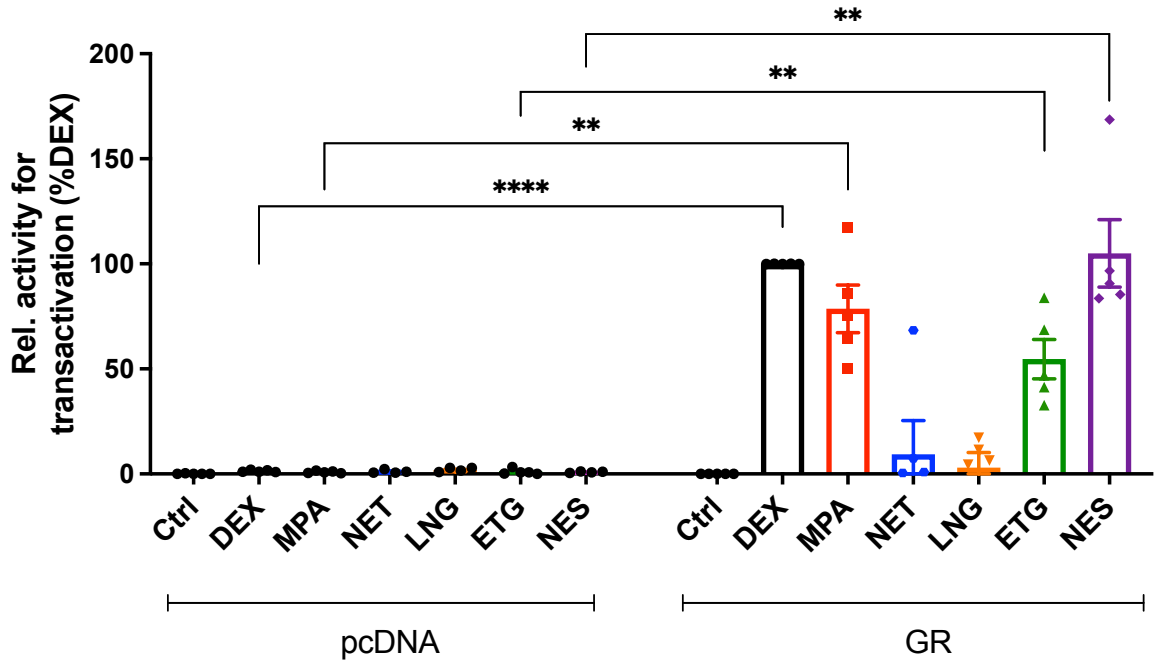


Figure 3.2.2.1: MPA, ETG and NES exhibit transcriptional activity via the GR on a synthetic GRE-promoter construct. COS-1 cells transfected with pGR or pcDNA and pGRE were treated for 24 hours with 10 μM of each ligand or 0.1% (v/v) EtOH (Ctrl). Luciferase activity was measured and normalised to protein concentration. Total activity for transactivation is expressed as DEX (set at 100%) and all other responses were set relative to DEX. The data represent five independent experiments plotted as mean ± SEM and each condition was performed in triplicate. One-way ANOVA and the Tukey (compares all pairs of columns: for this study, only stats comparing the progestins in the presence and absence of GR are shown) post-test were used for statistical analysis. Significant differences are indicated by asterisks where *, **, ***, **** represent $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively.

Next, COS-1 cells exogenously expressing pGR were incubated with increasing concentrations (1×10^{-10} M – 1×10^{-5} M) of DEX, MPA, ETG and NES for 24 hours. Dose-response analysis demonstrated that MPA, ETG and NES display partial GR agonist activity for transactivation of pGRE compared to DEX (**Figure 3.2.2.2A**). Relative to DEX, MPA was significantly more efficacious than ETG and NES;

whereas, ETG and NES exhibit statistically similar efficacies. While there were no detectable differences in potency between DEX and the progestins, MPA and ETG appeared less potent than DEX on the synthetic promoter construct. The relative efficacy and EC_{50} values for transactivation of the GRE-promoter construct via the GR in COS-1 cells are summarised in **Table 3.2.2**.

Next, progestin-induced transcriptional effects on an endogenous GR target gene, GILZ, were investigated. COS-1 cells exogenously expressing GR were stimulated with increasing concentrations (1×10^{-10} M – 1×10^{-5} M) of DEX, MPA, ETG and NES for 24 hours and the GILZ mRNA levels were measured and quantified using qRT-PCR. As shown in **Figure 3.2.2.2B**, MPA, ETG, and NES exhibited partial agonist activity for upregulating GILZ mRNA levels. Relative to DEX, ETG and NES were significantly less efficacious; whereas, MPA displayed statistically similar efficacy as ETG (**Table 3.2.2**). Unexpectedly, DEX, MPA, and ETG displayed statistically similar potencies that were significantly less potent than NES.

Based on the relative efficacy and EC_{50} values observed for the two promoters, the results suggest that MPA, ETG, and NES activate transcription via the GR in COS-1 cells. While these progestins exhibited variable potency, they showed no detectable differences in efficacy across both synthetic and endogenous GRE promoters.

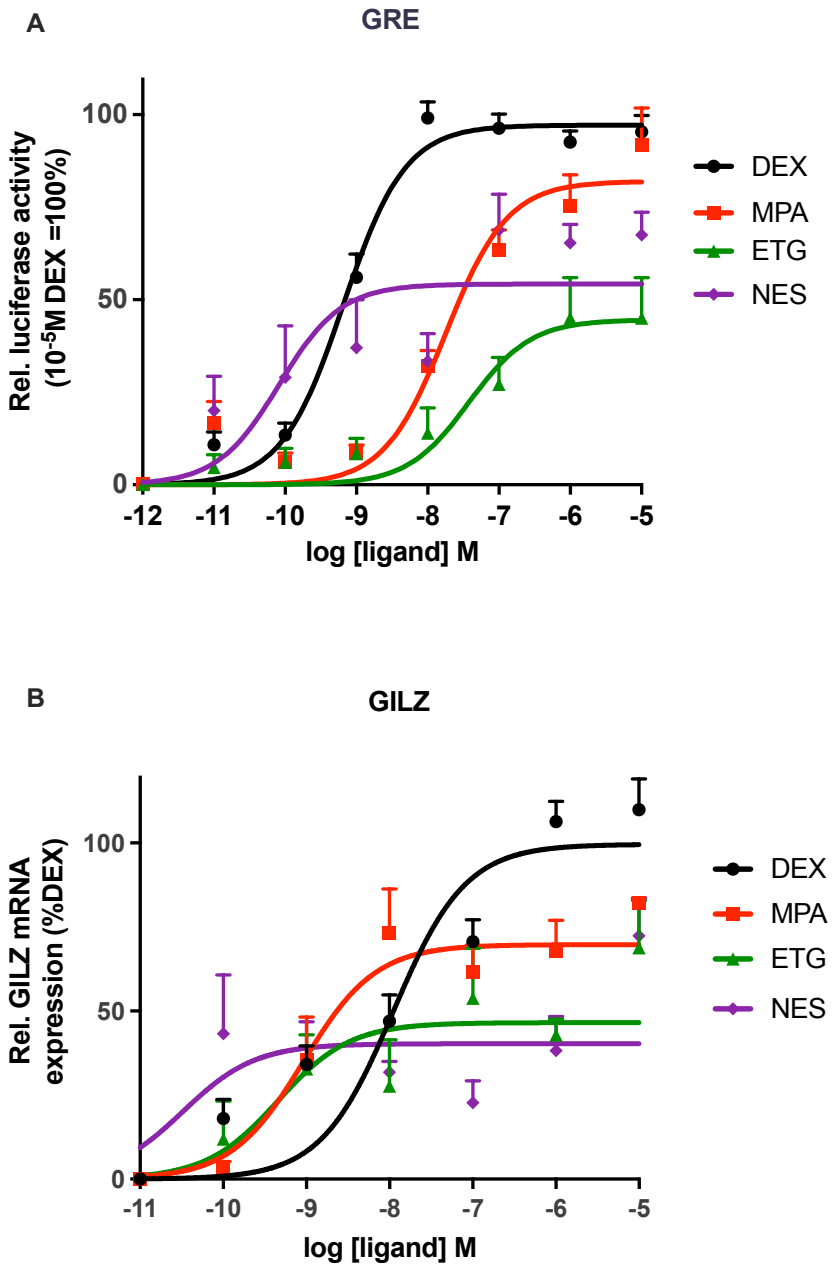


Figure 3.2.2.2: MPA, ETG and NES are partial agonist activity for transactivation of a GRE-promoter construct and GILZ mRNA levels in COS-1 cells. COS-1 cells transfected for 24 hours with (A) pGR and pGRE or (B) pGR only and treated with increasing concentrations (1 x 10^{-10} M – 1 x 10^{-5} M) of each ligand or 0.1% (v/v) EtOH (Ctrl). (A) Luciferase activity was measured and normalised to protein concentration determined using the Bradford method. (B) RNA was harvested, cDNA was synthesised and subjected to qRT-PCR using primer sets specific for GILZ and GAPDH. Levels of GILZ mRNA were normalised to GAPDH levels for each condition. The data represent at least three independent experiments expressed as mean ± SEM and each condition was performed in triplicate. Nonlinear regression and log(agonist) vs. response options were used for statistical analysis. Results are expressed as %

DEX, where the DEX maximal response was set to 100%, and all other responses were set relative to DEX.

Table 3.2.2: Table summarising the progestin-induced transcriptional effects (Rel. efficacy and EC_{50} values) for transactivation of the GRE-promoter construct and GILZ mRNA levels in COS-1 cells*

	GRE		GILZ	
Ligand	**MAX (%) ± SEM**	**EC_{50} (M) ± SEM**	**MAX (%) ± SEM**	**EC_{50} (M) ± SEM**
DEX	100 ± 2.17^{a}	$1.07 \pm 0.55 \times 10^{-9a}$	100 ± 4.86^{a}	$1.21 \pm 1.25 \times 10^{-7a}$
MPA	84.36 ± 3.99^{b}	$4.17 \pm 1.63 \times 10^{-8a}$	72.05 ± 5.42^{b}	$5.63 \pm 9.15 \times 10^{-8a}$
NET	ND	ND	ND	ND
LNG	ND	ND	ND	ND
ETG	45.88 ± 4.97^{c}	$3.19 \pm 1.21 \times 10^{-8a}$	$46.74 \pm 5.91^{b,c}$	$8.45 \pm 15.64 \times 10^{-9a}$
NES	55.81 ± 4.85^{c}	$1.85 \pm 0.77 \times 10^{-8a}$	34.25 ± 4.73^{c}	$2.63 \pm 3.31 \times 10^{-11b}$

*Data shown in Figure. 3.2.2.2A and B, were analyzed to obtain the efficacy, or maximal (MAX) effect as a percentage i.e. MAX (%) ± SEM, relative to DEX set at 100%. The potencies, i.e. EC_{50} (M) ± SEM values for each ligand for the GR are also shown.

a,b,c Statistical significance between treatment groups was determined using one-way ANOVAs with Tukey post-test. ND-not determined.

ND, not determined.

3.2.3 MPA acts as a full agonist; whereas, NES differentially regulates GILZ and IL-6 mRNA levels in HeLa cells

To determine whether the progestins exhibit cell-specific transcriptional effects via the GR, the progestin-induced regulation of endogenous GR target genes, GILZ and IL-6, was characterised in a cell line endogenously expressing the GR.

HeLa cells were incubated with 100 nM of the panel of progestins for 24 hours and GILZ and IL-6 mRNA levels were measured and quantified using qRT-PCR. Like DEX, MPA significantly induced GILZ mRNA levels compared to NET, LNG, and ETG. In contrast, no significant regulation by NES was detected, although a trend toward an increase was observed (**Figure 3.2.3.1A**). Relative to DEX, MPA acted as a full agonist; ETG and NES displayed similar partial agonist activity, yet both were significantly less efficacious than DEX. Interestingly, MPA and NES exhibited statistically similar relative efficacies. The relative efficacy values for the progestin-induced regulation of GILZ and IL-6 mRNA levels in HeLa cells are summarised in **Table 3.2.3**.

Figure 3.2.3.1B illustrates that MPA and NES, like DEX, displayed full agonist activity for repressing IL-6 mRNA levels; however, NET, LNG, and ETG did not induce detectable regulation (**Table 3.2.3**). When comparing relative efficacies, no significant differences in % repression of IL-6 mRNA levels were observed among DEX, MPA, and NES. In contrast, NET, LNG, and ETG demonstrated weak partial agonist activity with significantly less % repression of IL-6 mRNA levels compared

to DEX. These findings suggest that DEX and MPA may exhibit similar GC-like regulatory effects on GILZ and IL-6 genes, whereas MPA and NES display similar repressive effects via the GR.

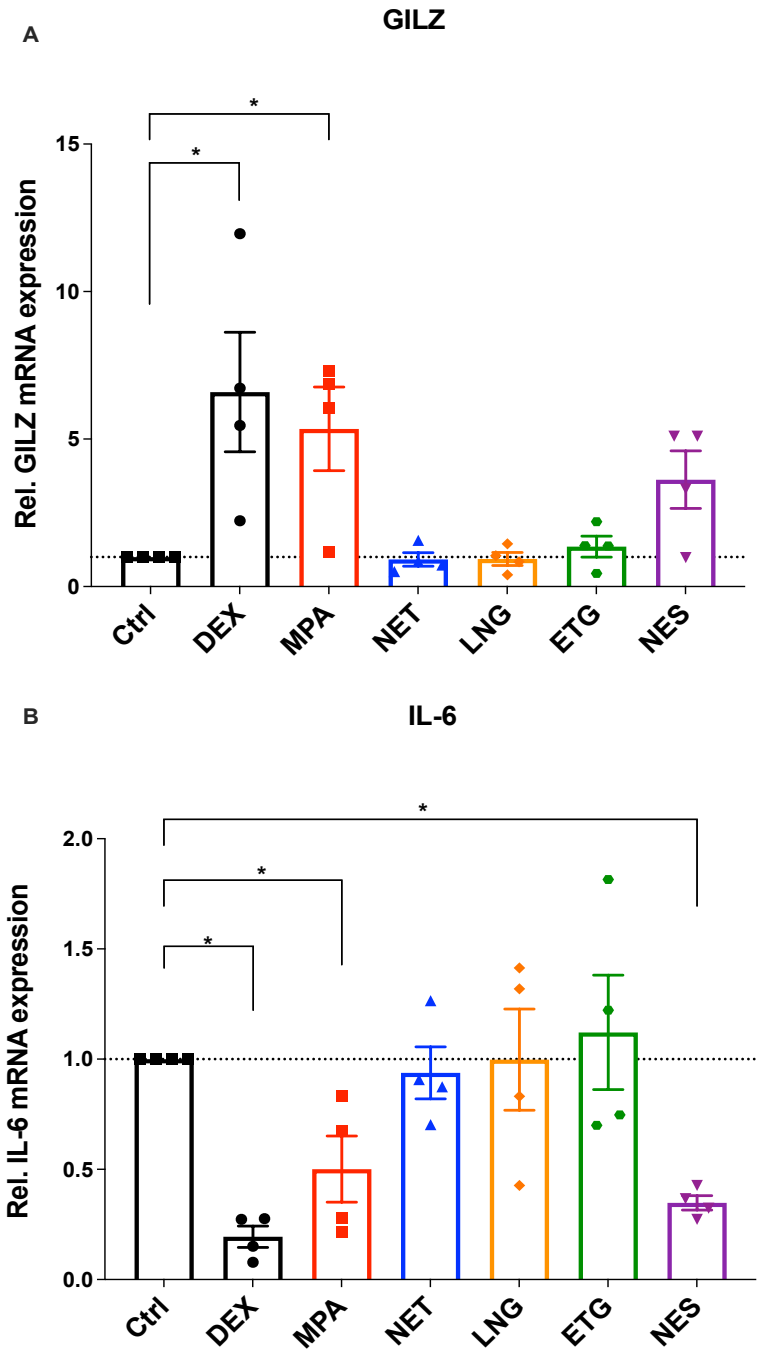


Figure 3.2.3.1: Like DEX, MPA is a full agonist, and NES differentially regulates GILZ and IL-6 mRNA levels expression in HeLa cells. HeLa cells were treated for 24 hours with 100 nM of each ligand or 0.1% (v/v) EtOH (Ctrl). RNA was harvested, cDNA synthesised, then subjected to qRT-PCR using primer sets specific for GILZ (A) or IL-6 (B) and GAPDH. GILZ or IL-6 mRNA levels were normalised to GAPDH levels for each condition. Figures represent four independent experiments shown as mean ± SEM. Results are expressed as fold differences relative to EtOH-only control (Ctrl) set to 1. Statistical analyses via one-way ANOVA with Dunnett post-test and Mann-Whitney t-tests were performed. Significant differences as determined by the Mann-Whitney t-

tests are indicated by asterisks where *, **, ***, **** represent $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively.

Table 3.2.3: Table showing the rel. efficacy values of the transcriptional effects of the progestins regulation GILZ and IL-6 mRNA levels in HeLa cells*

	GILZ	IL-6
Ligand	**MAX (%) ± SEM**	**MAX (%) ± SEM**
DEX	100 ± 2.02^{a}	100 ± 0.07^{a}
MPA	$81.48 \pm 1.42^{a, c}$	$62.80 \pm 0.15^{a, b, c}$
NET	16.70 ± 0.23^{b}	8.26 ± 0.12^{b}
LNG	15.26 ± 0.22^{b}	$-4.55 \pm 0.0.23^{b}$
ETG	$23.14 \pm 0.36^{b, d}$	$-16.00 \pm 0.26^{b, c}$
NES	$57.33 \pm 0.98^{c, d}$	$86.06 \pm 0.03^{a, b}$

***Data shown in Figure. 3.2.3.1A and B, were analysed to obtain the efficacy, or maximal (MAX) effect as a percentage i.e. MAX (%) ± SEM, relative to DEX set at 100%. Efficacy is based on the max responses at saturating concentrations of ligands (100 nM).

a,b,c,d Statistical significance between treatment groups was determined using one-way ANOVAs with Tukey post-test.

3.2.4 MPA, ETG and NES significantly regulate GILZ and IL-6 mRNA levels in PBMCs

Subsequently, the transcriptional effects of the progestins on systemic GR-target genes were investigated in PBMCs isolated from HIV-1-negative female donors of reproductive age. First, the cells were incubated using high progestin concentrations (100 nM) to simulate near-peak serum levels observed in users of injectable contraception. The cells were incubated for 48 hours with 100 nM of the progestins, then GILZ and IL-6 mRNA levels were measured and quantified using qRT-PCR.

Figure 3.2.4.1 illustrates that like DEX, MPA, ETG, and NES increased GILZ mRNA levels in PBMCs; whereas, MPA and NES significantly repressed IL-6 mRNA levels. These results suggest that MPA, and NES exert distinct gene-specific transcriptional effects by upregulating GILZ mRNA and repressing IL-6 mRNA levels; whereas, ETG selectively upregulates GILZ mRNA levels without regulating IL-6 mRNA levels in PBMCs. No detectable regulation by LNG and NET was observed on GILZ and IL-6 mRNA levels.

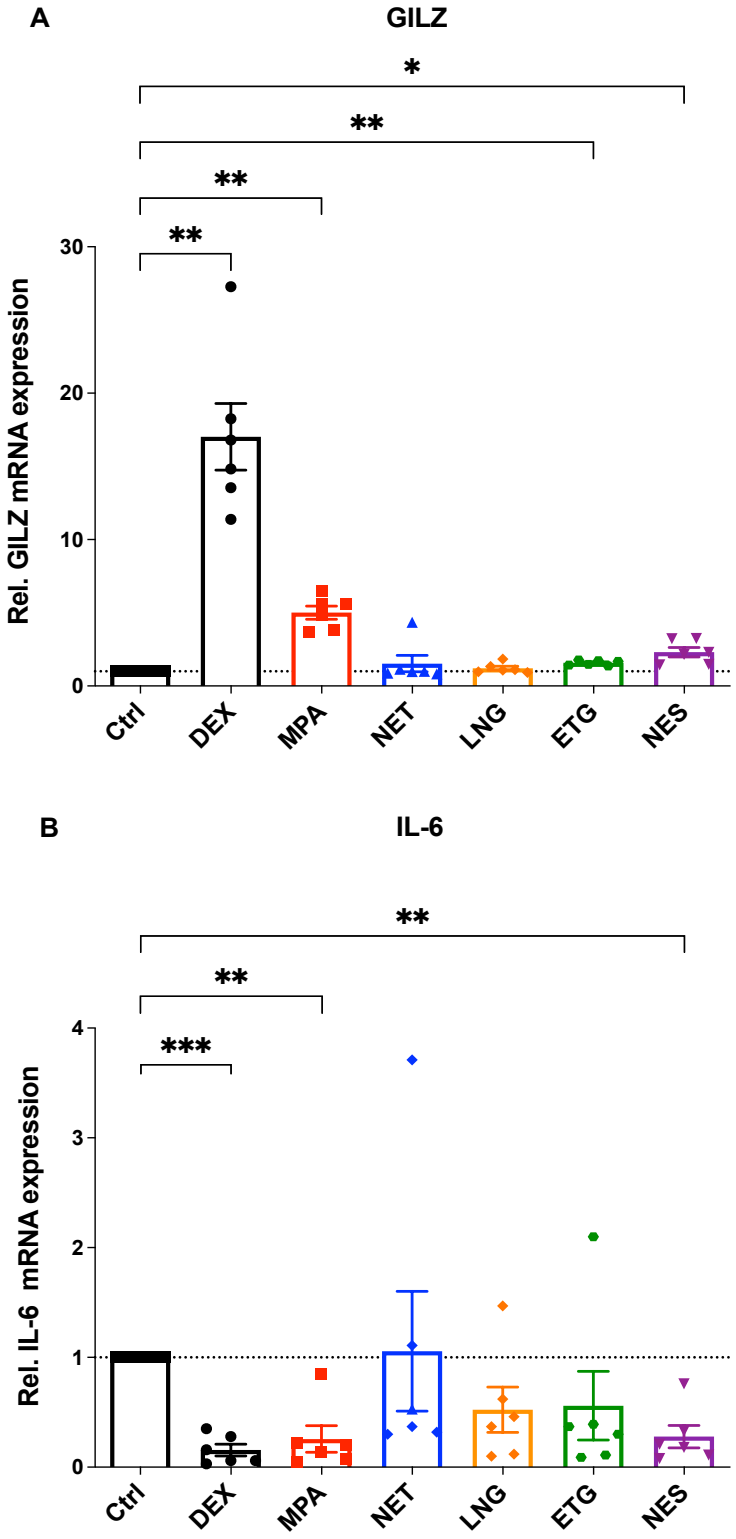


Figure 3.2.4.1: Unlike NET and LNG, MPA, ETG and NES differentially regulate GILZ and IL-6 mRNA levels in PBMCs. PBMCs were stimulated for 48 hours with 100nM of each ligand or 0.1% (v/v) EtOH (Ctrl). RNA was harvested, cDNA synthesised, then subjected to qRT-PCR using primer sets specific for GILZ (A) or IL-6 (B) and GAPDH. GILZ or IL-6 mRNA levels were normalised to GAPDH levels

for each condition. The data represent results from six individual donors shown as mean ± SEM. Results are expressed as fold differences relative to EtOH-only control (Ctrl) set to 1. Statistical analysis via ordinary one-way ANOVA with Dunnett post-test was performed. Significant differences are indicated by asterisks where *, **, ***, **** represent $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively.

3.2.5 MPA is a full agonist, while ETG and NES display partial agonist activity in regulating GILZ and IL-6 mRNA levels; whereas, LNG exhibits weak agonist activity for IL-6 repression in PBMCs

To characterise the progestin-induced transcriptional responses observed in **Figure 3.2.4.1**, the relative efficacy and potency of progestin-induced regulation of GILZ and IL-6 mRNA levels were evaluated in PBMCs incubated for 48 hours with increasing concentrations (1×10^{-10} M—1×10^{-5} M) of the progestins. Subsequently, GILZ and IL-6 mRNA levels were measured and quantified using qRT-PCR.

As shown in **Figure 3.2.5.1A**, dose-response analysis revealed that unlike NET, DEX and MPA displayed statistically similar full agonist activity for upregulation of GILZ mRNA levels. Relative to DEX, both ETG and NES displayed significantly less efficacious partial agonist activity for regulating GILZ mRNA expression, despite NES exhibiting significantly greater efficacy than ETG (**Table 3.2.2**). Non-linear regression analysis failed to determine the potency and efficacy of LNG, as GraphPad Prism 9 reported significant instability in the LNG dose-response curve. When comparing relative EC_{50} values, no significant differences were detected across progestins.

Except NET, MPA, LNG, ETG and NES displayed statistically similar agonist activity for transrepression of IL-6 mRNA levels in PBMCs compared to DEX (**Figure 3.2.5.1B**). However, the NET result (dotted line) was inconclusive due to considerable error in the data and the resulting curve instability (**Figure 3.2.5.1B**). In terms of potency, no significant differences were detected, although the

progestins appeared less potent than DEX, however, NES displayed an apparent higher potency than MPA and ETG (**Table 3.2.5**).

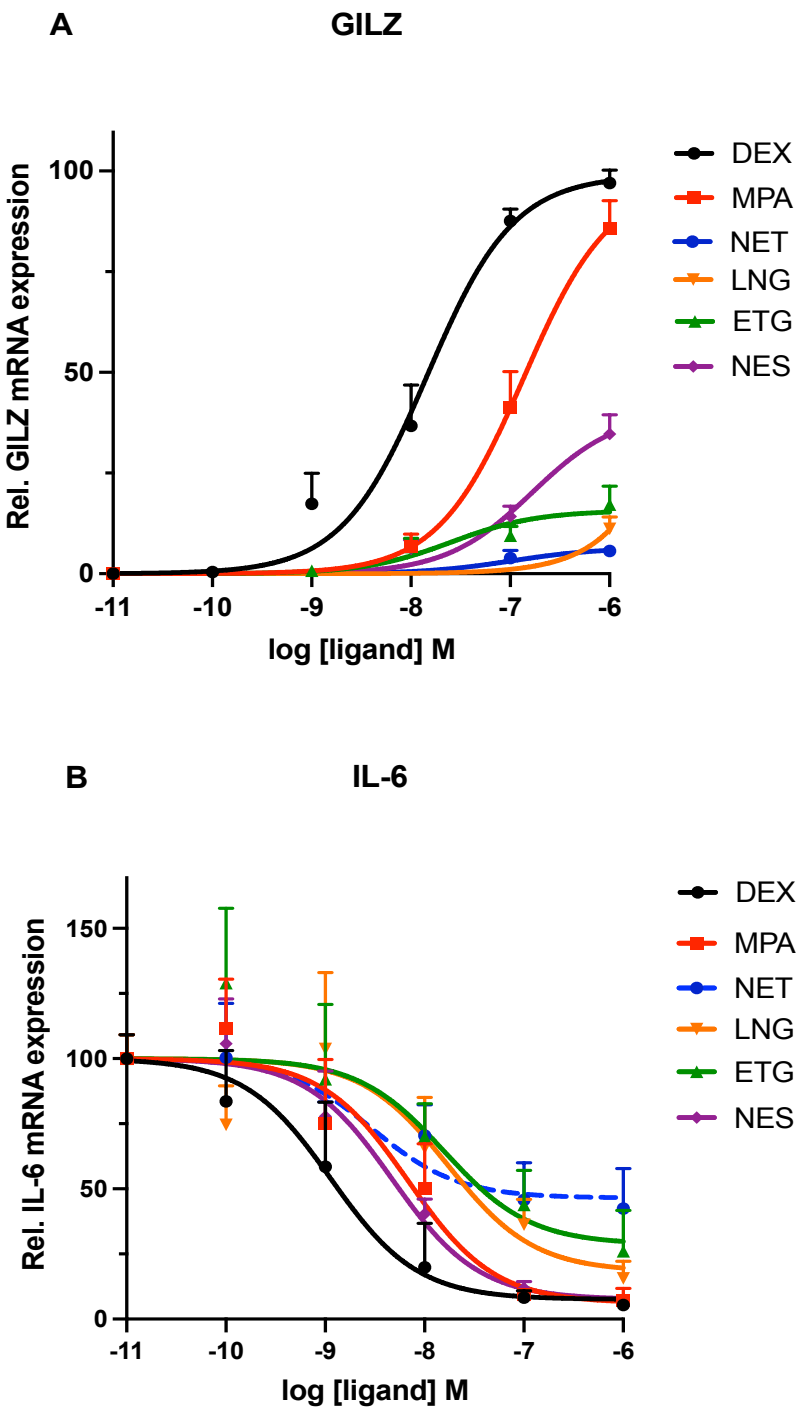


Figure 3.2.5.1: MPA, ETG and NES regulate GILZ mRNA levels and all progestins except NET are agonists for transrepression of the IL-6 gene in PBMCs. PBMCs were stimulated for 48 hours with increasing concentrations (1 x 10^{-10} M – 1 x 10^{-5} M) of ligands or 0.1% (v/v) EtOH (Ctrl). RNA was harvested, cDNA synthesised, then subjected to qRT-PCR using primer sets specific for GILZ (A) or IL-6 (B) and GAPDH. GILZ or IL-6 mRNA levels were normalised to GAPDH levels for each condition. The data represent results from six (A) and five (B) individual donors shown as mean ± SEM. Nonlinear regression and log(agonist) vs. response options were used for statistical analysis. Results are expressed as % DEX where the DEX maximal response was set to 100% and all other responses were set relative to DEX.

Table 3.2.5: Table showing rel. efficacy and EC_{50} values of the progestins for regulating GILZ and IL-6 mRNA expression in PBMCs*

	GILZ		IL-6	
Ligand	**MAX (%) ± SEM**	**EC_{50} (M) ± SEM**	**MAX (%) ± SEM**	**EC_{50} (M) ± SEM**
DEX	100 ± 4.87^{a}	$1.91 \pm 2.51 \times 10^{-8a}$	100 ± 9.16^{a}	$7.63 \pm 6.31 \times 10^{-9a}$
MPA	98.47 ± 7.09^{a}	$2.5 \pm 0.66 \times 10^{-7a}$	101.62 ± 11.50^{a}	$0.92 \pm 1.45 \times 10^{-8a}$
NET	6.50 ± 2.34^{b}	$5.12 \pm 9.91 \times 10^{-8a,\dagger}$	$57.95 \pm 9.55^{a,\ddagger}$	$4.66 \pm 4.32 \times 10^{-9a,\ddagger}$
LNG	ND	ND	88.53 ± 14.82^{a}	$2.95 \pm 6.27 \times 10^{-8a}$
ETG	15.85 ± 2.29^{b}	$2.04 \pm 3.19 \times 10^{-7a}$	77.19 ± 17.00^{a}	$2.44 \pm 0.10 \times 10^{-8a}$
NES	40.70 ± 4.40^{c}	$1.47 \pm 1.01 \times 10^{-7a}$	100.13 ± 7.92^{a}	$5.49 \pm 5.82 \times 10^{-9a}$

*Data shown in Figure. 3.2.5.1A and B, were analysed to obtain the efficacy, or maximal (MAX) effect as a percentage i.e. MAX (%) ± SEM, relative to DEX set at 100%. The potencies, i.e. EC_{50} (M) ± SEM values for each ligand for the GR are also shown.

a,b,c Statistical significance between treatment groups was determined using one-way ANOVAs with Tukey post-test.

†The EC_{50} values for NET should be interpreted with caution as very weak agonist activity was observed.

‡The MAX and EC_{50} values for NET should be interpreted with caution as Prism 9 deemed this curve unstable due to variable data.

ND-not determined.

3.2.6 RU486 significantly suppresses MPA- and NES-induced regulation of GILZ and IL-6 mRNA in PBMCs

PBMCs consist of a mixed population of cell types expressing varying levels of several SRs; however, the literature regarding SR expression in PBMCs remains inconsistent. While some studies indicate that whole PBMCs express ER, GR, and PR mRNA and protein with no detectable MR protein(Asin *et al.*, 2008; Cabrera-Munoz *et al.*, 2012; Tomasicchio *et al.*, 2013; Polikarpova *et al.*, 2019; Brundin *et al.*, 2021), others suggest that PR expression is low and findings differ across studies (Arruvito *et al.*, 2008; Cabrera-Munoz *et al.*, 2012; Tomasicchio *et al.*, 2013; Ray, 2015; Polikarpova *et al.*, 2019).

Despite this uncertainty, the data in the current study demonstrates that progestins elicit comparable transcriptional effects in PBMCs and COS-1 cells overexpressing the GR. Considering that the GR is the only functional SR in COS-1 cells, the observed transcriptional effects in this model are GR-mediated. Given the similarity in responses between PBMCs and COS-1 cells and literature suggesting low PR and negligible ER protein expression in PBMCs, these findings collectively suggest that GR may mediate the progestin-induced responses observed in PBMCs. However, further research is needed to fully elucidate the role of other SRs in this context.

To determine whether GR or PR mediate the progestin-induced regulation of GILZ and IL-6 mRNA expression, PBMCs were incubated for 48 hours with 100 nM of the

progestins in the absence and presence of the GR/PR antagonist RU486. Subsequently, the GILZ and IL-6 mRNA levels were measured and quantified.

In the absence of RU486, MPA and NES significantly upregulated GILZ mRNA levels in PBMCs (**Figure 3.2.6.1A**). However, co-stimulation with RU486 significantly reduced the upregulation, suggesting that MPA and NES likely regulate GILZ expression through a GR-dependent mechanism, although PR involvement cannot be fully excluded. Similarly, **Figure 3.2.6.1B** shows that, like DEX, both MPA and NES repressed IL-6 mRNA levels. However, in the presence of RU486, repression of IL-6 mRNA expression was significantly inhibited (**Figure 3.2.6.1B**). These results suggest that the GR most likely mediate the repression of the IL-6 gene by MPA and NES. Furthermore, NET, LNG, and ETG did not induce any significant regulation of GILZ and IL-6 mRNA expression. (**Figure 3.2.6.1**).

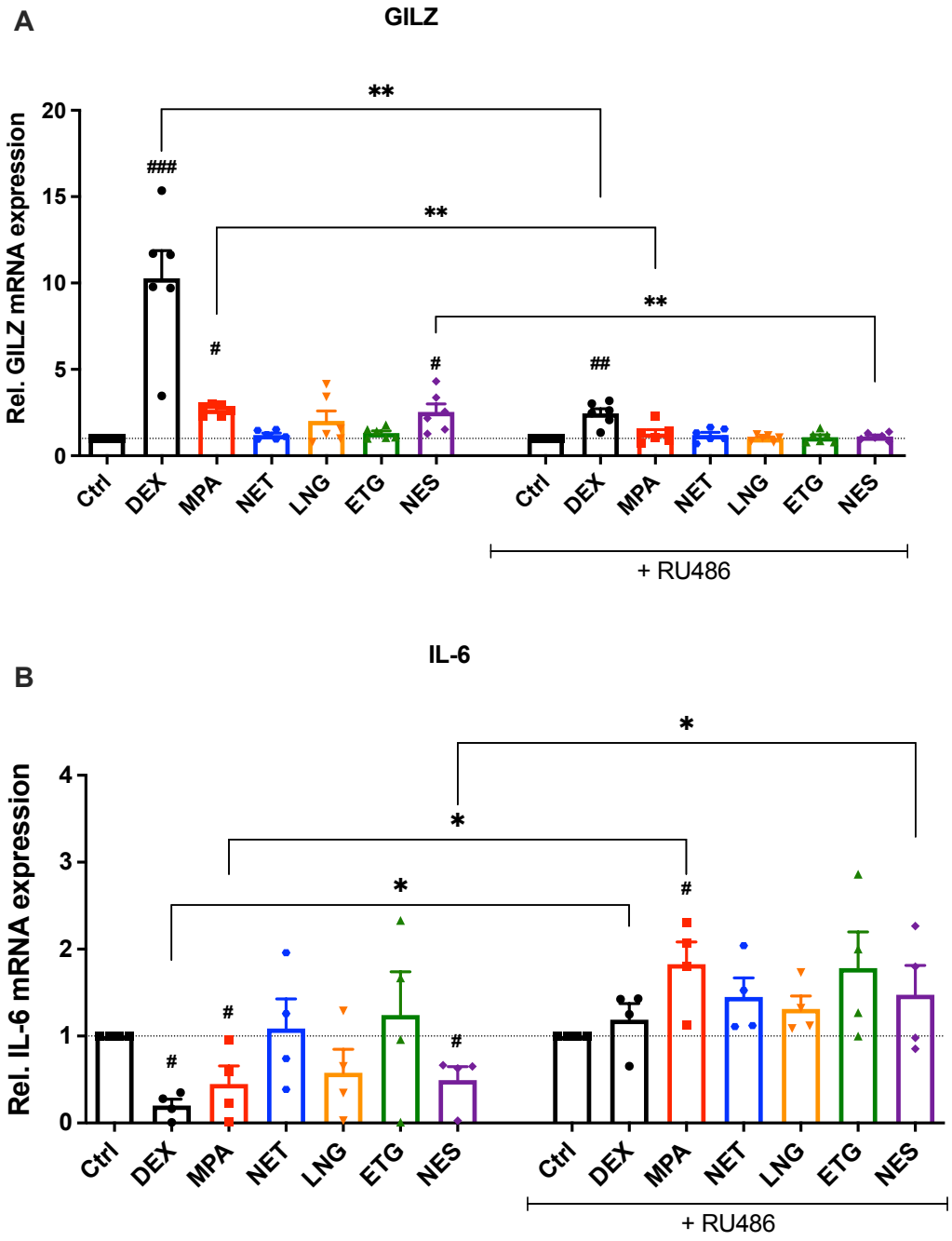


Figure 3.2.6.1: Co-treatment with the RU486 prevented the upregulation of GILZ and repression of IL-6 mRNA levels . PBMCs were isolated and treated for 48 hours with 100 nM ligands or 0.1% (v/v) EtOH (Ctrl) in the absence and presence of RU486 (100 nM). RNA was harvested, cDNA synthesised, then subjected to qRT-PCR using primer sets specific for GILZ (A) or IL-6 (B) and GAPDH. GILZ or IL-6 mRNA levels were normalised to GAPDH levels for each condition. Figures represent results from six (A) or four (B) PBMC donors shown as mean ± SEM. Results are expressed as fold differences relative to EtOH-only control (Ctrl) set to 1. Statistical analyses via one-way ANOVA with Dunnett post-test followed by Mann-Whitney t-tests were performed. Significant differences comparing each sample to

control are indicated by hashtags where #, ##, ### represent $p<0.05$, $p<0.01$, and $p<0.001$ respectively. Significant differences as determined by Mann-Whitney t-tests comparing each sample in the presence and absence of RU486 are indicated by asterisks where *, **, ***, **** represent $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively.

3.2.7 Progestin efficacy via the GR in COS-1 cells and IL-6 repression in PBMCs correlates with progestin RBA for the GR

Previous research has found a correlation between the transactivation potency of GR agonists and the RBA for the GR in COS-1 cells. However, no correlation was found for the same parameters for transrepression (Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009). Additionally, no correlation between the efficacy of GR agonists for transactivation and transrepression and the RBA for the GR (Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009). The previous study did not include LNG, ETG and NES, so little is known about the relationship between the respective binding affinity for the GR, potency, and efficacy for transactivation and transrepression. Next, the correlation between progestin-induced GR activity and binding affinity for the GR in COS-1 and PBMCs was investigated.

Figure 3.2.7.1 shows a significant positive correlation was detected between the binding affinity for the GR and the progestin efficacy via the GR on synthetic promoter in COS-1 cells. On the endogenous promoter in COS-1 cells, the same may be observed for all progestins except NES. However, in PBMCs, a significant correlation between affinity for the GR and regulation of GILZ expression was only detected for DEX and ETG (**Figure 3.2.7.2**). Except for NET and LNG, a significant correlation between affinity for the GR and the repression of IL-6 expression in PBMCs was detected for the other progestins (**Figure 3.2.7.3**).

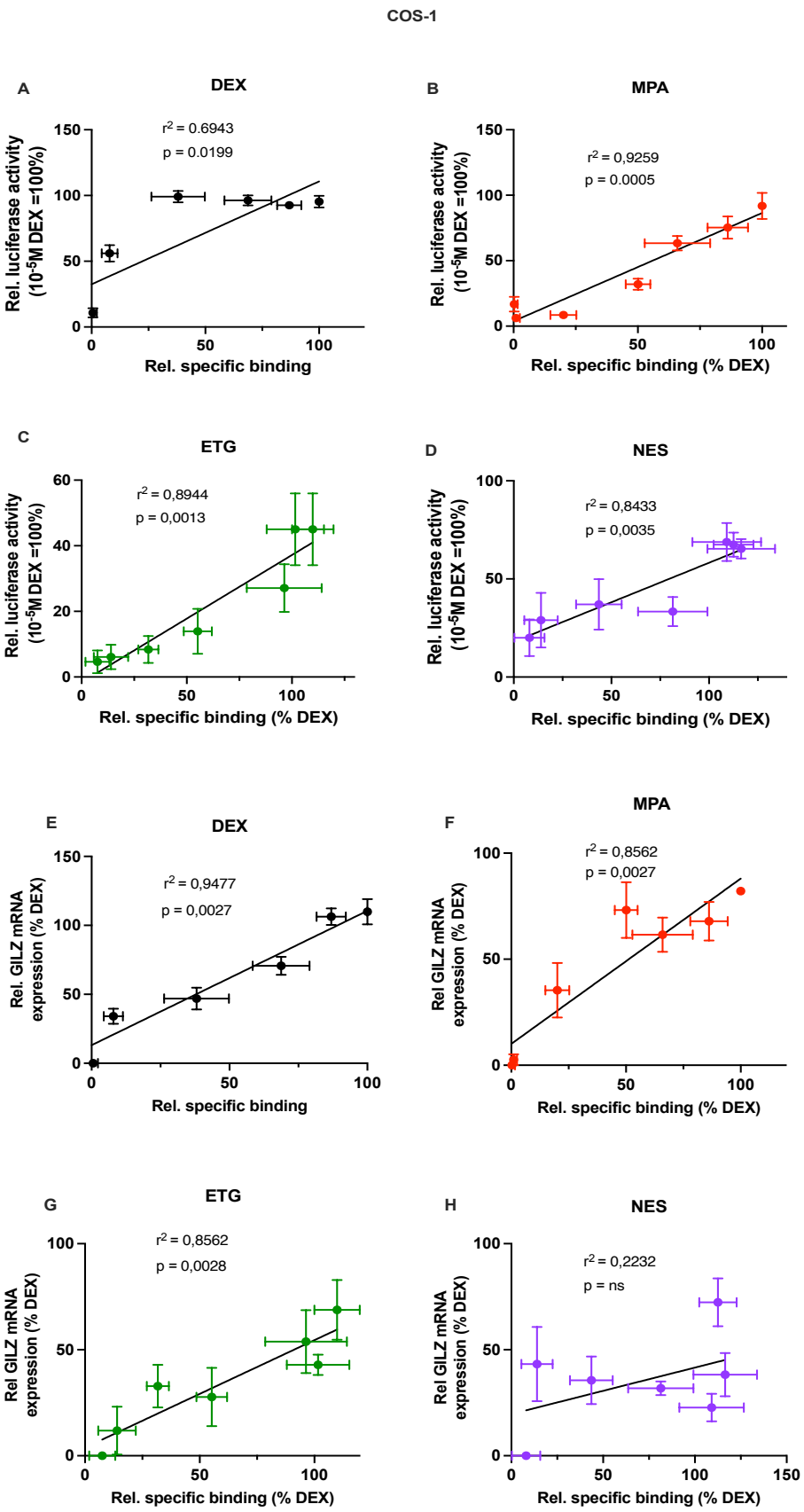

COS-1
A
DEX
r2 = 0.6943
p = 0.0199
Rel. luciferase activity (10-5M DEX =100%)
Rel. specific binding
B
MPA
r2 = 0,9259
p = 0.0005
Rel. luciferase activity (10-5M DEX =100%)
Rel. specific binding (% DEX)
C
ETG
r2 = 0,8944
p = 0,0013
Rel. luciferase activity (10-5M DEX =100%)
Rel. specific binding (% DEX)
D
NES
r2 = 0,8433
p = 0,0035
Rel. luciferase activity (10-5M DEX =100%)
Rel. specific binding (% DEX)
E
DEX
r2 = 0,9477
p = 0,0027
Rel. GILZ mRNA expression (% DEX)
Rel. specific binding
F
MPA
r2 = 0,8562
p = 0,0027
Rel GILZ mRNA expression (% DEX)
Rel. specific binding (% DEX)
G
ETG
r2 = 0,8562
p = 0,0028
Rel GILZ mRNA expression (% DEX)
Rel. specific binding (% DEX)
H
NES
r2 = 0,2232
p = ns
Rel GILZ mRNA expression (% DEX)
Rel. specific binding (% DEX)

Figure 3.2.7.1: Pearson correlation analyses revealed a significant correlation between binding affinity to the GR and progestin-induced transactivation on a synthetic and endogenous promoter in COS-1 cells for DEX and all progestins except NES. A - H are showing the Pearson correlation analysis between the relative luciferase activity obtained in **Figure 3.2.2.2** and relative specific binding (**Figure 3.2.1.2**) to GR on TAT-GRE and GILZ for DEX, MPA, ETG and NES in COS-1 cells.

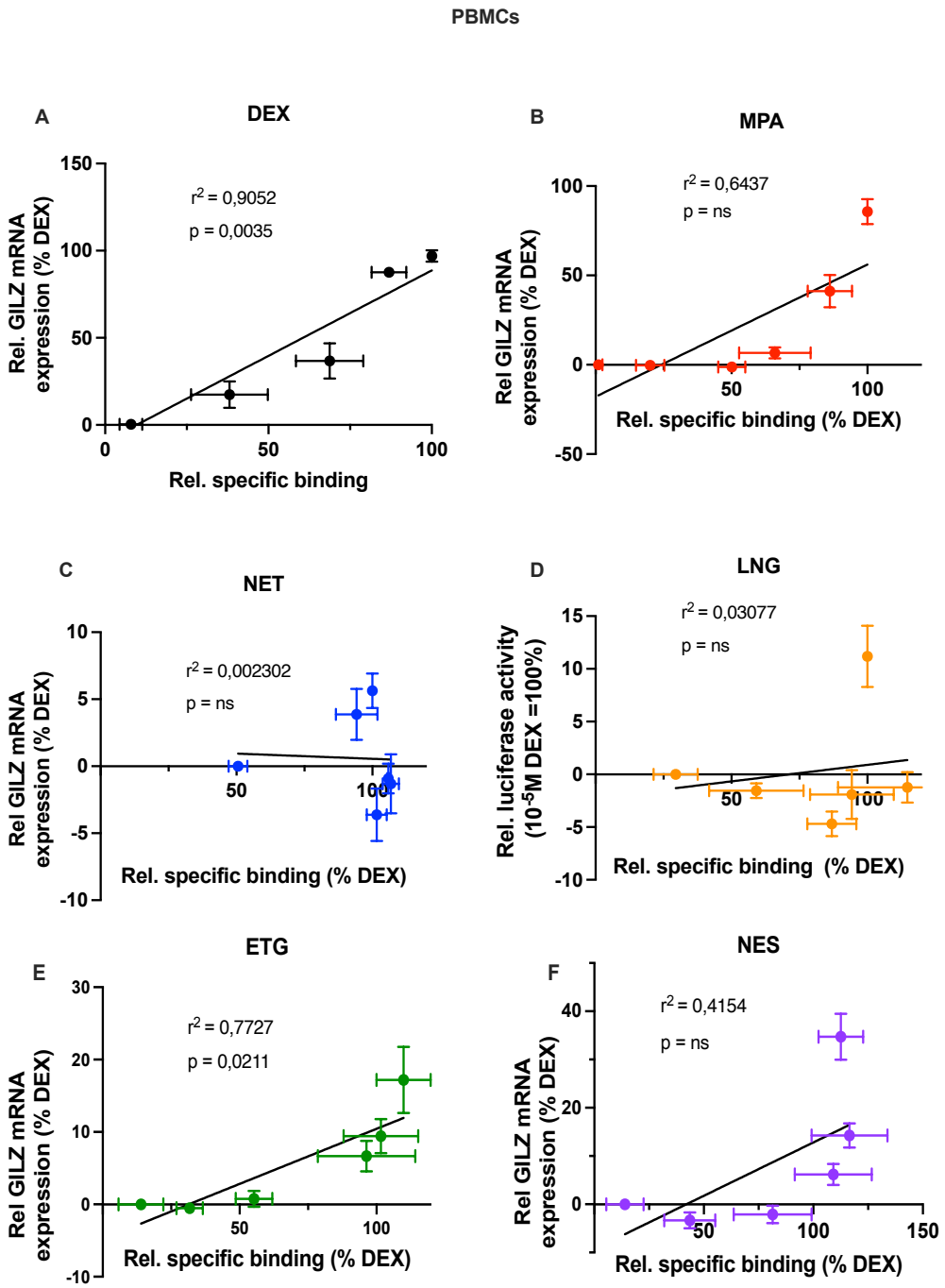


Figure 3.2.7.2: Pearson correlation analysis shows that all progestins except ETG do not show a significant correlation between binding affinity to the GR

and progestin-induced transactivation on GR target gene GILZ in PBMCs. A - F show the Pearson correlation analysis between the relative GILZ mRNA expression (**Figure 3.2.5.1A**) and relative specific binding (**Figure 3.2.1.2**) to GR for DEX, MPA, ETG and NES in PBMCs.

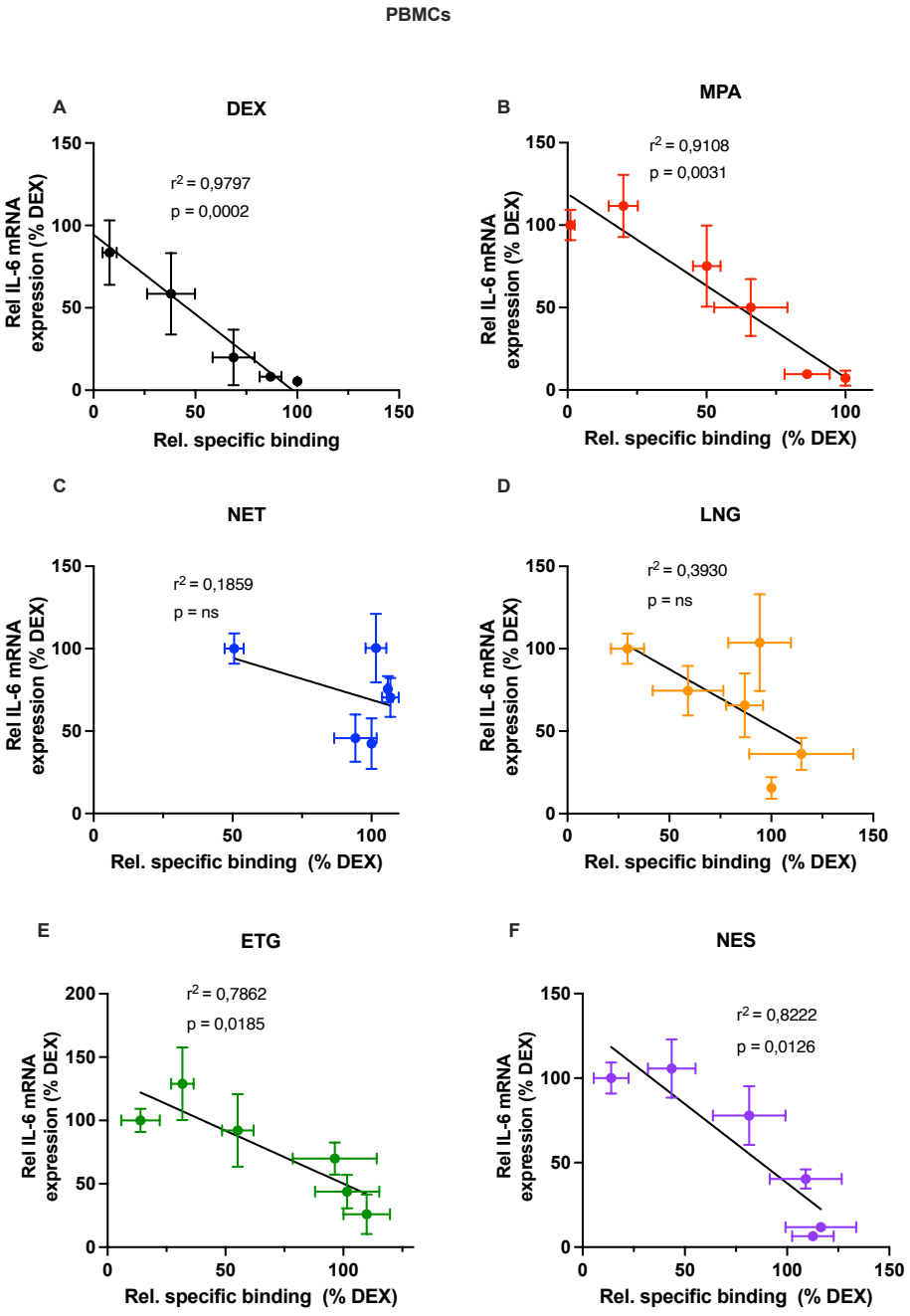


Figure 3.2.7.3: Pearson correlation analysis showing a significant correlation between binding affinity to the GR and progestin-induced the repression of IL-6 mRNA levels in PBMCs for DEX and all progestins except LNG and NET. A - F show the Pearson correlation analysis between the relative IL-6 mRNA expression obtained in **Figure 3.2.5.1B** and relative specific binding (**Figure 3.2.1.2**) to the GR for DEX, MPA, ETG and NES in PBMCs.

3.2.8 No significant correlation was detected between progestin potency and binding IC_{50} for the GR in COS-1 cells and PBMCs

Next the progestins potency via the GR and its binding IC_{50} for the GR were evaluated. Pearson correlation analysis revealed no significant correlation between potency and binding IC_{50} for the GR in COS-1 cells and PBMCs (**Figure 3.2.8.1**).

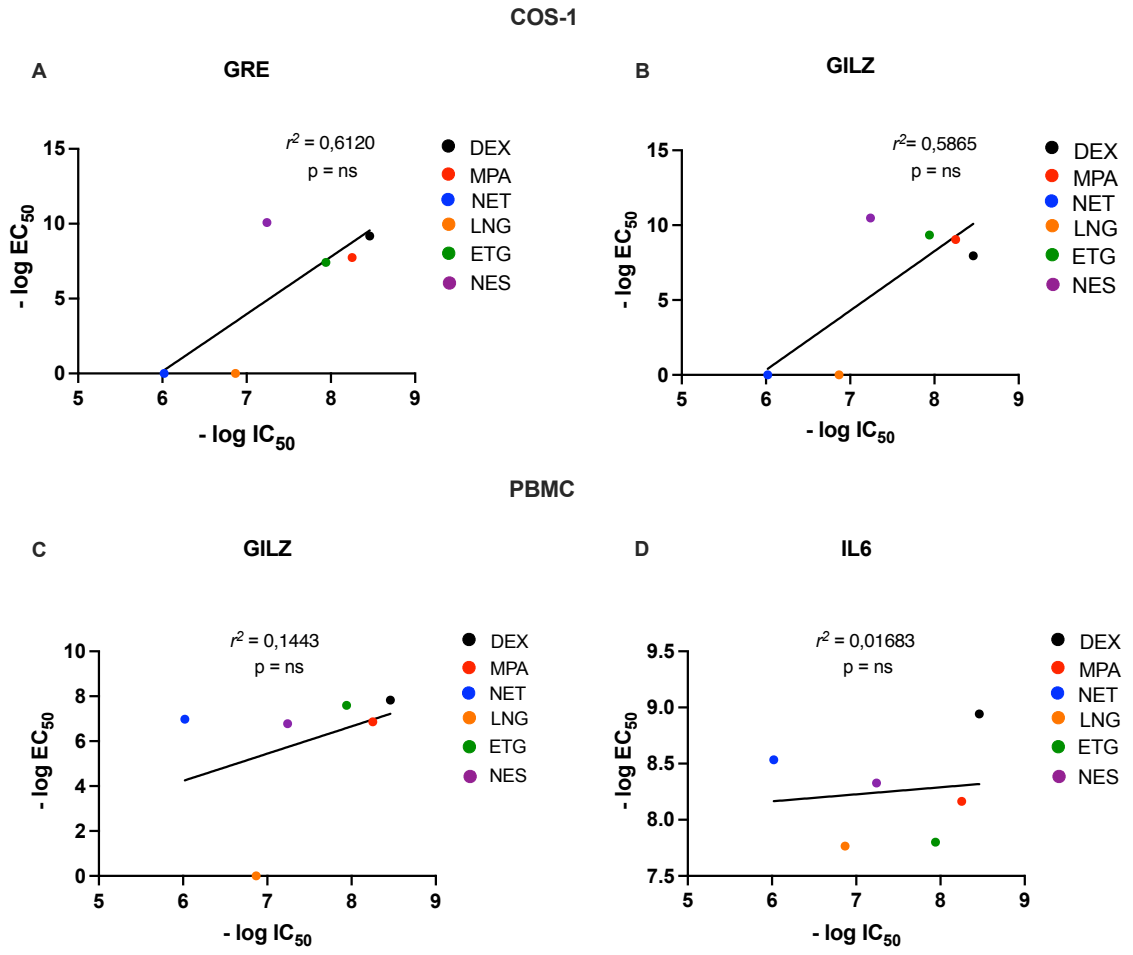


Figure 3.2.8.1: No correlation between progestin potency via the GR and binding IC_{50} for the GR in COS-1 and PBMCs was detected. Pearson correlation analysis between the relative -logEC_{50} (**Table 3.2.2** and **Table 3.2.5**) and relative -logIC_{50} (**Table 3.2.1**) for GR on (A) TAT-GRE and (B) GILZ in COS-1 cells, and (C) GILZ and (D) IL-6 in PBMCs, respectively.

This chapter revealed that MPA, ETG and NES bind to the GR with high affinity. These progestins act as partial GR agonists, regulate GILZ expression, and suppress IL-6 in COS-1 and HeLa cells. In PBMCs, MPA, ETG and NES significantly regulate GILZ and IL-6 mRNA levels, with MPA being a full agonist and ETG and NES being partial agonists on the respective promoters. The chapter also showed that the MPA- and NES-induced regulation of GILZ and IL-6 mRNA is GR-dependent. Pearson correlations revealed a significant correlation between progestin-induced GR efficacy and GR binding in COS-1 cells. However, no correlation between progestin potency and binding IC_{50} was observed in COS-1 cells and PBMCs. LNG and NET showed minimal to no regulation on either promoter in the different model systems. These findings provide insights into the effects of MPA, ETG and NES on regulating gene expression and GR activity.

Chapter 4

MPA and NES differentially regulate IFNγ mRNA and protein levels and increase HIV-1 infection in PBMCs: Possible role of the GR or PR

4.1 Background

The reported findings of the ECHO trial show that no substantial differences were detected in HIV-1 risk between DMPA-IM, LNG implant, and CU-IUD (Evidence for Contraceptive and Consortium, 2019). However, meta-analyses of higher quality observational studies detected a significant 40%–50% increased risk of HIV-1 acquisition associated with DMPA-IM use (Heffron *et al*., 2012; Polis *et al*., 2014; Morrison *et al*., 2015; Ralph, Gollub, and Jones, 2015; Polis *et al*., 2016). Considering these contrasting findings, it still remains valuable to elucidate the potential mechanisms by which MPA facilitates increased HIV-1 acquisition. Considering it has been proposed that MPA most likely facilitate increased HIV-1 acquisition by modulating soluble immune mediator levels (Blish and Baeten, 2011; Murphy, Irvin, and Herold, 2014; Hapgood, Kaushic, and Hel, 2018). This chapter will directly compare the effects of LNG, ETG and NES in parallel with MPA and NET on select immune gene mRNA and soluble immune mediator levels and the regulation of HIV-1 infection in PBMCs *ex vivo*. IFNγ was chosen because it is a

cytokine critical for innate and adaptive immunity against viral infections such as HIV-1, by inhibiting viral replication and enhancing immune cell activation (Dighe, Farrar, and Schreiber, 1993; Haase, 2005). CD4 and CCR5 expression were investigated because $CD4^+$ T cells are primary HIV-1 target cells, and CCR5 is a co-receptor that R5-tropic HIV-1 strains use to gain entry into cellular targets (Swanstrom and Coffin, 2012; Rottman *et al.*, 1997).

4.2 MPA and NES significantly repress IFNγ mRNA levels, with no detectable effect on CD4 and CCR5 mRNA levels in PBMCs

To investigate whether the progestins may regulate select immune function genes, PBMCs were isolated from female donors and incubated with 100 nM of the panel of progestins in parallel with DEX for 48 hours. Following RNA isolation, IFNγ, CD4 and CCR5 mRNA levels were measured and quantified using qRT-PCR.

Data from **Figure 4.2.1A** revealed that like DEX, MPA and NES significantly repressed IFNγ mRNA expression. Significant regulation of IFNγ expression by NET, LNG and ETG was not detected.

The effects of LNG, ETG, and NES on CCR5 and CD4 mRNA levels have not been thoroughly investigated. No significant CD4 and CCR5 mRNA regulation by MPA and the other panel of progestins was detected in PBMCs (**Figure 4.2.1B and C**).

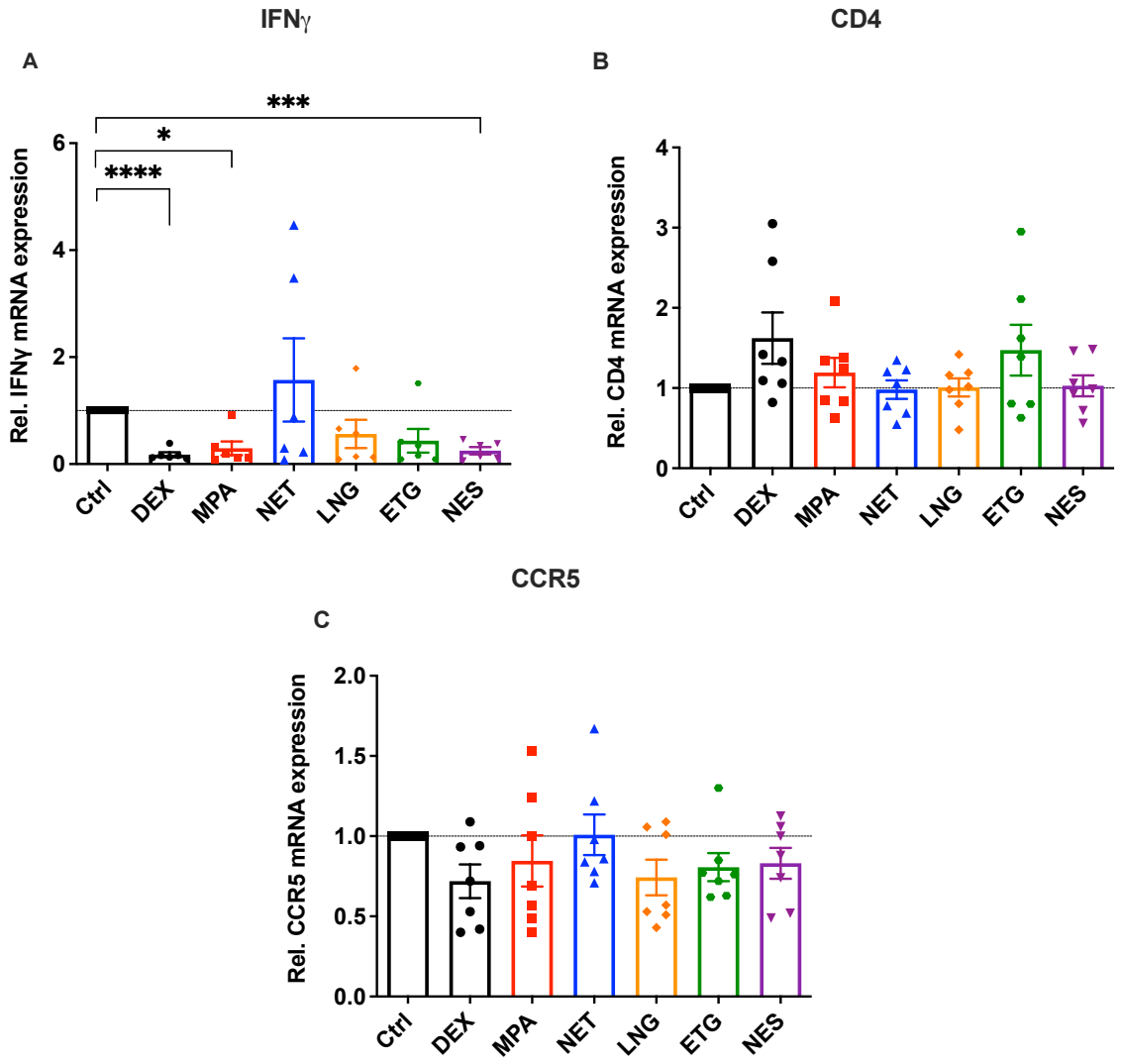


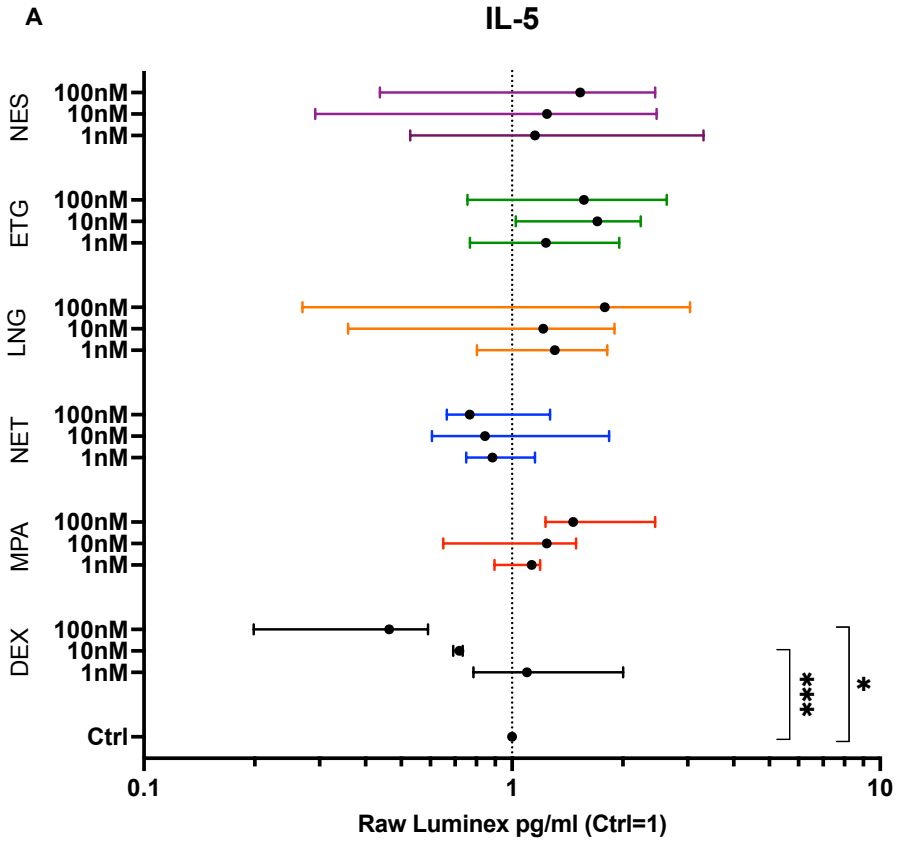
Figure 4.2.1: MPA and NES significantly repress IFNγ mRNA levels. PBMCs were stimulated with 100 nM ligand or 0.1% (v/v) EtOH (Ctrl) for 48 hours. RNA was harvested, cDNA was synthesised and subjected to qRT-PCR using primer sets specific for IFNγ (A) or CD4 (B) or CCR5 (C) and GAPDH. IFNγ, CD4, or CCR5 mRNA levels were normalised to GAPDH levels for each condition. Figures show pooled results from six or seven individual donors as mean ± SEM. Results are expressed as fold differences relative to EtOH-only control (Ctrl) set to 1. Statistical analysis via non-parametric Friedman test with Dunns multiple comparisons post-test. Significant differences are indicated by asterisks where *, **, ***, **** represent $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively.

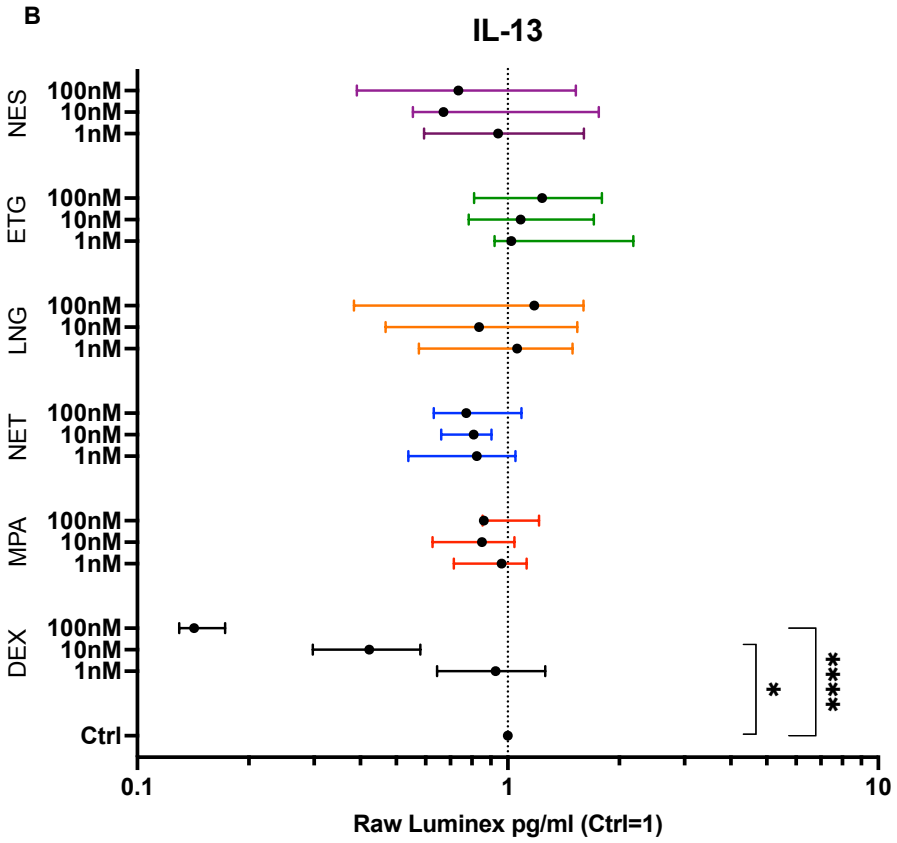
4.3 Except MPA, progestins do not regulate select soluble immune mediator protein levels of select cytokines in PBMCs

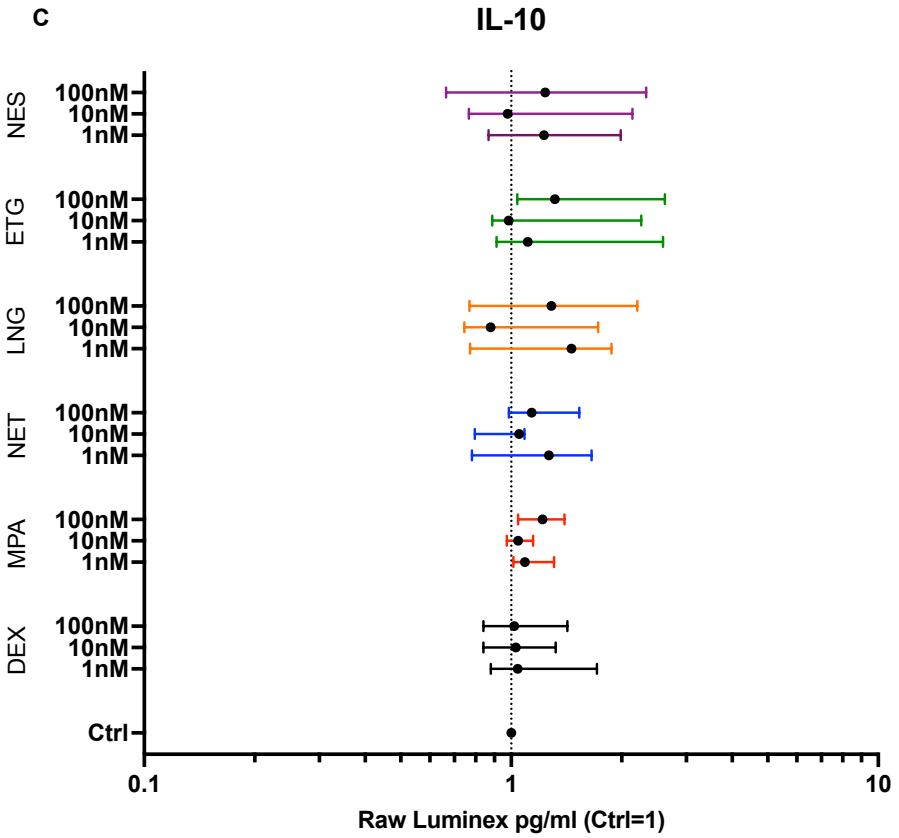
Next, the dose dependent progestin-induced regulation of secreted protein levels was investigated. PBMCs were isolated from female donors and incubated with MPA, NET, LNG, ETG, and NES in parallel with DEX. After 48 hours the supernatants from the PBMC dose-response gene expression experiments **(Section 3.2.4)** were harvested and a Luminex assay was conducted to measure the levels of secreted proteins. Thirteen cytokine expression levels were assessed. Of these 13, the levels of 3 cytokines were below the LOD **(Section 2.9.3)** and are not reported. Protein determination was possible for 10 of the cytokine expression levels assessed: IL-6, TNFα, IL-10, IL-1β, IL-1α, G-CSF, IFNγ, IL-13, IL-5 and IL-17.

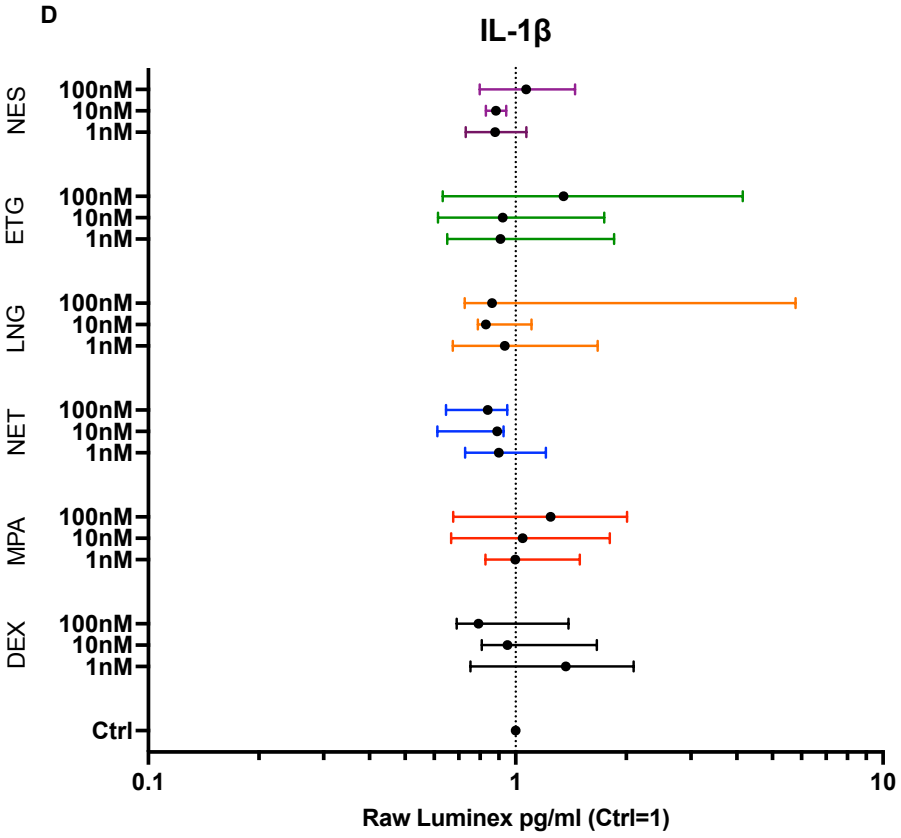
The results showed that IL-5 and IL-13 secreted protein levels were significantly repressed by DEX at 10 nM and 100 nM (**Figure 4.3.1A and B**). No significant regulation was detected; however, several trends were observed. MPA and NES appeared to induce a dose-dependent increase in IL-5 secreted protein levels (**Figure 4.3.1A**); whereas, it appeared as though ETG dose-dependently increased IL-13 and IFNγ secreted protein levels (**Figure 4.3.1B and F**). Interestingly, NET appeared to repress IL-5 protein levels in a dose-dependent manner (**Figure 4.3.1A**). Significant regulation was not detected, it appeared as though, DEX repressed, and MPA increased IL-1β levels in a dose-dependent manner (**Figure 4.3.1D**). Unlike the mRNA expression data (**Figure 4.2.1A**), MPA,

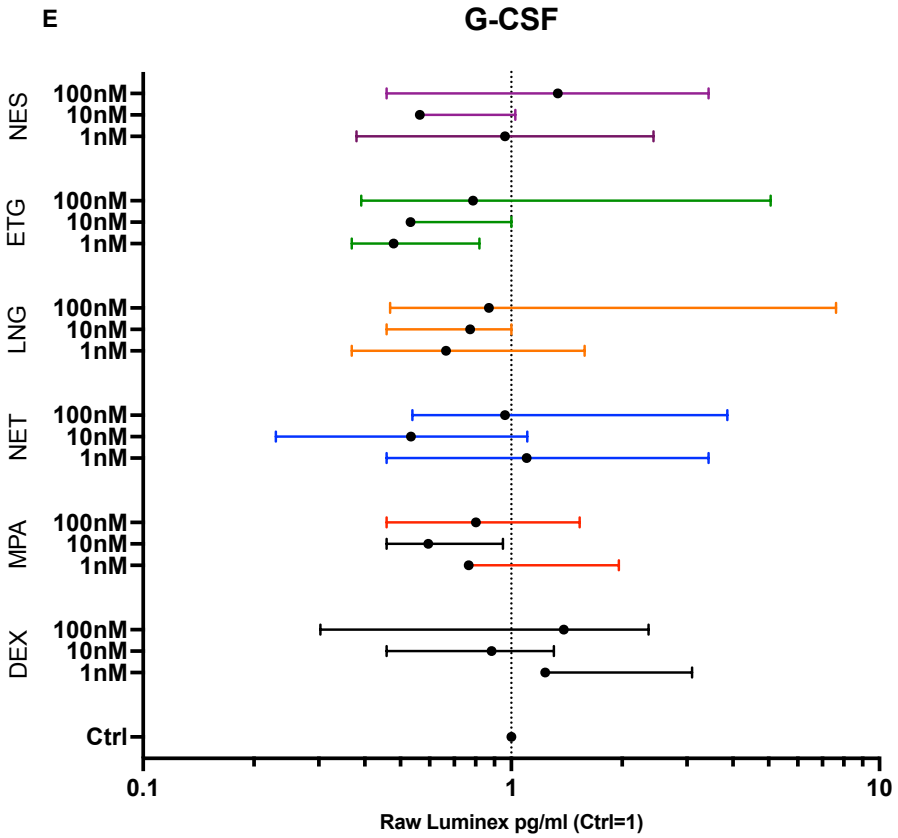
significantly increased secreted IFNγ protein levels at 100 nM (**Figure 4.3.1F**). Although no significant regulation was detected, it appeared as though DEX repressed TNFα, IL-6 and IL-17 protein levels in a dose-dependent manner (**Figure 4.3.1G, I and J**). The DEX repression of TNFα protein secretion at 10 nM and 100 nM approached significance with p-values of 0.0671 and 0.0504, respectively (**Appendix B, Table B1G**). Interestingly, LNG seemed to increase secreted TNFα protein levels in a dose-dependent manner. Overall, no significant effects were detected on the levels of secreted protein for the majority of the cytokines investigated, even at progestin concentrations of 100 nM.

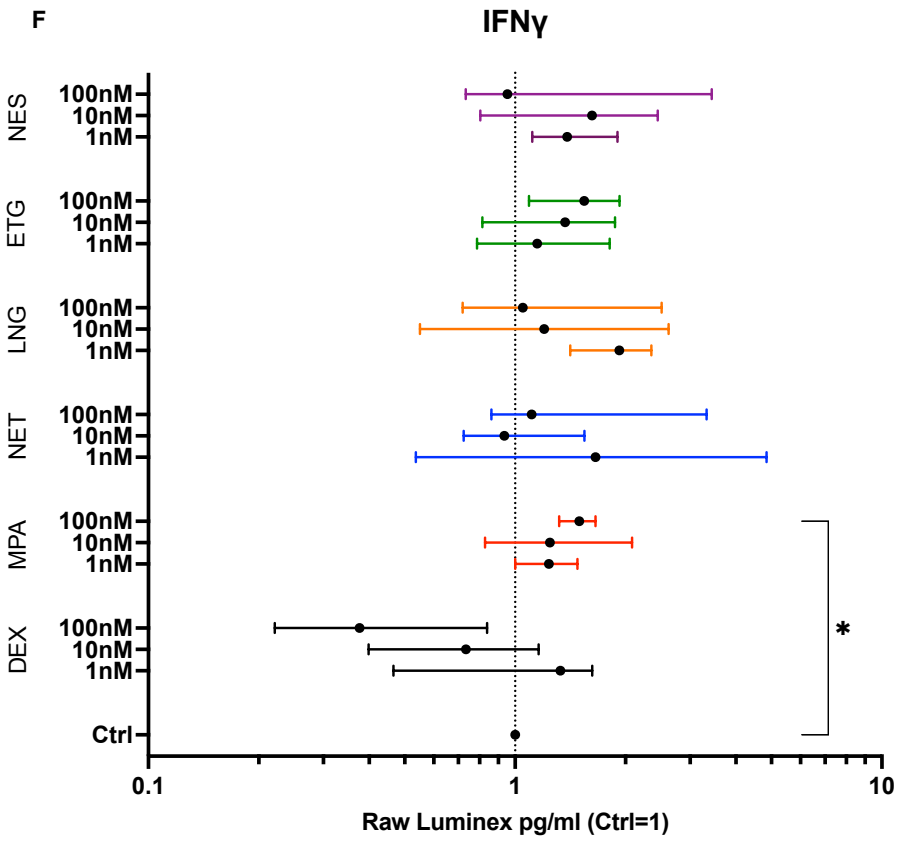

A
IL-5
NES
ETG
LNG
NET
MPA
DEX
100nM
10nM
1nM
Ctrl
***
*
0.1
1
10
Raw Luminex pg/ml (Ctrl=1)

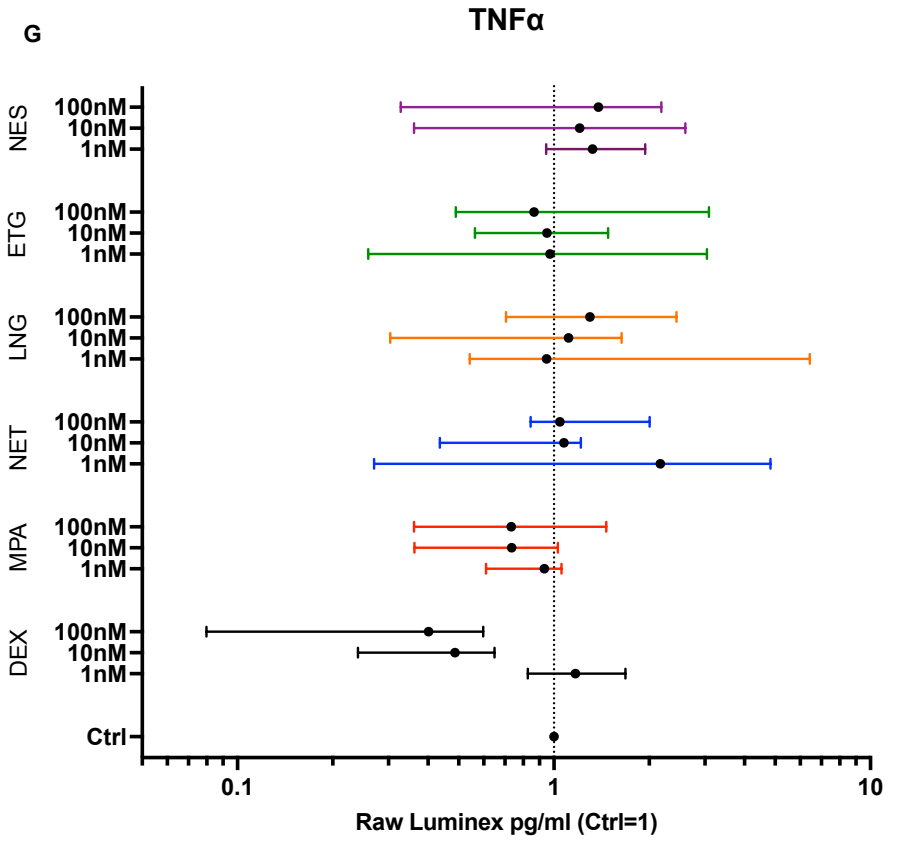

B
IL-13
NES
ETG
LNG
NET
MPA
DEX
100nM
10nM
1nM
Ctrl
*
****
0.1
1
10
Raw Luminex pg/ml (Ctrl=1)

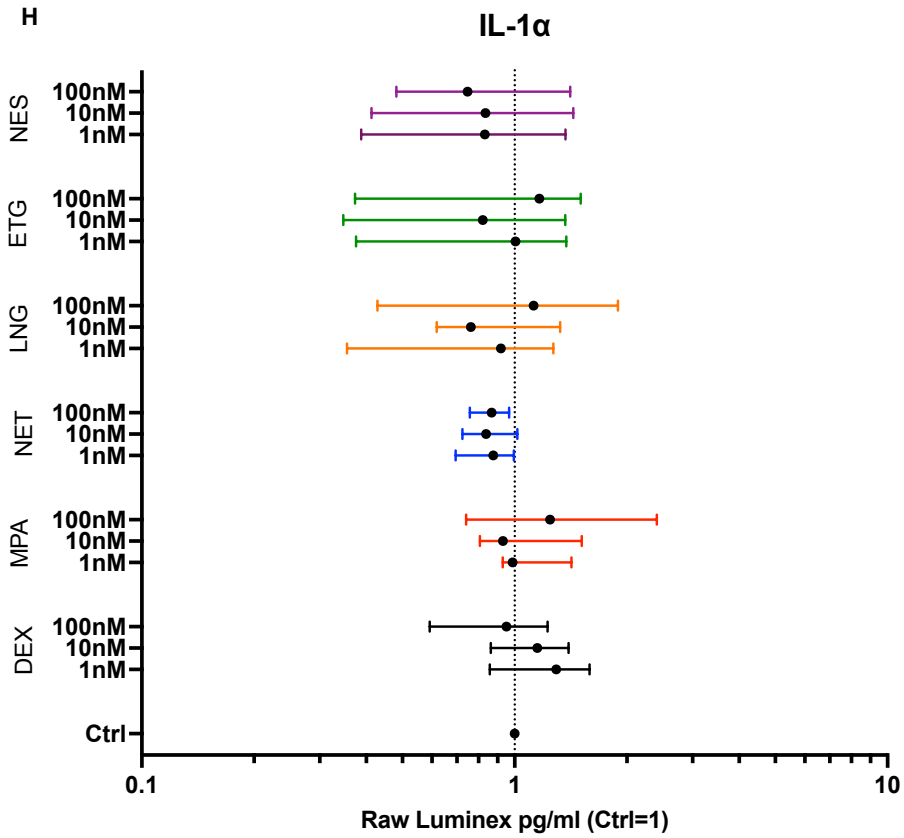

C
IL-10
NES
ETG
LNG
NET
MPA
DEX
100nM
10nM
1nM
Ctrl
0.1
1
10
Raw Luminex pg/ml (Ctrl=1)

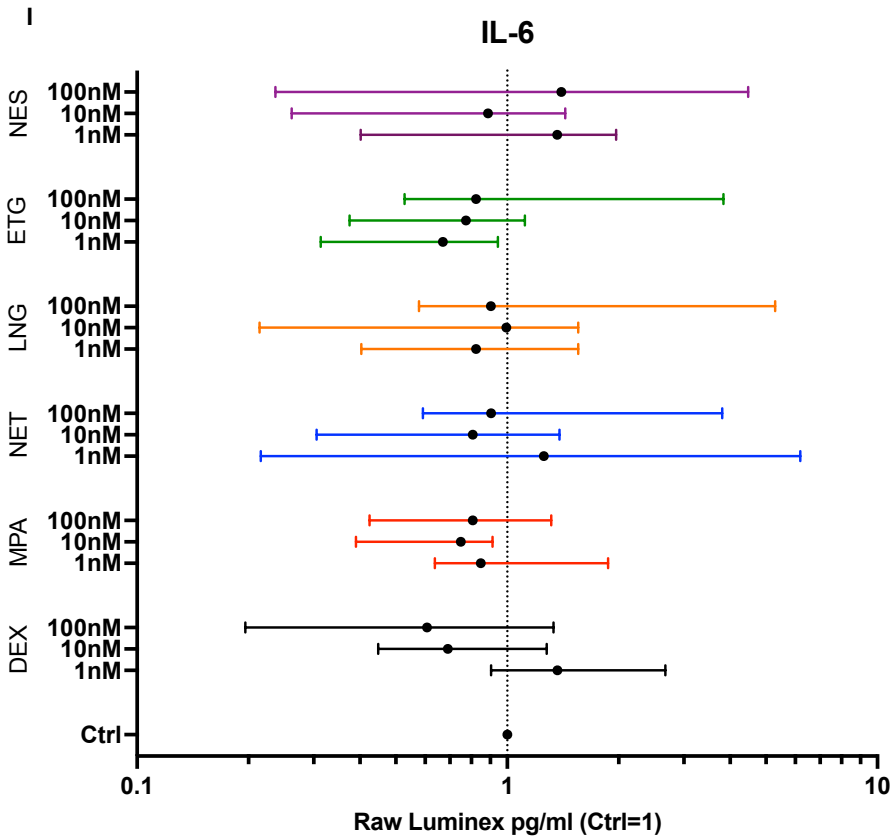

D
IL-1β
NES
ETG
LNG
NET
MPA
DEX
100nM
10nM
1nM
Ctrl
0.1
1
10
Raw Luminex pg/ml (Ctrl=1)


E
G-CSF
NES
ETG
LNG
NET
MPA
DEX
100nM
10nM
1nM
Ctrl
0.1
1
10
Raw Luminex pg/ml (Ctrl=1)


F
IFNγ
NES
ETG
LNG
NET
MPA
DEX
100nM
10nM
1nM
Ctrl
0.1
1
10
Raw Luminex pg/ml (Ctrl=1)
*


G
TNFα
NES
ETG
LNG
NET
MPA
DEX
100nM
10nM
1nM
Ctrl
0.1
1
10
Raw Luminex pg/ml (Ctrl=1)


H
IL-1α
NES
ETG
LNG
NET
MPA
DEX
100nM
10nM
1nM
Ctrl
0.1
1
10
Raw Luminex pg/ml (Ctrl=1)


I
IL-6
NES
ETG
LNG
NET
MPA
DEX
100nM
10nM
1nM
Ctrl
0.1
1
10
Raw Luminex pg/ml (Ctrl=1)

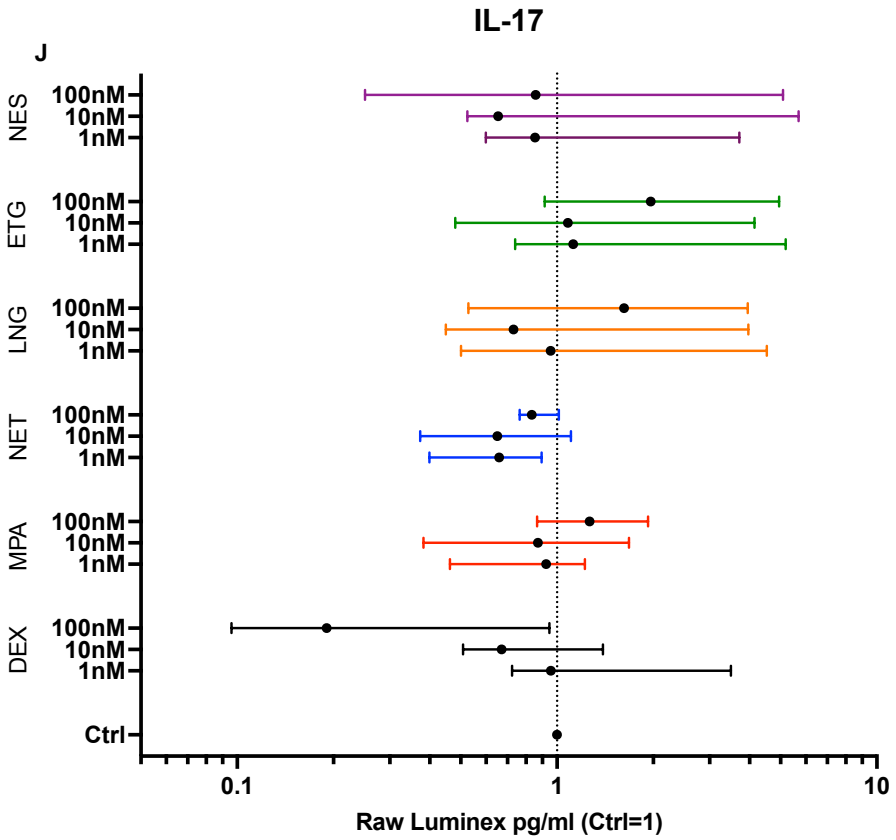

J
IL-17
NES
ETG
LNG
NET
MPA
DEX
100nM
10nM
1nM
Ctrl
0.1
1
10
Raw Luminex pg/ml (Ctrl=1)

Figure 4.3.1: MPA significantly increases secreted IFNγ protein levels in PBMCs. PBMCs were incubated with increasing amounts of DEX, MPA, NET, LNG, ETG, NES or 0,001% (v/v) EtOH (Ctrl) for 48 hours. Supernatants were harvested and processed for differential protein expression using a Miliplex® Human Cytokine/Chemokine magnetic bead panel (Merck, Germany). Relative protein expression levels of IL-5 (A), IL-13 (B), IL-10 (C), IL-1b (D), G-CSF (E), IFNγ (F), TNFα (G), IL-1a (H), IL-6 (I), and IL-17 (J) levels were normalised to EtOH control (Ctrl) set to 1. The graphs show pooled results of four independent experiments shown as median with range. Each data point at the different concentration points (1nM, 10 nM and 100 nM) is an average of results from four PBMC donors. Statistical analysis via one-way ANOVA with Dunnett post-test was performed. Significant differences are indicated by asterisks where *, **, ***, **** represent $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively.

4.4 MPA and NES increase HIV-1 infection in PBMCs *ex vivo* in a GR-dependent manner

Previous data from our research group shows that MPA but not NET, increases HIV-1 infection in the TZMbl cell line, PBMCs, and in cervical tissue explants (Bick *et al.*, 2021a; Maritz *et al.*, 2018; Ray *et al.*, 2019). The data also show that RU486 reduces the MPA-induced increase in HIV-1 infection. There is no *in vitro* information available about the effects of LNG, ETG and NES on HIV-1 infection.

Next, the potential effects of progestins on HIV-1 infection was investigated *ex vivo*. PBMCs were stimulated with 100 nM of each progestin for 48 hours. Thereafter, the samples were infected with 10 IU/ml HIV-$1_{BaL\_Renilla}$ and harvested after 5 days. Renilla luciferase readings revealed that MPA, LNG, ETG and NES, but not NET, significantly increased HIV-1 infection at 100 nM in PBMCs (**Figure 4.4.1A**).

To evaluate the involvement of the GR in the progestin-induced increase in HIV-1 infection, frozen PBMCs from the same donors were thawed and co-stimulated with or without the GR/PR antagonist RU486 for 48 hours, and infected 10IU/ml HIV-$1_{BaL\_Renilla}$ for 5 days. **Figure 4.4.1B** shows that MPA, NET, LNG and NES significantly increased HIV-1 infection in PBMCs relative to the control. The MPA- and NES-induced increase in HIV-1 infection was significantly reduced in the presence of RU486.

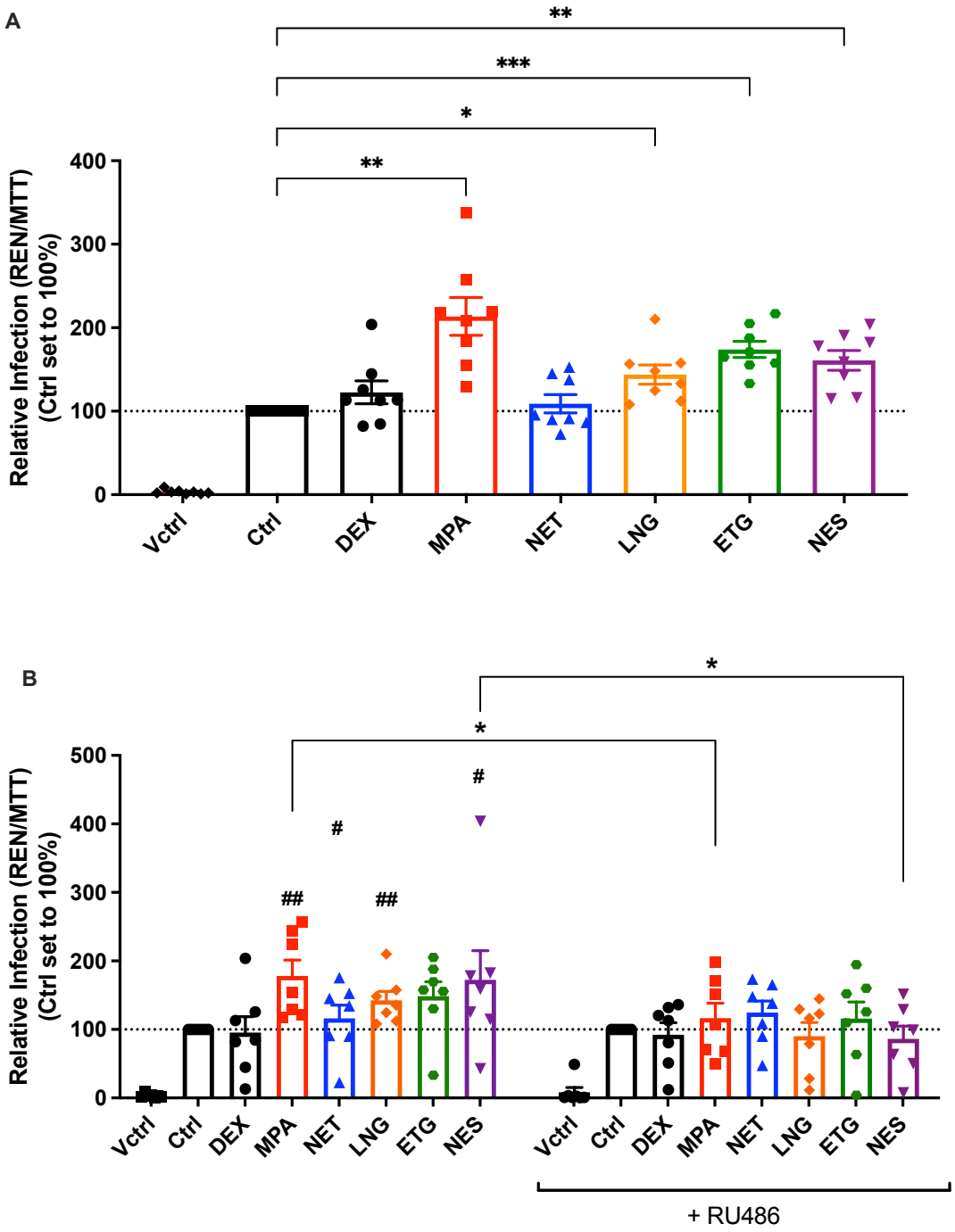


Figure 4.4.1: The MPA- and NES-induced increase in HIV-1 infection is repressed by GR/PR antagonist RU486. PBMCs stimulated with 100 nM of ligands or 0.1% (v/v) EtOH (Ctrl) (A), as well as in the absence and presence of RU486 (B) for 48 hours. Thereafter, PBMCs were infected with 10 IU/mL HIV-$1_{BaL\text{-}Renilla}$ IMC or equivalent volume of no-virus control for 2 hours, washed and maintained in ligand-free full RPMI supplemented with 30 IU/mL IL-2 for 5 days before harvesting for Renilla luciferase or MTT viability. Relative infection for each donor for progestins relative to EtOH control (Ctrl), where the only variable was the absence or presence of progestin, was calculated by normalizing RLU against corresponding MTT values.

Statistical analysis via one-way ANOVA with Dunnett post-test followed by Mann-Whitney t-tests was performed. Significant differences comparing each sample to control are indicated by hashtags where #, ##, ### represent $p<0.05$, $p<0.01$,and $p<0.001$ Significant differences as determined by Mann-Whitney t-tests comparing each sample in the presence and absence of RU486 are indicated by asterisks where *, **, ***, **** represent $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively. The results show pooled data from at least 8 (A) or 7 (B) independent PBMC donors ± S.E.M. Different sets of PBMC donors were used to conduct experiments in (A) compared to (B).

4.5 GR and PR are differentially expressed across PBMC cell subtypes

The results of the present study have shown that the progestins exhibit distinct RBAs for the GR and elicit GR-mediated transcriptional responses in COS-1 cells (**Figure 3.2.1.2–3.2.2.2**). In addition, the progestins exhibit a consistent rank order of efficacy in inducing GR-mediated transcriptional responses across COS-1, HeLa, and PBMC model systems (**Table 3.2.2-3.2.5**). Similarities between progestin-induced transcriptional responses in COS-1 cells exogenously expressing the GR in the absence of other SRs and HeLa cells endogenously expressing indicate a significant role for the GR. In contrast, pinpointing GR involvement in PBMCs is complicated by the mixed cell population and the variable expression of multiple SRs.

PR expression in PBMCs remains controversial due to inconsistencies in the available literature; whereas, there is more consistent data showing GR expression in PBMCs (Asin *et al.*, 2008; Arruvito *et al.*, 2008; Cabrera-Munoz *et al.*, 2012; Tomasicchio *et al.*, 2013; Polikarpova *et al.*, 2019). Notably, studies investigating SR expression within PBMC cell subtypes are limited and report contrasting findings. One study investigating SR gene expression in $CD4^+$ and $CD8^+$ T cells and $CD14^+$ monocytes found detectable levels of GR mRNA, but not PR mRNA (Brundin

et al., 2021). Using immunofluorescence, another study showed that $CD4^+$ T cells do not express detectable levels of PR; whereas, flow cytometry analysis showed that PR expression was detected at very low frequencies on $CD4^+$ and $CD8^+$ T cells (Arruvito *et al.*, 2008).

To date, only one study has reported detectable ER protein expression in whole PBMCs (Asin *et al.*, 2008), while others only detected ERα/ER and MR mRNA expression (Tomasicchio *et al.*, 2013; Brundin *et al.*, 2021) Given the limited evidence and the probability that relative ER and MR expression levels would be too low to mediate any progestin activity, ER and MR protein expression was not assessed in the present study. Furthermore, this panel of progestins have little to no binding affinity for the ERα and MR (Africander, Verhoog, and Hapgood, 2011; Stanczyk *et al.*, 2013; Africander, Louw, and Hapgood, 2013; Micks and Jensen, 2020).

While multiple studies have shown that MPA is a potent GR and PR agonist (Hapgood, Africander*, et al.*, 2014; Enfield *et al.*, 2020), previous *ex vivo* studies using the GR/PR antagonist RU486 suggest that the MPA regulates HIV-1 infection and immune gene expression through mechanisms likely involving the GR in PBMCs (Govender *et al.*, 2014; Hapgood, Ray*, et al.*, 2014; Maritz *et al.*, 2018; Bick *et al.*, 2022). However, RU486 is not a GR-specific antagonist and can diminish the effects of both the GR and PR. Furthermore, the variation in progestin-induced HIV-1 infection and immunomodulatory effects among PBMC donors in the present study, raises the possibility of inter-individual differences in GR:PR expression ratios

between individuals (**Figures 3.2.5.1-3.2.6.1B and 4.2.1-4.4.1**). To explore this and gain preliminary insight into the SRs potentially involved in mediating progestin responses in PBMCs, the GR and PR expression profiles were assessed using flow cytometry in $CD3^+$, $CD4^+$ and $CD8^+$ T cells, as well as $CD14^+$ monocytes from multiple donors.

PBMCs thawed overnight were stained with antibodies specific for the GR or the PR, as well as cell surface markers (see **Appendix A, Figure A1** for gating strategy). **Figure 4.5.1** summarises the median relative percentage expression of the GR and PR in the different T cells and monocytes in PBMCs isolated from eight donors. The GR and PR were undetectable in approximately 50% of $CD3^+$ and $CD8^+$ T cells (**grey**); whereas a considerable percentage of the cells expressed both the GR and PR (**yellow**) in $CD3^+$ (~44%), $CD4^+$ (~61%) and $CD8^+$ (~41%) T cells. Very few cells were detected expressing the GR only (**blue**) in $CD3^+$ (~7%), $CD4^+$ (~7%) and $CD8^+$ (~6%) T cells. In $CD14^+$ monocytes, both receptors were undetectable in approximately 64% of the cells (**grey**), while approximately 20% of the cells expressed both the GR and the PR (**yellow**). Interestingly, **Figure 4.5.1** shows that $CD14^+$ monocytes were detected to express the highest ratio (~14%) of GR^+PR^- cells (**blue**). Overall, a small proportion (~0-2%) of the T cells and monocytes expressed only the PR (**red**).

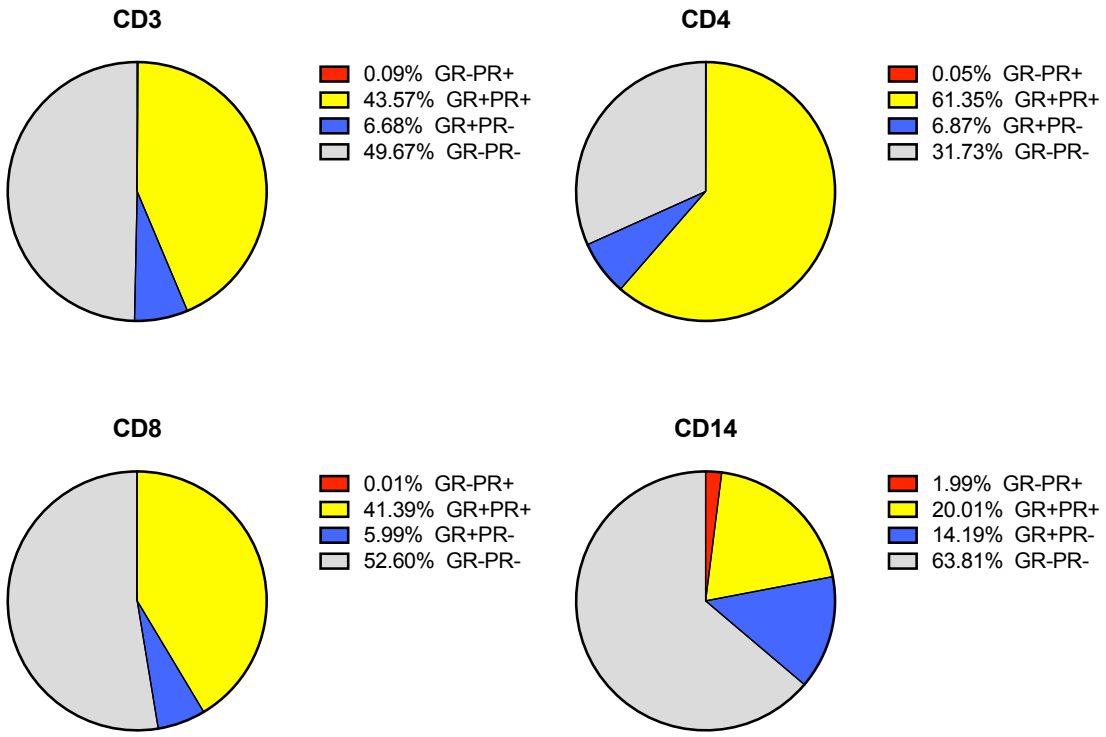


Figure 4.5.1: GR and PR are co-expressed in PBMC cell subtypes. PBMCs from eight independent donors were stained with cell surface markers and antibodies specific for the GR or the PR. The levels of GR and PR were determined in $CD3^+$, $CD4^+$, $CD8^+$ T cells and $CD14^+$ monocytes using flow cytometry. Results are shown as pie charts indicating the median relative % expression of the SR: PR only (**Red**); GR and PR (**Yellow**); GR only (**Blue**); None (**Grey**).

As shown in **Figure 4.5.2A**, the frequency of $CD4^+$ T cells co-expressing GR was the highest compared to the frequency of $CD3^+GR^+$, $CD8^+GR^+$ T cells and $CD14^+GR^+$ monocytes. While the frequency of $CD14^+GR^+$ monocytes appeared to be the lowest compared to the GR-expressing T cells, no significant differences in frequency were detected when comparing $CD14^+GR^+$ monocytes to $CD8^+GR^+$ T cells, and $CD3^+GR^+$ T cells to $CD8^+GR^+$ T cells. In terms of the PR, significant variability was observed across the frequency of PBMC cell subtypes co-expressing PR. The frequency of $CD14^+PR^+$ monocytes was significantly lower compared to the frequency of $CD3^+PR^+$, $CD4^+PR^+$ and $CD8^+PR^+$ T cells. The frequency of $CD4^+PR^+$

T cells expressing PR was significantly higher compared to $CD3^+PR^+$ and $CD8^+PR^+$ T cells, as well as $CD14^+PR^+$ monocytes (**Figure 4.5.2A**), reflecting a similar trend observed in the frequency of $CD4^+GR^+$ T cells.

In **Figure 4.5.2B,** significantly higher GR expression was observed in $CD4^+$ T cells compared to $CD3^+$ T cells; whereas, no significant differences in GR MFI were detected across $CD8^+$ T cells and $CD14^+$ monocytes. In contrast, PR expression was significantly different across the cell subtypes. **Figure 4.5.2B** shows PR expression was significantly higher in $CD14^+$ monocytes compared to PR^+CD3^+, PR^+CD4^+ and PR^+CD8^+ T cells. While the GR and PR expression levels are not directly comparable as different antibodies are used for detection, assuming the comparable antibody effectiveness, **Figure 4.5.2B** suggests that GR expression is higher than PR expression in $CD3^+$, $CD4^+$ and $CD8^+$ T cells, as well as $CD14^+$ monocytes. This observation is in line with the results obtained in **Figure 4.5.1**, where a relatively low proportion (~0-2%) of PR-only cells (**red**) were detected across the PBMC cell subtypes. These results indicate that, in cells expressing both the GR and PR (**yellow**), the relative abundance of the GR protein is most likely much higher than that of the PR, based on some assumptions. Although this is not easy to accurately quantify, the apparent abundance of receptor expression depends not only on biological target protein levels, but also on technical factors, including antibody affinity and specificity, epitope accessibility, and instrument sensitivity (Cossarizza *et al.*, 2021). An estimate of relative abundance of PR and GR assumes that the above factors do not vary, which may not be valid.

Antibody specificity in the flow cytometry experiments was demonstrated through the use of fluorescence minus one (FMO) controls (**Appendix A, Figure A1)**, which allowed accurate gate placement and distinction between true signal and background fluorescence. In addition, the inclusion of viability staining further minimized non-specific binding from dead or dying cells. While these approaches increased the reliability of the signal, a key limitation remains. Antibody cross-reactivity between GR and PR was not directly assessed in the present study. Definitive validation would require additional controls, such as reciprocal testing of antibodies in cells selectively expressing GR or PR, or genetic knockdown/knockout approaches, to exclude the possibility of cross-recognition (Appadoo, 2025).

Taken together, these results show that the GR and the PR are differentially expressed across the different PBMC cell subtypes. Additionally, the combined frequency of $CD3^+$, $CD4^+$, $CD8^+$ and $CD14^+$ cells expressing GR or PR, as well as the combined expression level (MFI) of GR or PR in these cells varied between donors (**Appendix B, Figure. B3**). For example, the combined/total frequency of GR-expressing cells in donor 8 (~ 250%) was about 1.6 times higher than that of donors 1 and 6 (**Appendix B, Figure B3A**). Interestingly, while donors 1 and 6 had similar combined frequency of $CD3^+$, $CD4^+$ and $CD8^+$ T cells, as well as $CD14^+$ monocytes expressing GR (**Appendix B, Figure B3A**), the combined expression (MFI) of GR was much higher in donor 1 compared to donor 6 (**Appendix B, Figure B3B**). Similarly, the combined frequency of $CD3^+$, $CD4^+$ and $CD8^+$ T cells, as well as $CD14^+$ monocytes expressing PR also varies between donors, while less

variation between donors is observed for combined PR expression (MFI) (**Appendix B, Figure B3**).

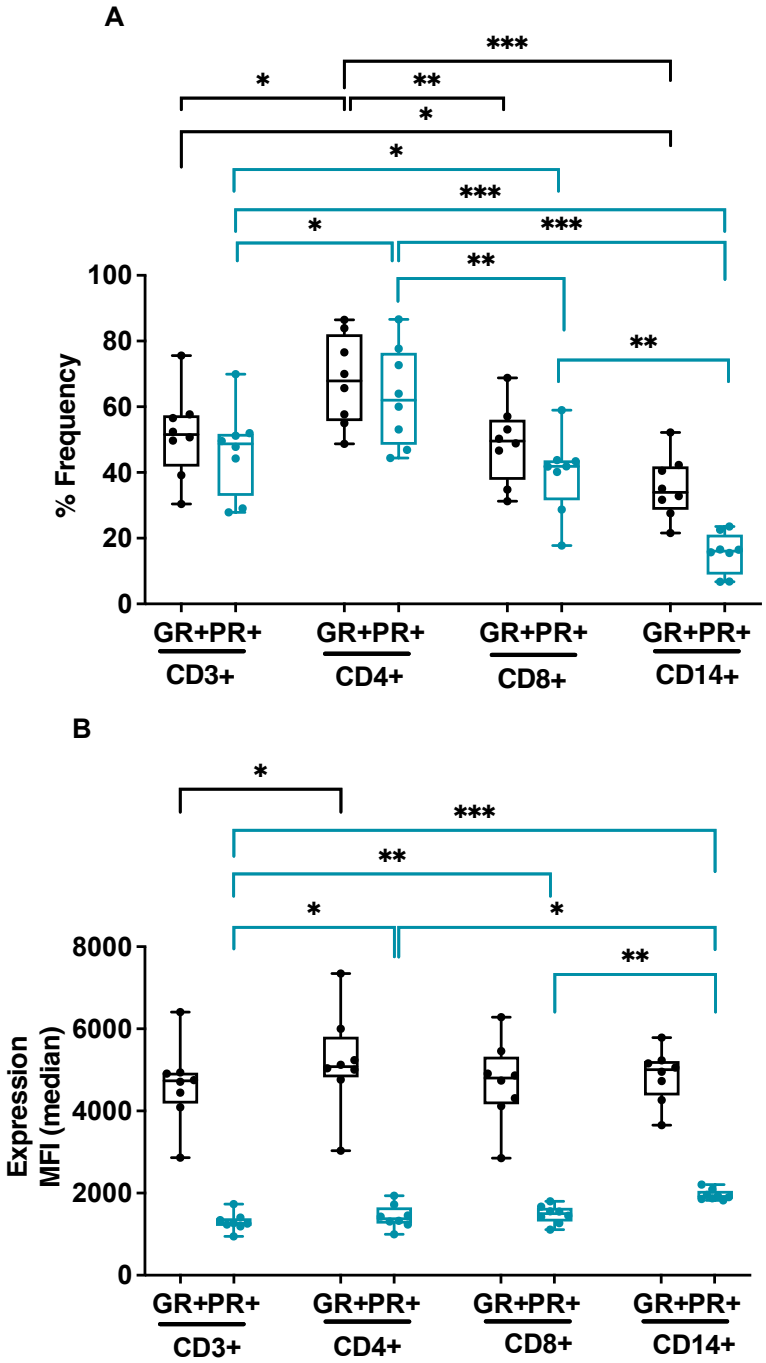


Figure 4.5.2: GR and PR are differentially expressed across PBMC cell subtypes: PBMCs were stained with cell surface markers and antibodies specific for the GR or the PR. The levels of GR (**black**) or PR (**blue**) were determined in $CD3^+$, $CD4^+$ and $CD8^+$ T cells, as well as $CD14^+$ monocytes using flow cytometry. Results shown as (A) % frequency of $CD3^+$, $CD4^+$, $CD8^+$ and $CD14^+$ cells co-expressing GR (**black**) or PR (**blue**) or (B) MFI of GR (**black**) or PR (**blue**) on $CD3^+$, $CD4^+$ and $CD8^+$ T cells, as well as $CD14^+$ monocytes. These data represent the mean ± SEM of eight donors. Statistical analysis via one-way ANOVA with Tukey post-test was performed on each data set, and significant differences are indicated by asterisks where *, **, ***, **** represent $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively.

4.6 GR and PR are differentially expressed in FGT tissue compartments

The results in **Figure 4.5.2** revealed that $CD4^+$ T cells express a higher % frequency of the GR and PR compared to $CD3^+$ and $CD8^+$ T cells, as well as $CD14^+$ monocytes. This most likely have important physiological implications since $CD4^+$ T cells are primary HIV-1 target cells (Rottman *et al.*, 1997; McDonald *et al.*, 2002). Given that the FGT consists of various cell types and is considered the primary site for HIV-1 infection (Ma *et al.*, 2020), understanding whether the GR and PR are co-expressed with CD4 cells in the FGT will give some insight into which receptor most likely be involved in the potential progestin-induced regulation of HIV-1 infection in the FGT. Studies have shown that along with the PR and ER, the AR, GR and MR are expressed in the FGT (Mertens *et al.*, 2001; Brosens *et al.*, 2004; Khan *et al.*, 2005; Ildgruben *et al.*, 2005; Ackerman *et al.*, 2016; Ray *et al.*, 2019). While MPA and NET can bind to the AR and MR, there is very limited and inconsistent literature on LNG, ETG and NES binding to the AR, ER and MR (Kumar *et al.*, 2000; Africander, Louw, and Hapgood, 2013; Africander, Storbeck, and Hapgood, 2014; Louw-du Toit *et al.*, 2017; Louw-du Toit, Hapgood, and Africander, 2020). Given that all the progestins act primarily through the PR and were shown to bind to and activate the GR in this study, as an initial qualitative view, only the GR and PR expression profile and whether these receptors are co-expressed with CD4 were investigated. Furthermore, investigating all SRs was beyond the scope of this thesis.

Ectocervical explant tissue from a single HIV-1 negative pre-menopausal woman was double stained with antibodies that recognise CD4 and the GR or PR. While

antibodies were not used to stain and identify epithelial or stromal cells, the basement membrane, a string of tightly packed cells at the bottom of the epithelium, was used to visually identify the epithelial layer (above the basement membrane) or the stromal cells (below the basement membrane) (See **Appendix B, Figure B4** for annotation). Secondary antibody control experiments were performed, where staining was done with both Cy3 anti-rabbit and Alexa 488 anti-mouse, in the absence of primary antibodies, in both the epithelial (**Panel A**) and stromal (**Panel B**) cells. In these control experiments, very little non-specific background signal was detected in the red channel, while non-specific background signal was undetectable in the green channel, in both the epithelial (**Panel A**) and stromal (**Panel B**) cells, (**Figure 4.6.1**). These results indicate negligible non-specific binding of both secondary antibodies. For the GR single stain control (mouse anti-GR primary with Alexa 488 anti-mouse and Cy3 anti-rabbit secondary antibodies), a very strong GR signal was obtained in the green channel, while very little non-specific signal was detected in the red channel (**Figure 4.6.2 Panel A**), consistent with negligible non-specific binding of the Cy3 anti-rabbit secondary antibody seen in **Figure 4.6.1**. Similar results were obtained in the PR single stain control (mouse anti-PR primary with Alexa 488 anti-mouse and Cy3 anti-rabbit secondary antibodies) (**Figure 4.6.2 Panel B**). Notably, in the majority of these cells, the GR and PR appear to be located in the nucleus (**Figure 4.6.2**). For the GR and the PR single stain control experiments, a single field of view with a strong GR or PR signal was chosen and cell type (epithelial or stromal) was not taken into consideration. For the CD4 single stain control (rabbit anti-CD4 primary with Alexa 488 anti mouse and Cy3 anti-rabbit secondary antibodies), strong CD4 signals were observed in both the epithelial and

stromal cells, hence two fields of view, one for each cell type, were chosen (**Figure 4.6.3**). **Figure 4.6.3** shows that strong CD4 signals were obtained in the red channels, but very little non-specific signal was detected in the green channels, which is consistent with the negligible non-specific binding of the Alexa 488 anti-mouse secondary antibody shown in **Figure 4.6.1**.

Taken together, these controls indicated minimum background non-specific staining and strengthen the results of the double stain experiments (CD4 and GR or CD4 and PR) as given below.

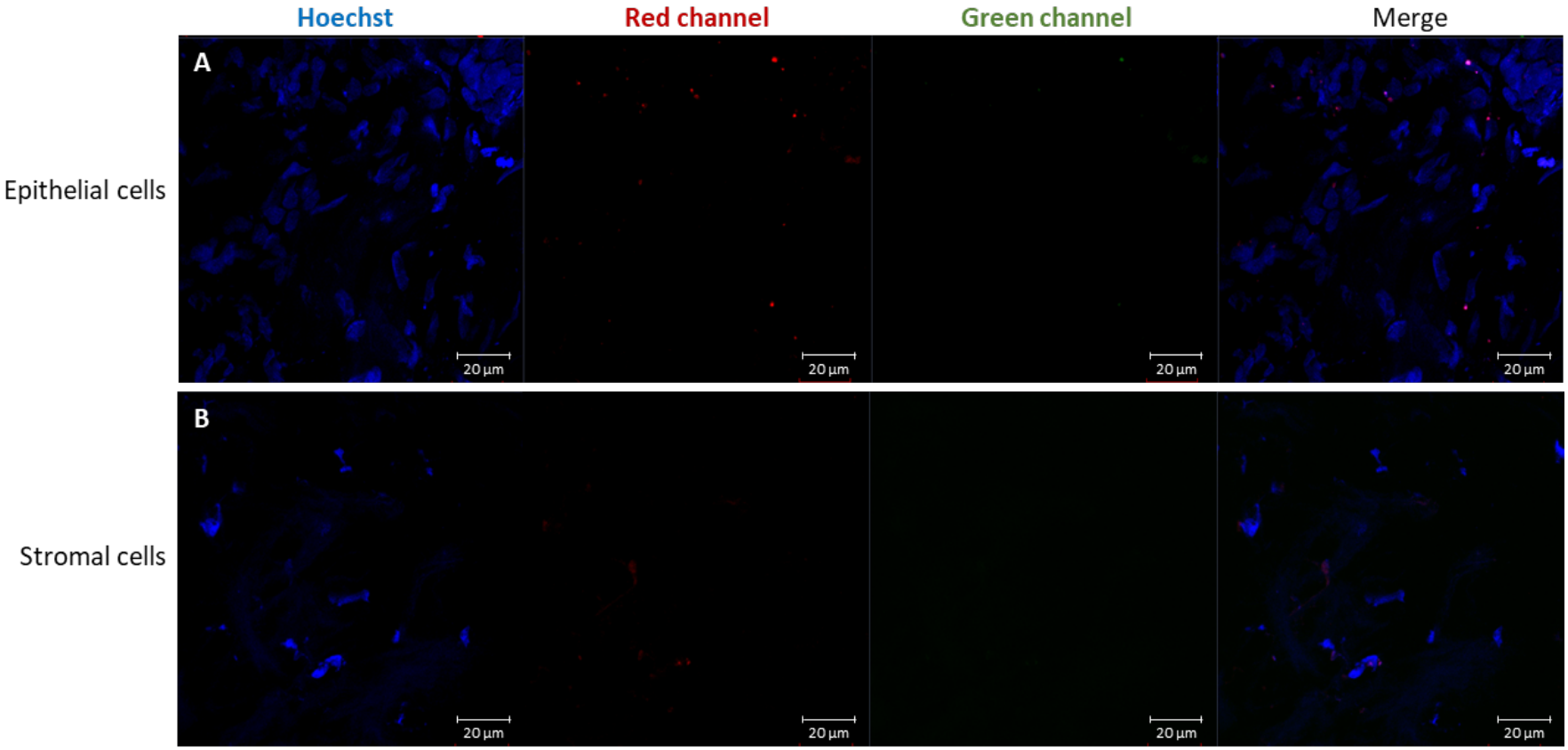


Figure 4.6.1. Secondary antibody control. Ectocervical tissue was fixed with 2% formaldehyde, washed, blocked and permeabilised as described in **Section 2.12** and stained with Cy3 anti-rabbit (red channel) and Alexa 488 anti-mouse (green channel) secondary antibodies in the absence of primary antibodies. Tissue was counterstained for nuclear material with Hoechst

(blue). Images represent the epithelial cells (**Panel A**), and stromal cells (**Panel B**) based on visual identification of the basement membrane cell layer.

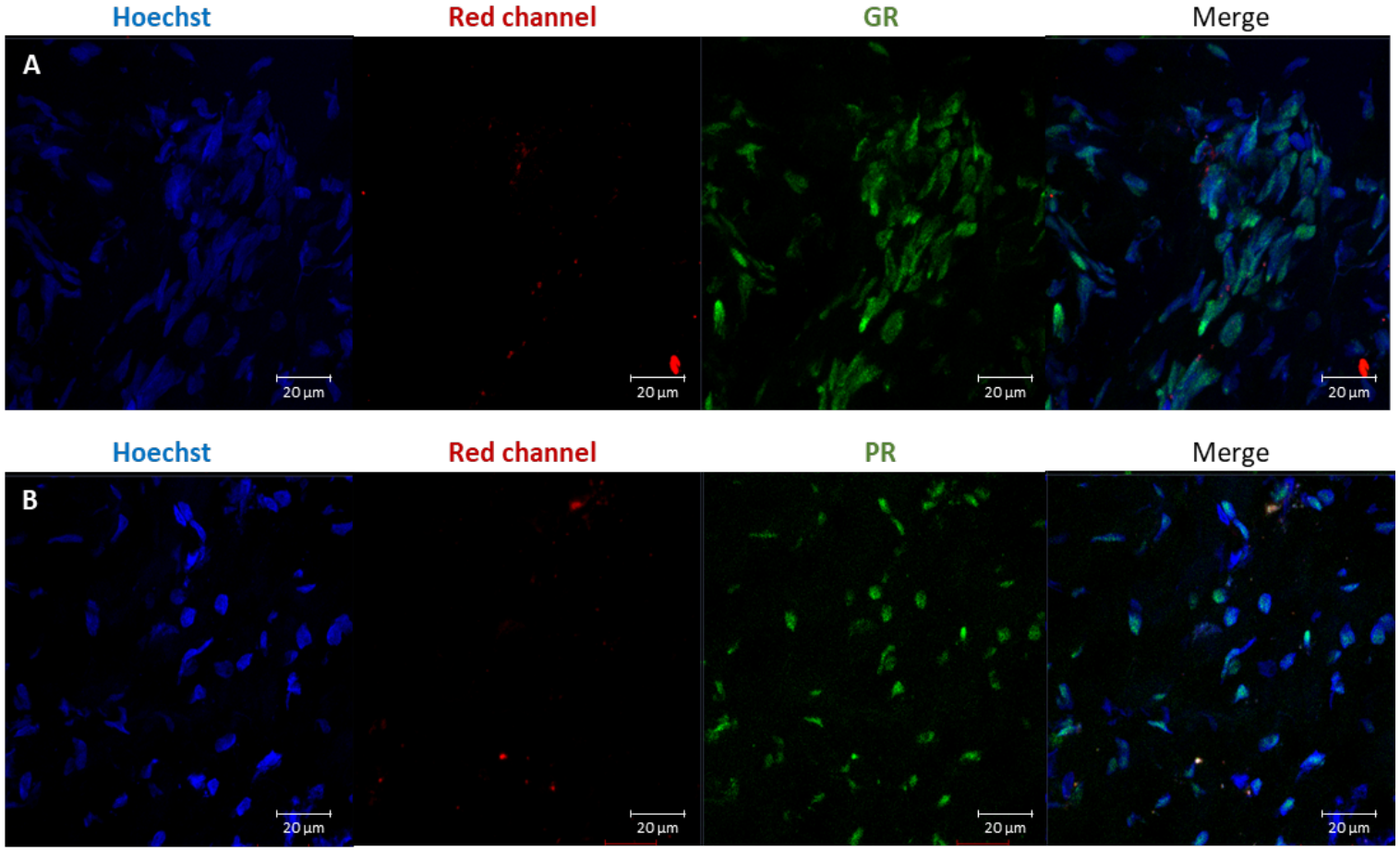


Figure 4.6.2. GR and PR single stain controls. Ectocervical tissue was fixed with 2% formaldehyde, washed, blocked and permeabilised as described in **Section 2.12**. Tissue was stained with a mouse GR antibody (**Panel A**) or mouse PR antibody (**Panel B**). In both panels tissue was stained with Cy3 anti-rabbit (red channel) and Alexa 488 anti-mouse (green channel) secondary antibodies. Tissue was counterstained for nuclear material with Hoechst (blue).

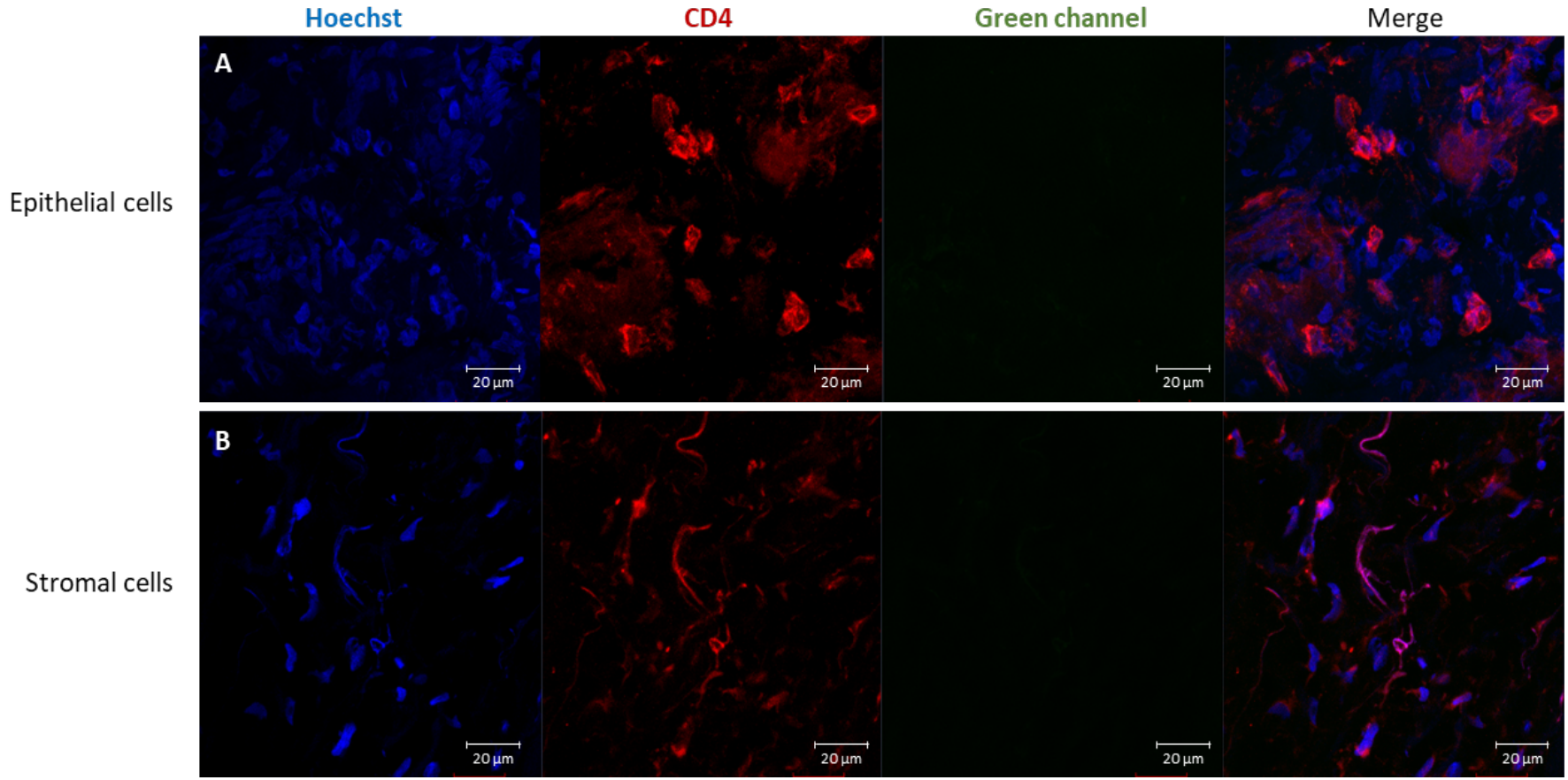


Figure 4.6.3. CD4 single stain control. Ectocervical tissue was fixed with 2% formaldehyde, washed, blocked and permeabilised as described in **Section 2.12**. Tissue was stained with a rabbit CD4 antibody (Panel A) and Cy3 anti-rabbit (red channel) and Alexa 488 anti-mouse (green channel) secondary antibodies. Tissue was counterstained for nuclear material with Hoechst (blue). Images represent the epithelial cells (**Panel A**), and stromal cells (**Panel B**) based on visual identification of the basement membrane cell layer.

Figure 4.6.4 is an immunofluorescent microscopy image depicting CD4- and GR-expressing cells in the epithelial layer (**Panel A**) and stroma (**Panel B**) of the ectocervix. The images show that both the GR and CD4 are expressed in the epithelial layer and stroma of the ectocervix with some cells indicating that the GR and CD4 are co-expressed. In **Figure 4.6.4** (**Panel C**), the PR and CD4 are both expressed in the ectocervix, with very few cells co-expressing the PR and CD4. While the PR does not appear to be expressed in the epithelial layer of the ectocervix, more PR expression was detected in stromal cells. On the other hand, the GR appeared to be expressed in both the epithelial layer and stroma of the ectocervix. These results suggest that the GR is possibly expressed throughout the ectocervix whilst PR expression most likely be localised in the stroma of the ectocervix. Additionally, it appears that a large proportion of cells expressing CD4 also express the GR; whereas, CD4 and PR co-expression seems relatively limited. These results should be regarded as qualitative and hypothesis-generating. Fluorescence intensity reflects both biological and technical variables. Therefore, to accurately assess relative GR and PR abundance, future research should employ more precise quantitative approaches, such as radioligand binding assays.

Flow cytometry analysis of PBMCs indicated that a subset of $CD4^+$ T cells co-expressed both GR and PR (**Figure 4.5.1**), however, immunofluorescent staining of ectocervical tissue suggested more limited PR co-localisation with CD4 in these cells (**Figure 4.6.4**). These apparent discrepancies likely reflect a combination of technical and biological factors. Flow cytometry provides high-throughput, quantitative single-cell measurements of receptor expression across large

populations, whereas immunofluorescence offers spatial information within intact tissue but is more susceptible to variability introduced by tissue architecture and staining conditions (Cossarizza *et al.*, 2021; Brundin *et al.*, 2021; North, 2006).

Technically, the two approaches differ in sensitivity and in their ability to detect cytoplasmic versus nuclear receptors; fixation and permeabilisation can alter antibody accessibility and thus influence detection patterns (North, 2006; Cossarizza *et al.*, 2021).

Biologically, PBMCs and ectocervical tissue represent distinct immunological compartments in which relative receptor expression may differ between systemic immune cells and mucosal tissue compartments. PR has been reported at low but detectable levels in peripheral T cells (Polikarpova *et al.*, 2019; Cabrera-Munoz *et al.*, 2012), whereas PR expression in ectocervical tissue is typically more restricted, heterogeneous and influenced by factors such as menstrual cycle stage and local microenvironment (Graham and Clarke, 1997; Tarini and Özardali, 2021). Taken together, these technical and biological differences likely explain the inconsistencies in CD4 receptor co-expression observed in the present study.

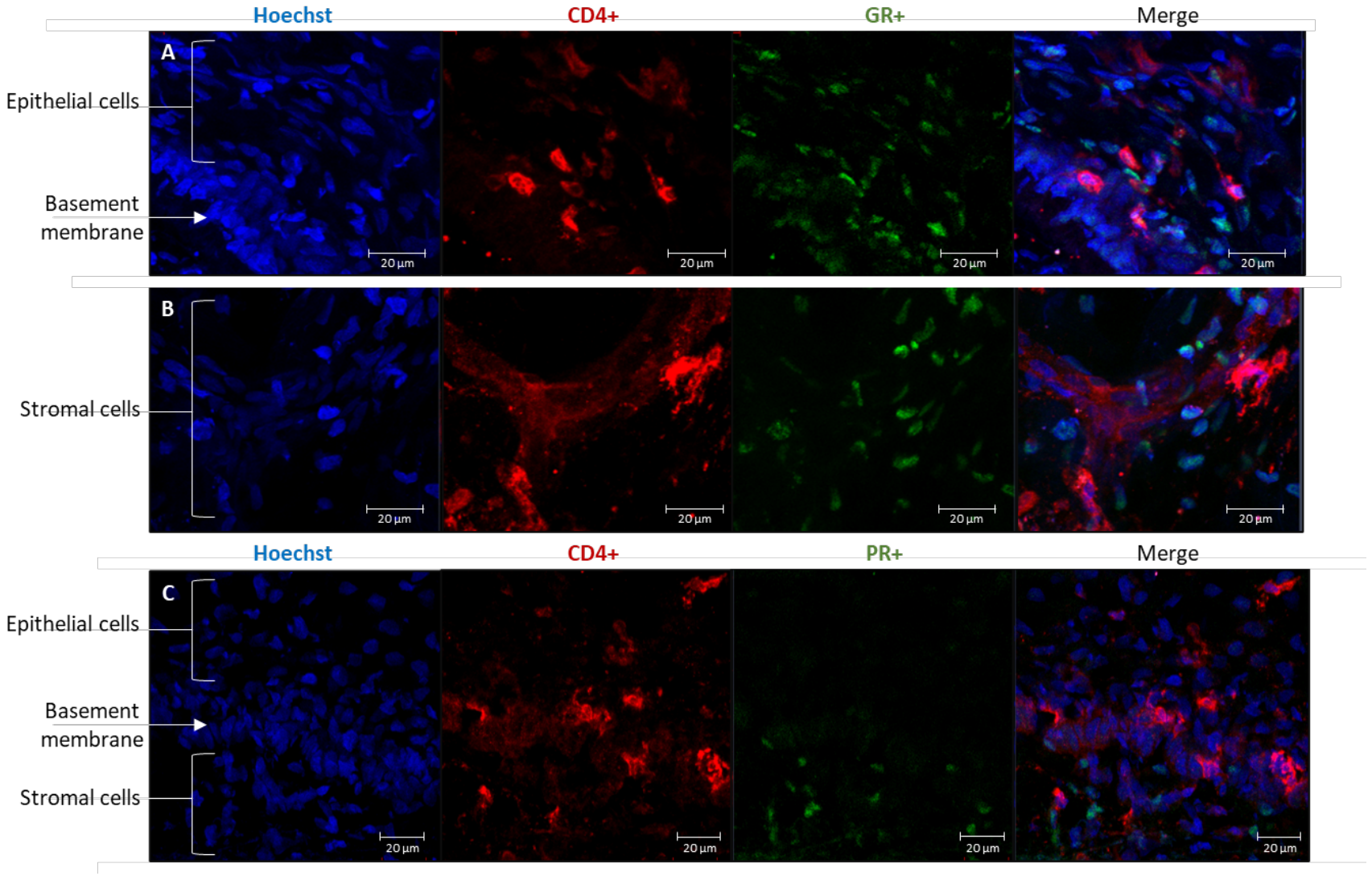

Hoechst
CD4+
GR+
Merge
A
Epithelial cells
Basement membrane
20 µm
B
Stromal cells
Hoechst
CD4+
PR+
Merge
C
Epithelial cells
Basement membrane
Stromal cells

Figure 4.6.4. A representative immunofluorescent microscopy image depicting the co-expression of the GR and CD4 (Panel A and B) or PR and CD4 (Panel C) in the epithelial and stromal layer of ectocervix tissue. Ectocervical tissue was fixed with 2% formaldehyde, washed, blocked and permeabilised as described in **Section 2.12**. Tissue was co-stained with a rabbit CD4 and mouse GR antibody (Panel A and B) or mouse PR antibody (Panel C). All tissue was stained with Cy3 anti-rabbit (red channel) and Alexa 488 anti-mouse (green channel) secondary antibodies. Tissue was counterstained for nuclear material with Hoechst (blue). Images represent the epithelial and stromal cells based on visual identification of the basement membrane cell layer. Ectocervical explant obtained from one patient.

Chapter 5

Progestins exhibit differential degrees of metabolism within and across different model systems

5.1 Background

To further elucidate the molecular mechanisms that may explain some of the differential effects of progestins in various model systems, progestin metabolism in PBMCs, COS-1, and HeLa cells was investigated. Thereafter, correlation analyses were conducted to assess whether a relationship exists between progestin efficacy and potency and progestin metabolism in the three model systems. The current author has contributed to a study in which some data from this chapter have been published in Skosana et al., 2019.

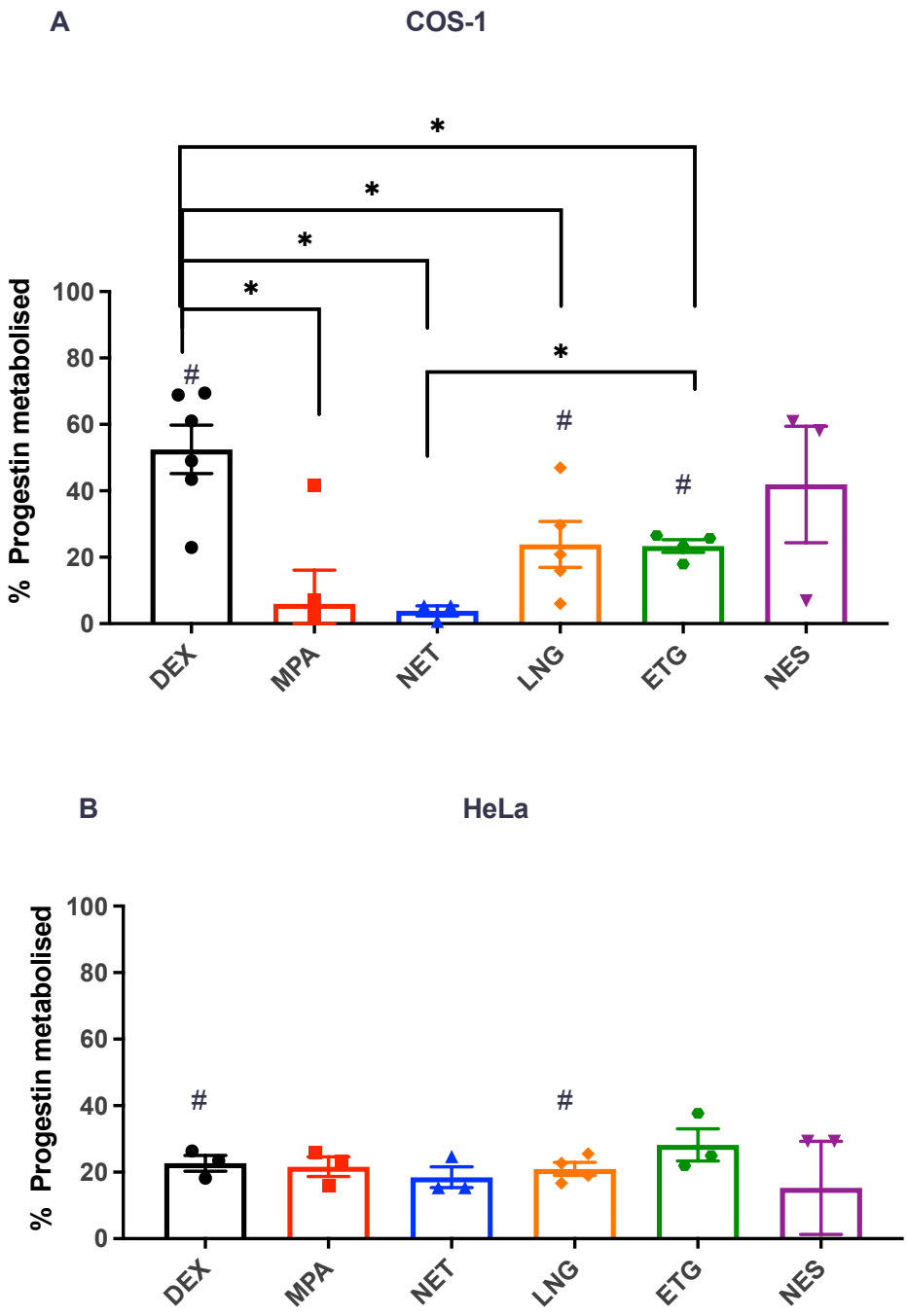
5.2 Progestins are differentially metabolised in a cell-specific manner

To investigate the metabolism of the panel of progestins in parallel with DEX, COS-1 and HeLa cells were seeded and stimulated with 100 nM of progestins for 24 hours. PBMCs were isolated and incubated with 100 nM of the panel of progestins for 48 hours. For each model system, a 'no cells' negative control for metabolism was included. The progestins were extracted from the supernatant using the methyl tert-butyl ether (MTBE) extraction method as described in **Section 2.11.3**. The cells were also lysed to investigate if the progestins were sequestered into the cells. The

concentration of progestin remaining in the samples was quantified using an optimised and validated UHPSFC-MS/MS method, summarised in **Appendix A, Table A1**. The percentage metabolism obtained is relative to a corresponding no-cell experiment which was set to 100% to account for adsorption loss (not shown).

The results in **Figure 5.2.1A** showed that after a 24-hour incubation in the absence and presence of COS-1 cells, DEX, LNG and ETG were significantly metabolized. All the progestins, but NES, were metabolized significantly less than DEX. MPA and NET were the least metabolised.

No significant differences in % metabolism were detected between the progestins in HeLa cells (**Figure 5.2.1B**). However, DEX and LNG showed significant % metabolism compared to no cells control. After a 48-hour incubation period, DEX exhibited substantial % metabolism in PBMCs compared to the progestins (**Figure 5.2.1C**). MPA was significantly less metabolised than DEX and ETG, with NES being the least metabolised in PBMCs. These results show that the progestins are differentially metabolised within each of the model systems.


A
COS-1
% Progestin metabolised
100
80
60
40
20
0
*
*
*
*
*
#
#
#
DEX
MPA
NET
LNG
ETG
NES
B
HeLa
% Progestin metabolised
100
80
60
40
20
0
#
#
DEX
MPA
NET
LNG
ETG
NES

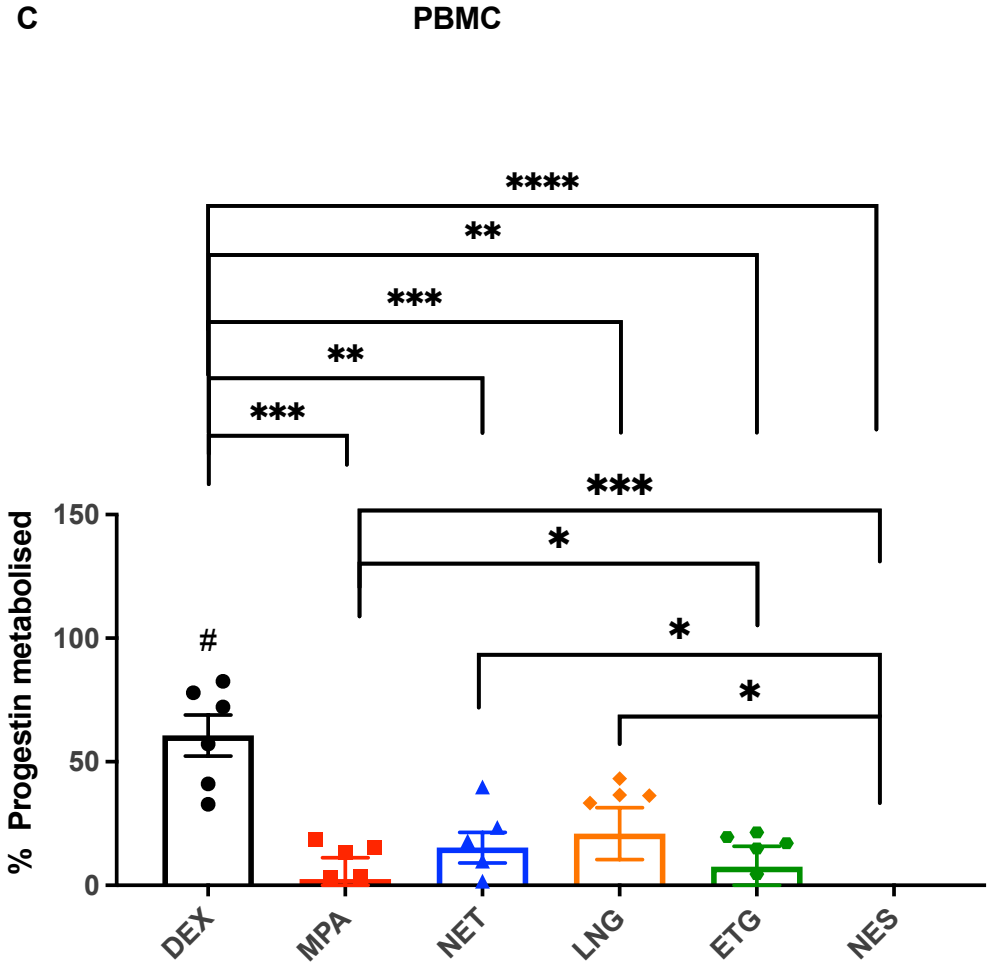


Figure 5.2.1. Progestins are differentially metabolised in COS-1, HeLa cells and PBMCs. COS-1 (A) or HeLa (B) cells or PBMCs (C) were seeded and stimulated with 100 nM progestins for 24 hours or 48 hours. These data represent the mean ± SEM of a minimum of three biological repeats. Statistical analysis for significant differences between the metabolism of each ligand relative to its no cell control was performed via paired t-tests. Significant differences are indicated by hashtags where #, ##, ### represent $p<0.05$, $p<0.01$ and $p<0.001$ respectively. To quantify whether the relative metabolism of two ligands within a cell line was significantly different, paired t-tests were performed between ligands. Significant differences are indicated by asterisks where *, **, ***, **** represent $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively.

When comparing how the progestins are metabolised across the three model systems, no statistical differences were detected for % metabolism of LNG and NET between the three model systems (**Figure 5.2.2**). DEX was significantly less

metabolised in HeLa cells compared to COS-1 and PBMCs. MPA and ETG were significantly less metabolised in COS-1 and PBMCs as to HeLa cells, whereas, NES was differentially metabolised across all three model systems, less so in PBMCs.

Taken together, these results show that the progestins are differentially metabolised in a cell-specific manner.

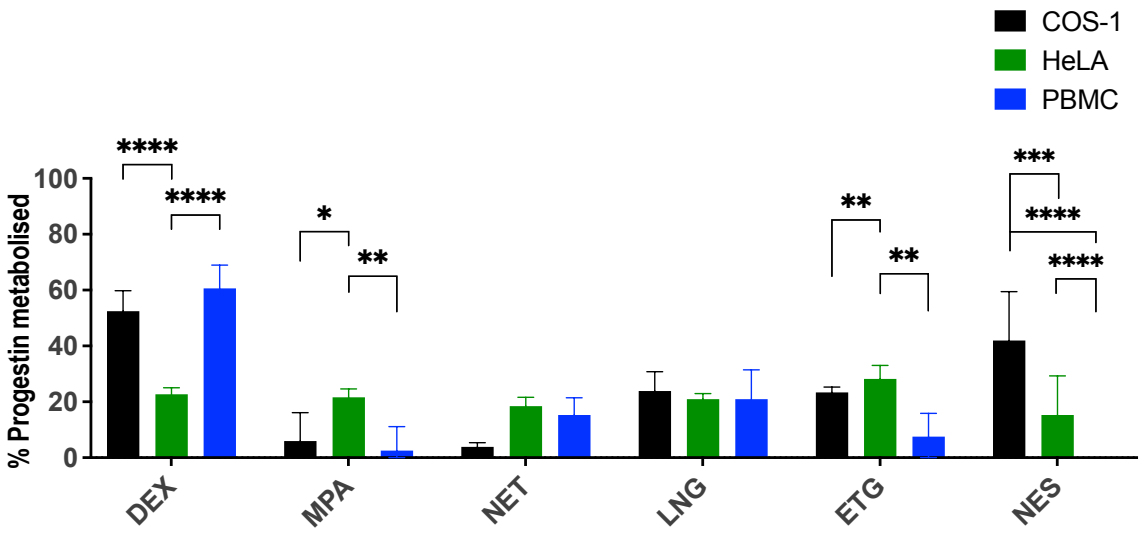


Figure 5.2.2. DEX, MPA, ETG and NES are differentially metabolized between the three model systems. Data from **Figure 5.2.1** were grouped, and a 2-way ANOVA with a Tukey post-test was used to determine statistical differences for the % metabolism of each progestin across the three model systems. These data represent the mean ± SEM of a minimum of three biological repeats. Significant differences are indicated by asterisks where *, **, ***, **** represent $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively.

5.3 Progestin metabolism does not correlate with progestin RBA for the GR or progestin-induced activity via the GR

Having identified that these progestins are differentially metabolised, a relationship between metabolism and progestin affinity for and activity via the GR was next

investigated. Considering that metabolism influences the concentration of added progestins, RBA, relative EC_{50} and/or efficacy values can be affected. First a Pearson correlation was performed to assess whether progestin metabolism correlated with progestin binding affinity for the GR. The RBAs (**Table 3.2.1**) were compared to the results obtained on progestin metabolism (**Figure 5.2.1**). **Figure 5.3.1** shows that there is no correlation between % metabolism of the progestins in COS-1 and HeLa cells and PBMCs and progestin binding affinity for the GR.

Next, a Pearson correlation analysis was performed to investigate whether progestin metabolism in COS-1 and HeLa cells and PBMCs correlates with either progestin efficacy or potency on synthetic or endogenous promoters for transactivation or transrepression. No correlation was found between progestin metabolism and how efficacious or potent the progestin is in any of the model systems on all promoters (**Figure 5.3.2**). This data indicates that the activity of progestins and affinity for the GR likely not be affected by the degree to which they are metabolised in a cellular or primary cell model system.

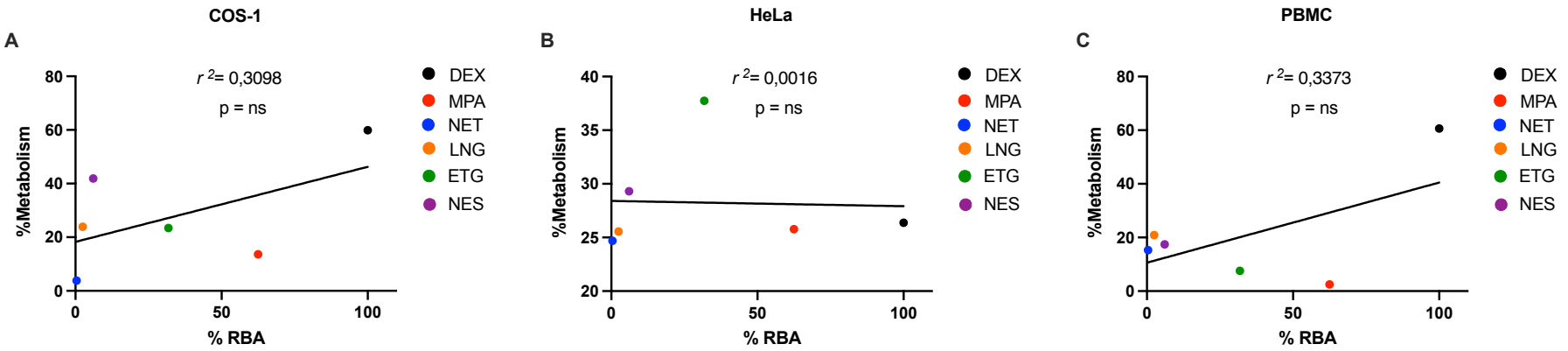
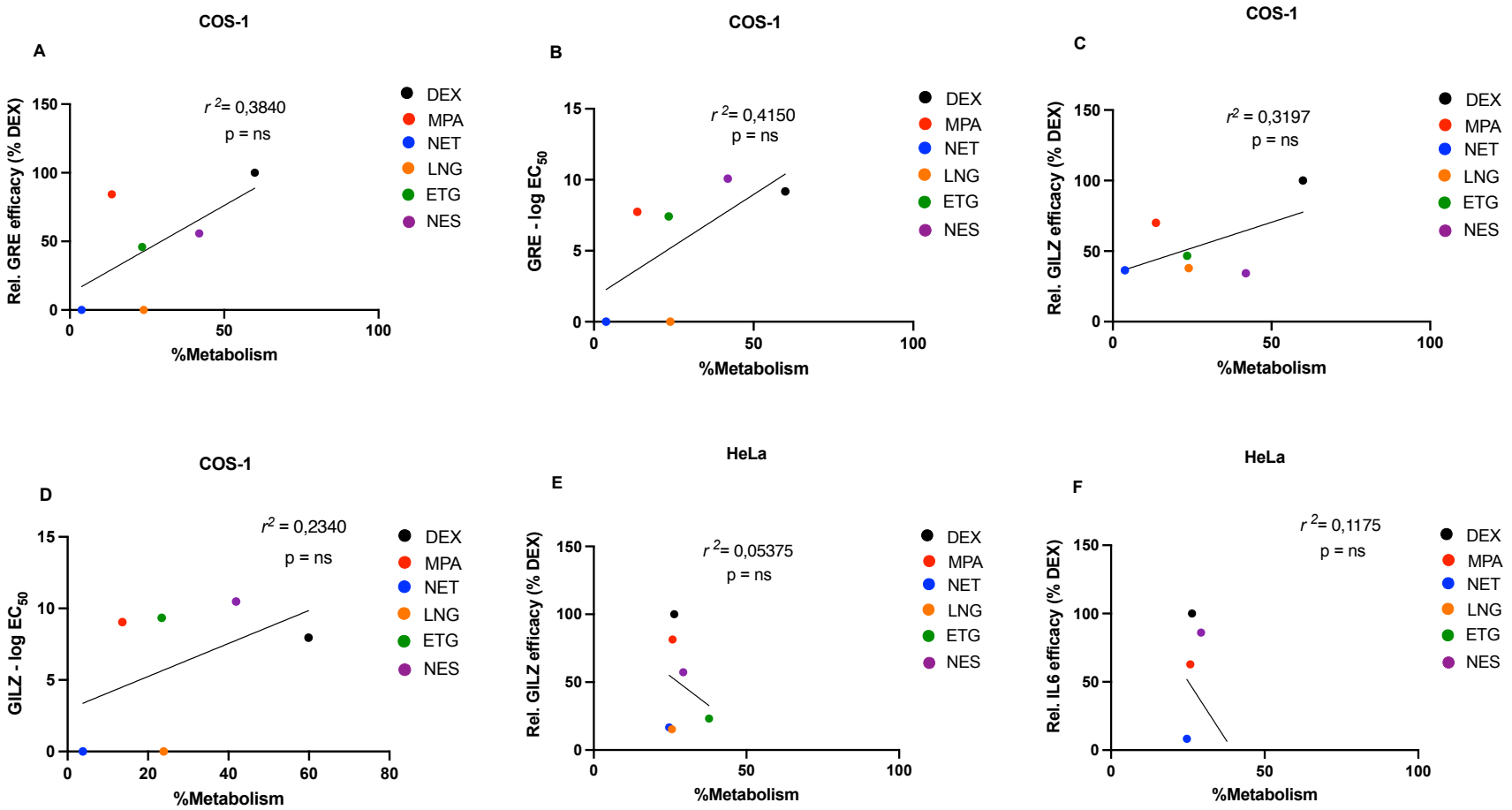


Figure 5.3.1. Pearson correlation analyses showing no correlation between % metabolism and progestin binding affinity for the GR in COS-1(A) and HeLa cells (B) and PBMCs (C). Pearson correlation analysis between % RBA obtained from **Table 3.2.1** and % metabolism from **Figure 5.2.1**. These data represent the mean of a minimum of three biological repeats.


COS-1
A
Rel. GRE efficacy (% DEX)
r^2= 0,3840
p = ns
%Metabolism
COS-1
B
GRE - log EC_{50}
r^2= 0,4150
p = ns
%Metabolism
COS-1
C
Rel. GILZ efficacy (% DEX)
r^2 = 0,3197
p = ns
%Metabolism
COS-1
D
GILZ - log EC_{50}
r^2 = 0,2340
p = ns
%Metabolism
HeLa
E
Rel. GILZ efficacy (% DEX)
r^2= 0,05375
p = ns
%Metabolism
HeLa
F
Rel. IL6 efficacy (% DEX)
r^2= 0,1175
p = ns
%Metabolism
DEX
MPA
NET
LNG
ETG
NES

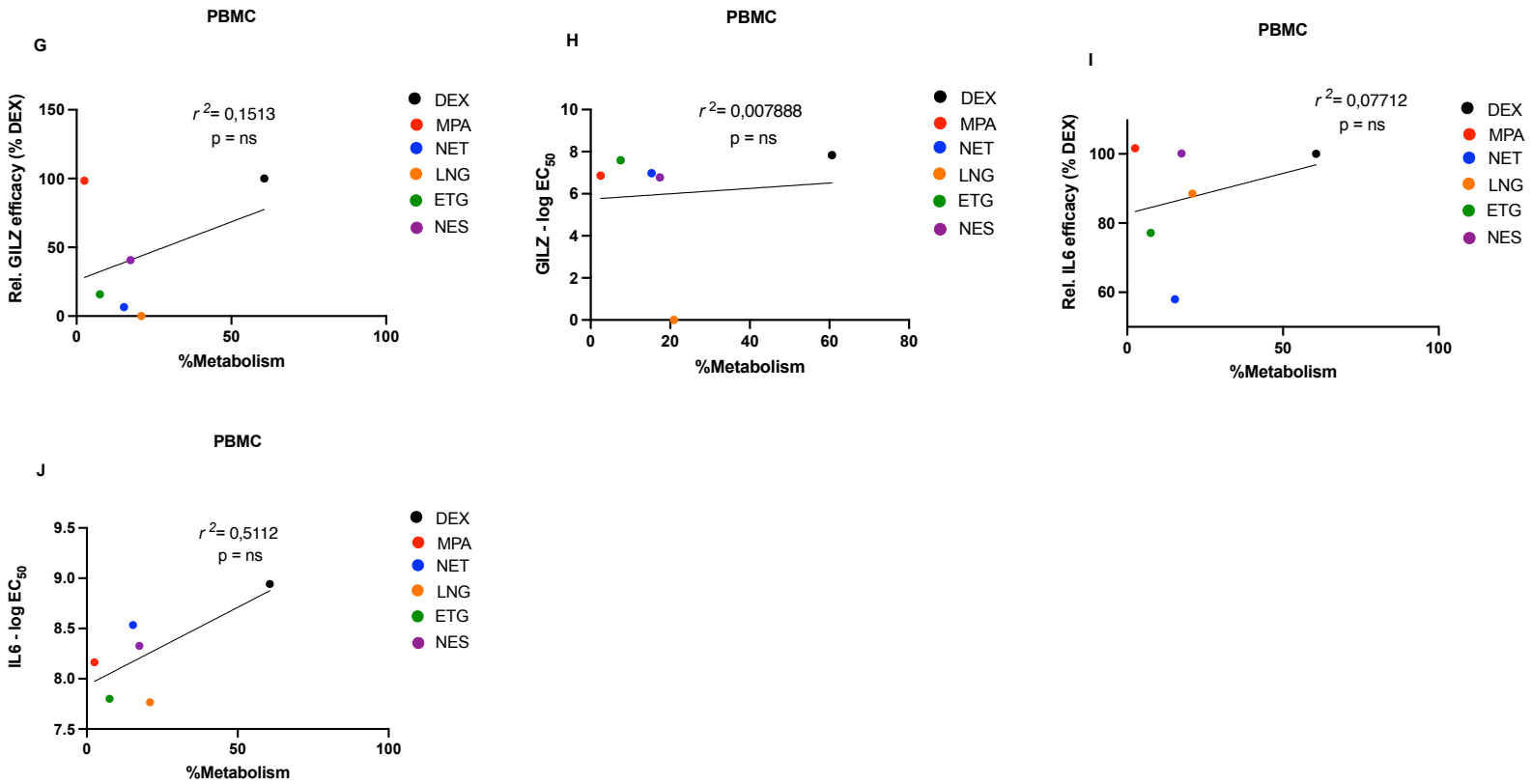


Figure 5.3.2 Pearson correlation analyses showing no correlation between % metabolism and progestin efficacy or potency via the GR in COS-1, HeLa cells or PBMCs. The graphs display Pearson correlation analyses between the % metabolism and relative efficacy of progestins on synthetic and endogenous promoters in COS-1 (A, C) and HeLa (E) cells as well as PBMCs (G, I). Furthermore, the graphs depict Pearson correlation analyses between % metabolism and relative – log EC_{50} (potency) values of progestins on synthetic and endogenous promoters in COS-1 (B, D) and HeLa (F) cells along with PBMCs (H, J). These data represent the mean of a minimum of three biological repeats.

Chapter 6

Discussion

6.1 Profiling the GC characteristics of synthetic progestins

In sub-Saharan Africa, long-acting progestin-only contraception methods such as injectables, IUDs, and implants are commonly used. These contraceptives are comprised of progestins synthesised to exert progestational effects primarily through the PR, but off-target interactions with other SRs, including the GR, have been reported in numerous studies. This highlights the importance of accurately profiling GR-mediated progestin activity, given that these off-target interactions will likely modulate critical physiological functions in tissues expressing the GR. The GR-mediated actions of MPA and NET are well documented; very limited information is available on the GR-mediated actions of LNG, ETG and NES, used in other contraceptives. However, due to varying in vitro experimental approaches across studies, previously published data on the GR-mediated actions of these progestins are not directly comparable. Since MPA, but not NET is known to bind to the GR with relatively high affinity and activate GR target genes as a starting point, the present study determined the GR affinity and activity of LNG, ETG and NES in parallel with MPA and NET. To the best of the present authors' knowledge, this is the first head-to-head study to characterise the GR-mediated affinity and activity of a wide panel of progestins in different model systems.

6.1.1 *Progestins exhibit differential binding affinity for the GR*

The first aim of this study was to investigate the RBA of the synthetic progestins MPA, NET, LNG, ETG, and NES for the GR. In pursuit of more reliable data on relative effects in the absence of confounding factors typical of non-parallel studies, the present study investigated the biological activity of the progestins in parallel and in a single model system.

The results in the present study indicate that MPA, and ETG bind to the GR with higher relative affinity than NET, LNG, and NES (**Figure 3.2.1.2**). Consistent with binding data conducted in animal and *in vitro* models, the present study reveals that MPA (62%) exhibits the highest affinity for the GR relative to DEX, when compared to NET, LNG, ETG and NES (Kontula *et al.*, 1983; Selman, 1996; Koubovec *et al.*, 2005; Schindler *et al.*, 2008).

Similar to the study by Fuhrmann *et al.* which reported an ETG RBA of 40%, the current study observed that ETG (30%) exhibited substantial binding to the GR; whereas, studies by Kumar *et al.*, Schindler *et al.*, Pollow *et al.*, and Kuhl *et al.* reported that ETG binds to the GR with low affinity (Fuhrmann, Slater, and Fritzemeier, 1995; Kumar *et al.*, 2000; Schindler *et al.*, 2008; Pollow *et al.*, 1992; Kuhl, 2005). Consistent with binding affinities reported by Schindler *et al.*, Pollow *et al.*, and Kumar *et al.*, LNG and NET exhibited little to no affinity for the GR (Pollow *et al.*, 1992; Schindler *et al.*, 2008; Kumar *et al.*, 2000).

Given that there are limited comparative studies available on the selected progestins affinity for the GR, there is not much data available with which to make direct comparisons. Some data reported the IC_{50}, while others reported the K_i, and some solely reported the RBA (Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009; Selman, 1996; Koubovec *et al.*, 2005; Schindler *et al.*, 2008; Fuhrmann, Slater, and Fritzemeier, 1995). Knowing the concentration at which the progestins likely to compete with endogenous GCs for binding to the GR is useful because these progestins are used in contraceptives and the GR is expressed in most tissues and cells, so the progestins binding to the GR likely result in GR-mediated side effects.

When comparing the IC_{50} values obtained in this study, MPA (5.58 nM) and ETG (11.5 nM) exhibited an IC_{50} statistically similar to DEX (3.46 nM). This result suggests that, similar to DEX, MPA and ETG likely exert GC-like effects at these concentrations. A study by Bick *et al.* reported the serum concentration range for intramuscular injection of MPA to be Cmax 2.6-99.6 nM and the serum concentration range for orally administered ETG to be 4.692-19.21 nM (Bick *et al.*, 2021a). As the IC_{50} values fall within the serum concentration range of contraceptive users (**Appendix B, Table B2**), this suggests that the MPA and ETG binding affinity for the GR may be physiologically relevant. Consistent with the findings by Kumar *et al.*, the present study demonstrates that NES exhibits an IC_{50} of 57 nM (Kumar *et al.*, 2000). However, these IC_{50} values, including, those of LNG and NET, suggest a significantly lower RBA to GR compared to MPA and ETG, and fall outside of the physiological serum concentration range observed in contraceptive users

(**Appendix B, Table B2**). As such, off-target GR interactions are not anticipated when these progestins are employed for contraception

According to Kontula *et al.,* pregnane derivatives exhibit substantial binding affinity for the GR; whereas, nortestosterone derivatives display very little binding affinity for the GR (Kontula *et al.*, 1983). This holds true for MPA, LNG, and NET; however, the present study showed that ETG, a nortestosterone derivative, binds to the GR with relatively high affinity. In contrast to the findings of Kuhl *et al.,* which reported a 38% binding affinity relative to DEX, this study found that NES, a pregnane derivative, exhibited a low RBA of only 6% compared to DEX for the GR (Kuhl, 2005). These data suggest that characterizing the affinity of a progestin for the GR based on its structural derivate alone may not be reliable. Conducting comparative whole cell binding assays within a uniform model system provides more reliable insight on the RBA of the progestins for the GR.

The GR exists in multiple conformations and a study by Veleiro *et al.* hypothesised that the GR may be able to accommodate different ligands due to the flexible conformation of its LBD (Veleiro *et al.*, 2010). The authors also suggested that the displacement of amino acids and rearrangement of hydrogen bonds may play a role in a ligand's affinity for the GR (Veleiro *et al.*, 2010). All the progestins in this study exhibited significantly different binding affinities. This may be due to the variable conformations the GR LBD assumes to accommodate the different progestin structures.

The differences in the progestin RBAs for the GR reported in this study compared to previous work may be due to the differences in model systems. Some of the previous studies were conducted *in vivo,* where the expression of other steroid receptors cannot be excluded (Selman, 1996; Fuhrmann, Slater, and Fritzemeier, 1995; Kumar *et al.*, 2000; Kontula *et al.*, 1983; Pollow *et al.*, 1992). Also, the *in vitro* studies by Koubovec *et al.,* and Ronacher *et al.,* do not report RBAs for ETG, LNG or NES (Koubovec *et al.*, 2005; Ronacher, Hadley, Avenant, Stubsrud, Simons Jr.*, et al.*, 2009). Additionally, there is no literature available where this entire panel of progestins is directly compared in parallel. The power in this study is that for the first time, all the progestins are investigated in parallel for more accurate comparisons using a model system expressing no SRs other than the human GR.

These results support the first aim of the first hypothesis that the progestins exhibit differential binding to the GR.

6.1.2 *Progestins exhibit gene-and cell-specific transcriptional activity via the GR*

Multiple studies show that the affinity a progestin has for a steroid receptor is not a direct indication of its biological activity through the receptor (Hapgood, Africander*, et al.*, 2014). The second aim of the first central hypothesis was to determine MPA, NET, LNG, ETG and NES responses via the GR in COS-1 and HeLa cells and PBMCs. For the first time, the potency, efficacy and resultant biocharacter for transactivation and transrepression of GR-regulated genes for each progestin are reported in this study.

6.1.2.1 *Transactivation*

The data in the present study show that MPA displays partial to full agonist activity for transactivation of a synthetic GRE-containing promoter and endogenous GR-regulated gene, GILZ in COS-1 (**Figure 3.2.2.2**), and HeLa cells (**Figure 3.2.3.1A**) and PBMCs (**Figure 3.2.5.1A**); whereas, NET exhibits little to no detectable activity. These results are consistent with multiple *in vitro* studies in similar model systems that report on the GR responses generated by MPA and NET on promoter-reporter and endogenous genes (Koubovec *et al.*, 2004; Koubovec *et al.*, 2005; Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009; Govender *et al.*, 2014; Hapgood, Ray*, et al.*, 2014).

ETG, NES and LNG responses on endogenous and synthetic GRE-promoters in COS-1 and HeLa cells, as well as PBMCs, are reported for the first time in the present study.

The present study consistently shows that LNG exhibits no activity for transactivation on both GRE-promoters in COS-1 cells or on the GILZ gene in HeLa cells and PBMCs. This result is consistent with an *in vivo* bioassay showing that LNG does not exhibit GC activity in rat livers (Kumar *et al.*, 2000); however, these results are not directly comparable due to study design differences. Thus, without reliably comparable literature, it is difficult to draw definitive conclusions on the transcriptional activity of LNG via the GR.

The present study found that ETG displayed differential transcriptional responses via the GR in a model system-dependent manner. Dose-response analyses in HeLa cells were not performed; however, the present study shows that 100 nM of ETG does not regulate GILZ mRNA levels in HeLa cells. This is consistent with a review by Schindler *et. al.* which reported that ETG does not exhibit GC activity (Schindler *et al.*, 2008). In contrast, the present study also demonstrates that ETG exhibits partial agonist activity for transactivation of both the synthetic and endogenous GRE-promoter in COS-1 cells and PBMCs. Although there are differences in study design, the weak partial agonist activity displayed by ETG on the GILZ gene in PBMCs is consistent with results from another *in vitro* study which reported that ETG displayed a weak transcriptional response via the GR (Fuhrmann, Slater, and Fritzemeier, 1995). Considering that the study by Fuhrmann *et. al*, is the only comparable study available in the literature and the authors do not report on the reproducibility of the observed transcriptional activity of ETG (Fuhrmann, Slater, and Fritzemeier, 1995). Whether the observed *in vitro* effects would occur *in vivo* is undetermined.

The present study shows that NES displays partial agonist activity for transactivation of the synthetic GRE-promoter in COS-1 cells and the GILZ gene in COS-1 cells and PBMCs. Although not significant, there appeared to be a NES-induced trend for increased GILZ mRNA levels in HeLa cells. Collectively, these results are different to one study by Kumar *et al.,* which found that NES did not exhibit GC activity in rat liver (Kumar *et al.*, 2000). To the best of the present authors' knowledge, the study by Kumar *et al.,* is the only published study investigating the effect of NES on GC-

specific responses (Kumar *et al.*, 2000). Due to the limited research on the GC activity of NES, it is difficult to draw firm conclusions on whether the observed effect *in* vitro would occur in different target cells *in vivo*.

However, a notable difference between the two studies is the model system. Unlike the rat liver model used in the Kumar *et al.* study, the present study utilized a cell line model which provided a more controlled environment to study the direct effects of NES, eliminating potential confounding factors like the presence of other SRs at varying levels as well as rapid metabolism of NES observed in the liver (Kumar *et al.*, 2000).

When comparing the relative potencies between the progestins in each model system, no significant differences were detected in the relative potencies of the progestins on the synthetic GRE promoter in COS-1 cells; however, NES was the most potent than DEX, MPA and ETG, in regulating GILZ mRNA levels in COS-1 cells (**Table 3.2.2**). This suggests that NES may elicit potent transcriptional responses on endogenous GR target genes compared to MPA and ETG. Collectively, these results indicate that apart from NES, MPA and ETG exhibit comparable efficacy and potency, with similar rank order on both synthetic and endogenous GRE promoters in COS-1 cells (**Table 3.2.2**). Multiple copies of the GRE sequence are present in both the promoter-reporter and the endogenous GILZ gene (Ayroldi and Riccardi, 2009), which may explain the similar effects of the progestin on both promoters. Interestingly, no significant differences in potencies were detected between the progestins in PBMCs, suggesting that the progestins

may generate similar transcriptional responses on GR-regulated genes in PBMCs (**Table 3.2.5**). However, factors such as high inter-donor variability may influence the observed responses with the likelihood of differences beyond the statistical power of the assay occurring. The relative potencies were not determined in HeLa cells.

When comparing the progestin potencies via the GR on GILZ in COS-1 cells and PBMCs, the progestins were less potent in PBMCs. The differences might be due to variations in GR expression levels between the cell types, as SR expression levels can influence potency and may fluctuate between experiments in different cell lines. For example, a study by Zhao *et al.,* found that progestins may cause a more potent GR response in an environment with high GR levels (Zhao *et al.*, 2003). This suggests that the GR expression levels in COS-1 cells may have been relatively higher than those in PBMCs in the present study. Given that GILZ is a GC-regulated anti-inflammatory gene, the regulation of this gene by these progestins suggests that they may have GC-like properties. If these responses fall within the range of serum concentrations of contraceptive users, this may have physiological implications.

Taken together, the data in the present study suggest that in contraceptive users, MPA, ETG and NES may exert physiologically relevant biological effects via the GR (**Table 3.2.3** and **Appendix B, Table B2**).

Dose-response analyses are important for determining whether the progestin-induced responses via the GR are likely to be relevant at physiological doses. However, the extent to which these findings translate to *in vivo* settings remains uncertain, as *in vitro* and *in vivo* progestin responses may be both tissue- and cell-specific in similar or distinct ways. The clinical relevance of the observed *in vitro* effects require further investigation.

6.1.2.2 *Transrepression*

The present study shows that at saturated concentrations i.e. concentrations when all the receptors are bound to ligand, MPA and NES significantly repress IL-6 mRNA levels in HeLa cells and PBMCs. The response of MPA and NES on the IL-6 gene was as efficacious as the DEX-induced repression of IL-6 mRNA expression. Consistent with the results on the MPA-induced inhibition of IL-6 mRNA expression in the present study. Multiple studies have reported the transrepressive effects of MPA on synthetic and endogenous genes involved in inhibition of immune function. Previous studies by Govender *et. al.,* and Hapgood *et al.,* have reported on the immunosuppressive effects of MPA on IL-6 mRNA levels in End1/E6E7, HeLA cells and PBMCs (Govender *et al.*, 2014; Hapgood, Africander*, et al.*, 2014). Promoter-reporter studies in HEK293 and COS-1 cells report that unlike NET, MPA is a full agonist for transrepression on both AP-1 and NFκB (Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009; Koubovec *et al.*, 2005; Komane *et al.*, 2022).

While available literature suggests that NES does not exhibit detectable GC activity (Kumar *et al.*, 2000), the present study shows for the first time, that NES represses

IL-6 mRNA expression in a manner similar to the synthetic GC, DEX. This implies that like MPA, NES may exert immunosuppressive effects at high concentrations.

Using dose response analysis in PBMCs, the present study shows that all the progestins except NET, are agonists for transrepression of the endogenous IL-6 gene. This data is similar to the results obtained by Hapgood *et al.,* and Govender *et al.,* for the dose-dependent MPA-induced repression of IL-6 (Govender *et al.*, 2014; Hapgood, Ray*, et al.*, 2014) , except that in the current study, NET exhibits very weak agonist activity. Different model systems, donor-specific effects, or varying progestin incubation times may have an impact on the variations in these results.

When comparing the potency of the progestin-induced repression of IL-6, no statistical differences were detected. The EC_{50} values of all the progestins except ETG, fall within the serum concentration range of contraceptive users (**Table 3.2.5**). However, the EC_{50} values for NET should be interpreted with caution as Prism 9 deemed this curve unstable due to variable data. While no studies have directly compared the transrepressive effects of ETG, LNG and NES on IL-6 mRNA expression, this data suggests that it is possible that NES, like MPA, may exert immunosuppressive effects that may inhibit immune function *in vivo*, and the possible effects of NET and LNG are likely to be negligible as they display weak agonist activity on the IL-6 promoter.

Taken together, these results show for the first time that MPA, ETG and NES, but less so for LNG and NET, induce transcription of the GRE-promoter construct in COS-1 cells, and regulate GILZ and IL-6 mRNA levels in COS-1 and HeLa cell lines and PBMCs. The progestins may have gene-specific responses, based on the variable efficacies and potencies. It's worth noting that progestin activity in repressing transcription appears to be more potent than for transactivation. These results suggest that MPA, ETG and NES are more likely to exert biological effects through the transrepression of GR-mediated genes as opposed to transactivation.

Investigating the relative efficacies of the progestin-induced GR activity in different model systems and on varying promoters provides information that assists with elucidating the biocharacter of each progestin. The progestins exhibited a consistent rank order of efficacy for eliciting. GR transcriptional responses across COS-1, HeLa, and PBMC model systems When comparing the progestins, **Table 3.2.2** shows that the progestins exhibit differential biocharacter for activity via the GR. In COS-1 cells, the progestins displays the same biocharacter on the synthetic promoter-reporter and endogenous promoter (**Table 3.2.2**). MPA, ETG and NES are partial agonists; whilst the biocharacter for NET and LNG could not be determined. In PBMCs, MPA is a full agonist on both the GILZ and IL-6 promoters (**Table 3.2.5**); whereas, ETG and NES exhibit different biocharacter on the promoters. Interestingly, NET is a weak partial agonist on the GILZ promoter and LNG is a partial agonist on the IL-6 promoter in PBMCs.

Determining the relative EC_{50} values of the progestin responses via the GR provides insight into whether progestin activity falls within the serum concentration of contraceptive users. **Appendix B, Table B2** showed that in COS-1 cells, the MPA-induced regulation of the synthetic GRE-promoter and GILZ gene falls within the serum concentration of women using the MPA-containing intramuscular injection administered at 150mg. Additionally, the potency of the ETG-induced increase in GILZ mRNA levels falls within the serum concentration of women on COC containing ETG. Interestingly, in PBMCs, EC_{50} values for the IL-6 regulation for all the progestins, except ETG, fall within the serum concentration of contraceptive users (**Appendix B, Table B2**). These results suggest that progestin activity via the GR may exert distinct physiologically relevant biological effects.

6.1.2.3 *Gene- and cell-specific relationship between the relative efficacies and potencies for transactivation and transrepression, as well as progestin binding affinity for the GR*

In the present study, the progestins exhibit differential effects on the transcriptional activity of the GR. To get a better understanding about the potential factors that may influence the differential effects observed, the fourth aim of this study determined whether the progestin-induced transcriptional activity of the GR is influenced by the progestin affinity for the GR. The current study builds upon previous research by Ronacher *et al.* which investigated progestin-induced GR activity on synthetic promoters in COS-1 cells (Ronacher et al. 2009). In the present study, transcriptional assays were conducted in COS-1 cells and PBMCs, and on a synthetic GR promoter and endogenous GR target genes.

A study conducted by Ronacher *et al.* investigated progestin-induced transcriptional activity of the GR and found that there was no correlation between the progestin efficacies for transactivation and transrepression via the GR and the progestin RBA for the GR (Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009). However, Ronacher *et al.* did find a correlation between the progestin RBA for the GR and progestin potencies for transactivation via the GR but not transrepression (Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009). Contrary to the findings reported by Ronacher *et al.*, the data in this study showed that there is variable correlation between the progestin RBA and efficacy for transactivation and transrepression via the GR and that it is gene- and cell-specific (Ronacher, Hadley, Avenant, Stubsrud, Simons*, et al.*, 2009) (**Figure 3.2.7.2–3.2.7.3**). DEX, MPA, ETG, and NES showed a positive correlation between RBA for GR and efficacy on a synthetic GRE promoter in COS-1 cells, whereas, only DEX, MPA, and ETG show a positive correlation between RBA and GR efficacy in the same cell line but on an endogenous GR promoter. Furthermore, only DEX and ETG showed a positive correlation between RBA for GR and efficacy on the same endogenous promoter in PBMCs. Also, DEX, MPA, ETG, and NES affinity for the GR in PBMCs correlates negatively with efficacy for IL-6 gene transrepression.

Overall, these results demonstrated that, just as progestins exhibit differential binding and transcriptional effects via the GR, the relationship between progestin affinity for the GR and the efficacy and potency of the progestin-induced GR responses are equally variable. This is highlighted by the discordant relationship

observed when comparing ETG and NES. While ETG displayed higher affinity for the GR than NES, NES exhibited more potent transcriptional and immunomodulatory effects compared to ETG. This disparity may be explained by ligand-specific conformational changes in the GR. Different progestins can induce unique receptor conformations, which in turn influence the recruitment of co-regulators and alter DNA-binding dynamics (Veleiro *et al.*, 2010; Weikum, Liu, and Ortlund, 2018). This selective GR modulation underscores the complexity of progestin signalling.

Moreover, given that the downstream transcriptional effects of SR-ligand complexes are dependent on the availability of cell- and promoter-specific cofactors and coregulators (Katzenellenbogen, O'Malley, and Katzenellenbogen, 1996; Hapgood, Africander*, et al.*, 2014), the relative efficacy and potency values of progestin-induced GR responses will likely vary between COS-1 and HeLa cells and PBMCs. Future studies should also investigate the role of GR cofactors in COS-1 and HeLa cells, as well as in PBMCs to determine how coregulator availability and recruitment influence the observed cell- and gene-specific progestin responses (Ronacher, Hadley, Avenant, Stubsrud, Simons Jr.*, et al.*, 2009; Hapgood *et al.*, 2005).

These results support the first central hypothesis that the progestins exhibit differential affinity for the GR, and gene-specific transcriptional effects via the GR.

6.2 Progestins regulate select immune mediators and HIV-1 infection *ex vivo*

Investigating the immunomodulatory effects of the progestins is important for understanding whether the progestins may indirectly facilitate increased HIV-1 infection. DMPA-IM use has been associated with an increased risk of HIV-1 acquisition, and several biological mechanisms, including effects on the levels of soluble immune modulators and frequency of HIV-1 target cell populations, whereby MPA may facilitate HIV-1 acquisition in the FGT, have been proposed (Hapgood, Kaushic, and Hel, 2018) (**Figure 1.6.1**).

Multiple clinical and limited preclinical studies support the hypothesis that unlike NET and LNG, MPA may facilitate HIV-1 acquisition through modulating immune gene expression and the levels of soluble immune modulators ; however, very little is known about the immunomodulatory effects of ETG and whether NES regulates immune function is yet to be determined.

The first aim under the second central hypothesis determined whether LNG, ETG, and NES, in parallel with MPA and NET, regulate mRNA and secreted protein levels of select immunomodulatory genes in PBMCs.

6.2.1 *MPA and NES exert distinct regulatory effects on select immune genes and soluble immune mediators ex vivo*

The present study showed that like MPA, NES significantly represses the expression of IFNγ mRNA (**Figure 4.2.1A**); whereas, MPA increases the secreted protein levels of IFNγ (**Figure 4.3.1A)** in PBMCs *ex vivo*. This is the first study to investigate the progestin-induced regulation of IFNγ. Most available clinical studies measure progestin-induced changes in FGT levels of secreted IFNγ protein in women on DMPA-IM reported contrasting results (Morrison *et al.*, 2014; Deese *et al.*, 2015; Michel *et al.*, 2015; Ngcapu *et al.*, 2015; Francis *et al.*, 2016; Molatlhegi *et al.*, 2020; Achilles *et al.*, 2020; Dabee *et al.*, 2021; Haddad *et al.*, 2023). Results from some clinical studies reported immunosuppressive effects on secreted IFNγ protein levels in the FGT (Michel *et al.*, 2015; Ngcapu *et al.*, 2015; Molatlhegi *et al.*, 2020; Achilles *et al.*, 2020; Haddad *et al.*, 2023), and in select PBMC cell populations (Huijbregts *et al.*, 2013; Matubu *et al.*, 2021) in women on DMPA-IM compared to women using non-hormonal contraception methods; whereas, some clinical studies have reported pro-inflammatory effects (Morrison *et al.*, 2014; Deese *et al.*, 2015; Francis *et al.*, 2016). Interestingly, *in vitro* studies in PBMCs have also reported that MPA suppressed secreted IFNγ protein levels (Kleynhans *et al.*, 2013; Huijbregts, Michel, and Hel, 2014; Piccinni *et al.*, 2019). In contrast to the previously published studies in PBMCs (Kleynhans *et al.*, 2013; Huijbregts *et al.*, 2013; Huijbregts, Michel, and Hel, 2014; Piccinni *et al.*, 2019; Matubu *et al.*, 2021), the present study shows that MPA increases secreted IFNγ protein levels in PBMCs. Currently no available

studies have examined the immunomodulatory effects of MPA on both mRNA and protein levels in the same study.

The results in the present show that NET does not significantly regulate IFNγ mRNA levels in PBMCs *ex vivo* (**Figure 4.2.1A**), although there appears to be a trend towards an increase in gene expression. There are no comparable studies available on the effect of NET on IFNγ mRNA expression; therefore, whether NET regulates IFNγ gene expression is yet to be determined. Consistent with a previous *ex vivo* study that did not detect significant effect on IFNγ protein levels in systemic T cell populations with exogenous NET (Huijbregts, Michel, and Hel, 2014; Matubu *et al.*, 2021),the present study shows that NET does not regulate secreted IFNγ protein levels in PBMCs. In contrast, some clinical studies investigated the effect of NET-EN on secreted protein levels of select immune mediators in the FGT and reported that NET-EN exerts pro-inflammatory effects compared to control subjects (Deese *et al.*, 2015; Achilles *et al.*, 2020). The differences in NET–EN-induced immunomodulatory effects between the FGT and PBMCs suggest that NET-EN may exert cell- and tissue-specific effects on secreted soluble immune mediators.

It is important to note the differences in study design between the present study, which investigated the effects of progestins on the levels of secreted IFNγ in PBMCs, and previous studies that assessed secreted IFNγ protein levels in systemic T cell populations incubated with exogenous MPA (Huijbregts, Michel, and Hel, 2014) or systemic T cell populations from women on NET-EN (Matubu *et al.*, 2021).

The results from the present study show that LNG and ETG, like NET, do not regulate IFNγ mRNA and protein levels in PBMCs *ex vivo*. These results are consistent with clinical findings, which reported that LNG and ETG do not significantly regulate secreted systemic IFNγ protein levels in women using LNG and ETG implants compared to women using non-hormonal methods (Achilles *et al.*, 2020; Matubu *et al.*, 2021). Similarly, an *in vitro* study by Huijbregts *et al.,* did not detect significant modulation of secreted IFNγ protein levels in systemic T cell populations by exogenous LNG (Huijbregts, Michel, and Hel, 2014). It is noteworthy that some clinical studies in women using the LNG-implant have also reported increased levels in select soluble immune mediators in the FGT compared to control subjects (Shanmugasundaram *et al.*, 2016; Radzey *et al.*, 2022).

The present study shows for the first time that NES significantly repressed IFNγ gene expression in PBMCs but did not significantly modulate secreted systemic IFNγ protein levels *ex vivo*. There are no published *in vivo*, *ex vivo,* or *in vitro* studies assessing the effect of NES on immunomodulatory genes and soluble immune mediators thus, whether NES exerts regulatory effects remains unknown.

Taken together, the results from the present study are supported by some findings from the literature. Overall, the results from this study show for the first time that MPA and NES significantly repress IFNγ mRNA levels in PBMCs, suggesting that it is probable that the immunosuppressive effects of DMPA-IM on IFNγ protein levels *in vivo* (Huijbregts *et al.*, 2013; Michel *et al.*, 2015; Ngcapu *et al.*, 2015; Molatlhegi *et al.*, 2020; Achilles *et al.*, 2020; Matubu *et al.*, 2021; Haddad *et al.*, 2023) may be

due to a direct repressive effect of MPA on IFNγ gene expression in PBMCs. Notably, it is difficult to directly compare *in vitro* results to *in vivo* effects due to multiple confounding factors. Thus, it remains unclear whether the *in vivo* effects occur via this mechanism.

Additionally, besides the MPA regulation of secreted IFNγ, the present study suggest that MPA, NET, LNG, ETG and NES do not change the levels of secreted IL-6, TNFα, IL-10, IL-1β, IL-1α, G-CSF, IFNγ, IL-13, IL-5 and IL-17 in PBMCs *ex vivo* (**Figure 4.3.1**). It is important to note that the lack of cytokine modulation in the absence of exogenous immune stimulation is consistent with describing the stimulus-dependent nature of GR-mediated cytokine regulation. GR's immunomodulatory effects are strongly influenced by cell type, activation state, and microenvironment (Horton and Remick, 2010; Pavlik *et al.*, 2023). In resting or unstimulated PBMCs, where basal cytokine production is low, GR activation typically results in minimal changes. In contrast, under inflammatory stimulation, when cytokine transcription is robustly induced, GR activation exerts more pronounced suppressive or modulatory effects (Cain and Cidlowski, 2017; Pavlik *et al.*, 2023). Thus, the limited cytokine changes observed in **Figure 4.3.1** likely reflect the quiescent state of the PBMCs in these assays. Within this context, the observed progestin-induced increases in HIV-1 infectivity (**Figure 4.4.1**), are likely to be independent of broad shifts in the levels of secreted cytokines. Instead, GR activation may directly modulate several host processes relevant to HIV-1 infection, including the regulation of entry and activation markers (e.g., CCR5, CD69) that affect target-cell susceptibility, the induction of selective transcriptional modulators

such as GILZ that alter T-cell responses and promote HIV-1 replication even in the absence of cytokine changes, and direct proviral effects of GR signalling, influencing replication and transcription (Hapgood and Tomasicchio, 2010; Maritz *et al.*, 2018; Vetillard and Schlecht-Louf, 2018; Bick *et al.*, 2022). Collectively, these mechanisms provide a plausible explanation for the increased infectivity observed despite the limited alterations in baseline cytokine profiles.

Moreover, the present study suggests that MPA, and possibly NES, elicit distinct regulation of IFNγ mRNA and protein levels in PBMCs. It remains unclear why MPA represses IFNγ mRNA levels while increasing IFNγ protein levels in PBMCs.

While some differences were observed, the small sample size and subsequent data variability, likely due to inherent inter-donor variability, and technical inconsistencies, may have limited the ability to detect significant differences in the effects of progestins on systemic soluble mediators. Overall, the results on the immunomodulatory effects of progestins in the present study should be interpreted with caution and further research is required to understand the mechanisms involved in the diverse regulatory effects of progestins on soluble immune mediators.

6.2.2 *No progestin-induced regulation of CD4 and CCR5 mRNA expression was detected in PBMCs ex vivo*

The present study shows that progestins do not significantly alter CD4 and CCR5 mRNA levels in PBMCs *ex vivo* (**Figure 4.2.1**). This result is different from an *in vitro*

study in a cervical cell line model which reported that MPA increased CD4 and CCR5 but NET decreased CD4 and CCR5 mRNA levels (Maritz *et al.*, 2018). Currently, there are no studies investigating the effect of LNG, ETG and NES compared to MPA and NET on CD4 and CCR5 mRNA levels in PBMCs.

Considering that MPA potentially increases HIV-1 acquisition by increasing the frequency of HIV-1 target cell populations, some *ex vivo* studies in PBMCs have reported contrasting findings on the effect of MPA on the expression of CCR5 and frequency of $CD4^+$ T cells (Maritz *et al.*, 2018; Bick *et al.*, 2022) compared to the undetectable effects of progestins on CD4 and CCR5 mRNA levels in PBMCs observed in the present study. While mRNA levels do not directly correlate to protein levels due to multiple cellular regulatory processes, relatively consistent with the present study, previous *ex vivo* studies in PBMCs did not detect significant changes in the frequency of $CD4^+$ and $CD4^+CCR5^+$ T cells with exogenous MPA (Sampah *et al.*, 2015; Maritz *et al.*, 2018; Bick *et al.*, 2022). It is worth noting that other studies only detected changes in $CD4^+$ T cell populations in PBMCs after a 7-day ex vivo incubation with MPA (Maritz *et al.*, 2018; Bick *et al.*, 2022); whereas, the current study conducted a 48-hour incubation with MPA. These results imply that a longer exposure with MPA may be required when investigating effects on PBMCs *ex vivo.* In addition, the *ex vivo* findings in PBMCs were relatively comparable to some clinical studies in DMPA-IM users which observed no significant changes in the frequency of HIV-1 target cell populations including, $CD4^+$ and $CD4^+CCR5^+$ T cells in PBMCs (Achilles *et al.*, 2020; Omollo *et al.*, 2020; Sciaranghella *et al.*, 2015; Li *et al.*, 2019; Tasker *et al.*, 2017) as well as in FGT samples (Achilles *et al.*, 2020; Lajoie

et al., 2019; Omollo *et al.*, 2020; Bunjun *et al.*, 2022; Smith-McCune *et al.*, 2017) compared to baseline controls or women using non-hormonal contraception.

Notably, the relationship between cell frequency and MFI remains unclear, as some studies have observed increased CCR5 expression in $CD4^+$ T cells without a corresponding increase in the frequency of $CD4^+$ T cells (Maritz *et al.*, 2018; Bick *et al.*, 2022). This suggests that MPA may selectively increase CCR5 MFI to potentially increase HIV-1 susceptibility in PBMCs. In a relatively comparable manner, some clinical studies detected increased CCR5 expression in HIV-1 target cell populations such as, $CD4^+$ T cells in PBMCs (Sciaranghella *et al.*, 2015) and FGT samples (Byrne *et al.*, 2016; Tasker *et al.*, 2020) from DMPA-IM users compared to women on non-hormonal contraception. One study showed LNG-IUD users expressed higher levels of $CCR5^+CD4^+$ T cells compared to women not using hormonal contraception (Sciaranghella *et al.*, 2015).

The results in the present study show that NET, LNG, ETG and NES do not regulate CD4 and CCR5 mRNA levels in PBMCs. As previously mentioned, while it is challenging to directly compare mRNA expression data with protein expression data, the undetectable effects of NET on CD4 and CCR5 mRNA expression are relatively consistent with an *ex vivo* study in PBMCs incubated with exogenous NET which did not detect significant changes in the frequency of $CD4^+$ T cells (Maritz *et al.*, 2018). There are no comparable studies examining the direct effects of LNG, ETG and NES on mRNA or protein levels of HIV-1 target cell populations. While clinical studies in NET-EN, LNG-implant and -IUD, as well as ETG-implant and -IVR users

report contrasting findings, the data consistently show no detectable changes in the overall frequency or cell numbers of HIV-1 target cell populations including $CD4^+$ and $CD4^+CCR5^+$ T cells in PBMCs (Achilles *et al.*, 2020; Li *et al.*, 2019; Haddad *et al.*, 2020) and FGT samples (Michel *et al.*, 2015; Li *et al.*, 2019; Haddad *et al.*, 2020; Achilles *et al.*, 2020) compared to control subjects. There are currently no comparable clinical studies on the effect of NES on the HIV-1 target cell populations. Taken together, these results suggest that MPA and LNG, unlike NET and ETG, may increase the frequency and infectivity of HIV-1 target cells, thus increasing the risk of HIV-1 acquisition.

Overall, it is challenging to directly compare *ex vivo* gene expression data from the present study with *in vivo* effects. However, these data may provide valuable mechanistic insights on the direct effects of progestins in cells, considering that some clinical and *ex vivo* data demonstrated that DMPA-IM or MPA potentially increases HIV-1 acquisition by directly increasing CCR5 expression on systemic HIV-1 target cells (Sciaranghella *et al.*, 2015; Omollo *et al.*, 2020; Maritz *et al.*, 2018; Bick *et al.*, 2022) and MPA significantly increased CD4 and CCR5 mRNA levels *in vitro* (Maritz *et al.*, 2018). Given that HIV-1 acquisition in women primarily occurs within the FGT, and considering the differences in immune cell distribution and immune responses between the FGT and PBMCs, it is difficult to directly link changes in systemic HIV-1 target cell populations to HIV-1 acquisition in comparison to the FGT. However, PBMC data can provide valuable insights into potential systemic immune responses, immune cell function, and biomarkers for HIV-1 susceptibility, ultimately contributing to our understanding of HIV-1 acquisition in the

FGT. Further comparative studies are needed to better understand the effects of NET, LNG, ETG and NES on systemic immune cell populations involved in HIV-1 infection.

6.2.3 *MPA, LNG, ETG, and NES, unlike NET, significantly increase HIV-1 infection ex vivo*

Having investigated proposed mechanisms through which MPA may affect HIV-1 infection, the present study assessed whether the progestins can directly modulate HIV-1 infection in PBMCs *ex vivo*. The current study found that MPA, LNG, ETG, and NES, unlike NET, significantly increase HIV-1 infection in PBMCs *ex vivo*. (**Figure 4.4.1A**). The current study is the first to report the LNG-, ETG-, and NES-induced increase in HIV-1 infection in parallel to MPA and NET in PBMCs *ex vivo.* The MPA-induced increase in HIV-1 observed in this study is consistent with other results from *in vitro* and *ex vivo* studies conducted in PBMCs and cervical tissue explants (Huijbregts *et al.*, 2013; Sampah *et al.*, 2015; Maritz *et al.*, 2018; Ray *et al.*, 2019). This result is also consistent with clinical studies and epidemiological observational meta-analyses which showed increased HIV-1 incidence among DMPA-IM users (Tasker *et al.*, 2017; Morrison *et al.*, 2015; Polis *et al.*, 2016). The recent ECHO results stated that no substantial differences were detected in HIV-1 risk between DMPA-IM, LNG implant, and CU-IUD, however, these results did not inform on the effect of DMPA-IM-use on HIV-1 infection compared to no contraception or condom use (Evidence for Contraceptive and Consortium, 2019; Hapgood, 2020). Additionally, the ECHO trial was designed to detect only a 50% or

greater difference in HIV-1 risk between methods. Thus, relative risks of less than 50% may have occurred but would not have been detected as significant. It is interesting to note that a that a near significant increase in HIV-1 risk for DMPA-IM was reported in this trial when comparing DMPA-IM and LNG implant ($p = 0.060$) (Evidence for Contraceptive and Consortium, 2019; Hapgood, 2020), suggesting that more statistical power in the trial may have found this to be a significant effect.

Given that the findings from the present study showed that LNG exhibits low GR binding affinity and minimal GR activity, it is possible that LNG modulates HIV-1 infection primarily via binding to and activating the PR or AR, or by directly regulating immune genes through non-genomic signalling cascades, which involve rapid activation of membrane-associated PR or AR and downstream kinase pathways such as MAPK (Hapgood, Kaushic, and Hel, 2018; Medina-Laver *et al.*, 2021). Currently, no *in vitro* studies have examined the direct effects of LNG on HIV-1 infection. However, unlike what is suggested from the *ex vivo* findings of the present study, clinical studies on LNG-IUD and implant users did not detect significant increases in the risk of HIV-1 acquisition (Lavreys *et al.*, 2004; Wall *et al.*, 2015; Curtis *et al.*, 2020) compared to baseline samples.

Consistent with other *ex vivo* results from studies by Maritz *et al.,* and Ray *et al.*, the present study showed that NET did not affect HIV-1 levels in PBMCs (Maritz *et al.*, 2018; Ray *et al.*, 2019). These results align with clinical studies and epidemiological meta-analyses which reported that unlike DMPA-IM, NET-EN use was not

associated with increased HIV-1 risk (Ralph, Gollub, and Jones, 2015; Noguchi *et al.*, 2015; Morrison *et al.*, 2015).

Currently, there are no published studies available to compare with the *ex vivo* ETG- and NES- induced increase in HIV-1 infection observed in the present study.

The MPA- and LNG- induced increase in HIV-1 infection levels observed in **Figure 4.4.1A,** falls within reported serum concentrations ranging from 2.6–99.6 nM and 0.3-616.4 nM, respectively (Sivin *et al.*, 1997; Jensen, 2005; Bick *et al.*, 2021a) (**Appendix B, Table B2**). Considering peak progestin concentrations (100 nM) employed in the present study, these data indicate that observed regulatory effects on HIV-1 infection may have physiological relevance for DMPA-IM, LNG-implant and -IUD users. In contrast, the published serum concentration range for contraceptives containing ETG (0.28-19.1 nM) and NES (0.18-27.3 nM) indicate that the observed effects of ETG and NES on HIV-1 infection in the present study likely fall outside these reported serum concentration ranges in contraceptive users, thus the physiological relevance is less certain. However, since the work in the present study was completed, a more recent clinical study reported median peak serum concentrations of 6.59 nM (IQR 4.80-8.70 nM) for MPA and 13.6 nM (IQR 9.28-18.5 nM) for NET, providing further context for interpreting the *in vitro* findings (Avenant *et al.*, 2023).

Taken together, given the available supporting evidence, these results imply that MPA and possibly LNG, but not NET, may directly modulate HIV-1 infection. These

results suggest that the presence of MPA and LNG during HIV-1 acquisition may enhance HIV-1 infection. Given the lack of available literature on the association between ETG and NES implants and HIV-1 acquisition, further investigation is needed to determine whether these progestins may directly influence HIV-1 risk. The reported serum concentration ranges provide qualitative insights to contextualise the relevance of the findings in the present study; however, it is important to note that these data may be influenced by differences in study design, including methods of detection, variations in study populations or sampling times. Investigating the dose-response of progestins on HIV-1 infection to obtain the EC_{50} values, would provide more comparable data to assess the physiological relevance of the observed progestin effects on HIV-1 infection.

Current evidence suggests that most contraceptives are highly effective and the WHO considers a small increase in HIV-1 risk (less than 50% between methods) associated with certain contraceptives is outweighed by the benefits of preventing unintended pregnancies, which significantly reduce maternal mortality and improve socioeconomic outcomes (World Health Organization, 2019; Evidence for Contraceptive and Consortium, 2019; Hapgood, 2020; Tepper *et al.*, 2020). Additionally, high coverage of effective antiretroviral therapy further mitigates HIV-1 transmission risks, underscoring the need for a balanced approach when evaluating contraceptive safety (Oldenburg *et al.*, 2018). These considerations should guide future research priorities to ensure informed public health recommendations.

6.3 MPA and NES regulate immune gene expression and HIV-1 infection in a GR-dependent manner

6.3.1 *MPA- and NES-induced regulation of GILZ and IL-6 mRNA levels is mediated by the GR*

The third aim of the first central hypothesis was to determine the role of the GR in mediating the MPA-, ETG-, and NES-induced responses on GILZ and IL-6 mRNA levels in PBMCs. The GR/PR antagonist RU486 was used to gain mechanistic insight and investigate the involvement of the GR in mediating the progestin-induced gene regulation.

The present study demonstrates that, except for ETG, co-stimulation with RU486 diminishes the MPA- and NES-induced regulation of GILZ and IL-6 in PBMCs *ex vivo* (**Figure 3.2.6.1**). While these effects are consistent with GR involvement as described in previous *in vitro and ex vivo* studies (Hapgood, Africander*, et al.*, 2014; Govender *et al.*, 2014), the use of RU486 does not allow definitive discrimination between GR- and PR-mediated pathways. Moreover, as the GR may not exclusively regulate GILZ and IL-6, PR involvement cannot be entirely ruled out. These results should therefore be interpreted as indicative of GR-related activity, while acknowledging that PR-mediated mechanisms may also play a role.

Overall, the results of this thesis suggest that, similar to MPA, NES may exhibit GC-like properties by regulating the expression of GR-associated target genes, such as

GILZ and IL-6, in PBMCs. However, further studies are required to confirm the receptor-specific pathways involved.

6.3.2 *MPA- and NES-induced increase in HIV-1 infection is mediated by the GR*

The second aim of the second central hypothesis assessed whether the progestins directly modulate HIV-1 infection with a requirement for the GR.

Using the GR/PR antagonist, RU486, the present study observed a significant repression of the MPA- and NES-induced increase in HIV-1 infection in PBMCs *ex vivo*. The observed reduction in the MPA-induced increase in HIV-1 infection is consistent with previous research by Maritz *et al.*, and Bick *et al.,* showing that MPA increases HIV-1 infection through a GR-dependent mechanism in PBMCs (Maritz *et al.*, 2018; Bick *et al.*, 2022). The present study shows for the first time that like MPA, NES increases HIV-1 infection in a GR-dependent manner in PBMCs.

Intriguingly, in the present study, the GR agonist, DEX, did not potentiate HIV-1 infection (**Figure 4.4.1**). Previous studies have reported that DEX significantly reduces proliferation and induces apoptosis in total PBMCs (Smits *et al.*, 1998; Totino *et al.*, 2006; de Almeida, Dantas, and Pereira, 2019). One *ex vivo* study in $CD4^+$ T cells reported that HIV-1 enhanced the DEX-induced increase in apoptosis (Tomasicchio *et al.*, 2013).This result gives a plausible reason for the limited DEX effect observed in the present study. Notably, this finding is consistent with results from a similar study conducted in PBMCs from HIV-negative women, which also

reported minimal effects of DEX under comparable conditions (Ray, 2015). Tomasicchio *et. al.,* also observed a GR-mediated pro-apoptotic effect induced by MPA (Tomasicchio *et al.*, 2013). However, the MPA-induced apoptosis in $CD4^+$ T cells reported by Tomasicchio et al. may be cell-specific, as the present study, using the MTT assay, did not observe any impact of MPA on total PBMC viability in the presence of HIV-1 (data not shown). Overall, these findings suggest that the limited DEX effect observed in the present study may be attributed to GR-mediated suppression of cell viability in specific PBMC subsets, with HIV-1 potentially enhancing DEX-induced apoptosis.

Interestingly, compared to the results in **Figure 4.4.1A**, when determining GR involvement in mediating the progestin-induced increase in HIV-1 infection the progestins exhibited differential regulation of HIV-1 infection in the absence of RU486 (**Figure 4.4.1B**). Unlike in **Figure 4.4.1A**, NET but not ETG, significantly increased HIV-1 replication. The reasons behind this are unclear but likely reflect a combination of factors. In **Figure 4.4.1A,** the small sample size and inter-individual variability among PBMC donors may have limited the statistical power to distinguish true biological effects from random variation. Furthermore, as the experiments in **Figure 4.4.1A** and **Figure 4.4.1B** not performed in parallel, the high inter-individual variability between PBMC donors may have contributed to differences in results across studies, particularly given the small sample sizes.

Overall, using a GR/PR antagonist makes it difficult to definitively distinguish whether the progestin-induced increases in HIV-1 infection and immunomodulatory

gene regulation are mediated by the GR or PR. While MPA is a known potent PR agonist, substantial evidence suggests that MPA predominantly exerts its immunomodulatory effects through the GR, particularly in systems where PR expression is low or absent (Govender *et al.*, 2014; Hapgood, Ray*, et al.*, 2014; Maritz *et al.*, 2018; Bick *et al.*, 2022). In the present study, RU486 effectively suppressed MPA- and NES-mediated responses (**Figure 3.2.6.1** and **Figure 4.4.1B**), thus, it is plausible that NES, like MPA, primarily regulates immune gene expression and enhances HIV-1 infection through the GR. However, the available literature regarding PR expression in PBMCs is inconclusive so PR-mediated actions cannot be entirely ruled out (Asin *et al.*, 2008; Arruvito *et al.*, 2008; Cabrera-Munoz *et al.*, 2012; Tomasicchio *et al.*, 2013; Polikarpova *et al.*, 2019).

To definitively attribute the observed progestin effects to the GR, future studies should utilise more selective approaches. This includes gene silencing techniques, such as siRNA and CRISPR-Cas9, as well as next-generation selective GR antagonists like CORT125281 (exicorilant) and CORT125134 (relacorilant) (Kroon *et al.*, 2018; Koorneef *et al.*, 2020; Pivonello *et al.*, 2021), which were specifically designed to avoid cross-reactivity with the PR, thereby offering a more reliable means of delineating GR-specific pathways.

6.4 Pinpointing the role of the GR

The third aim of the second central hypothesis was to determine relative GR and PR expression across immune cell types in PBMCs and ectocervical tissue explants. Since GR and PR are expressed in both PBMCs and the FGT, evaluating the SR expression profile may provide insight on the primary SR potentially involved in mediating progestin responses observed in the present study.

6.4.1 *The GR and PR expression profile in PBMC cell subtypes*

To investigate whether variability in progestin-induced effects among PBMC donors may be associated with differences in GR:PR expression ratios, and the fact that RU486 is not a GR-specific antagonist, potentially antagonising the progestin-induced effects on immune gene expression and HIV-1 infection observed in **Figure 3.2.6.1 and Figure 4.4.1** via the PR and/or the GR, the relative GR and PR levels were assessed in PBMC cell subtypes.

The present study shows for the first time the GR and PR expression profiles (measured as GR^+PR^-, PR^+GR^- and GR^+PR^+) in different PBMC cell populations ($CD3^+$ vs $CD4^+$ vs $CD8^+$ vs $CD14^+$). Interestingly, a considerable median relative % of both GR and PR expression was detected in the T cells, specifically a significant population of GR^+PR^+ T cells, while a lower frequency of GR^+PR^- and PR^+GR^- T cells were detected within these cell populations. (**Figure 4.5.1**). The rank order for the highest to lowest relative % expression of GR^+PR^+ is $CD4^+ > CD3^+ > CD8^+ >$

$CD14^+$ (**Figure 4.5.1**). These results are different to a previous study which detected a low frequency of PR in systemic $CD3^+$ and $CD4^+$ T cells using flow cytometry (Arruvito *et al.*, 2008). Currently, there are no comparable studies on the frequency of GR across PBMC cell subtypes.

The present study also detected higher GR MFI in $CD4^+$ T cells compared to the other immune cell populations; whereas PR MFI (albeit low), was higher in $CD14^+$ monocytes compared to other immune cell populations (**Figure 4.5.2B**). There are currently no studies available that assess GR MFI in different immune cell types. While mRNA levels do not directly correlate to protein levels due to multiple cellular regulatory processes, relatively consistent with the present study, a previous study reported high GR mRNA levels in $CD4^+$ and $CD8^+$ T cells and $CD14^+$ monocytes (Brundin *et al.*, 2021). In agreement, previous studies have reported detectable levels of GR mRNA and protein in total PBMCs *ex vivo* (Bamberger *et al.*, 1999; Tomasicchio *et al.*, 2013). In contrast to the present study, one study using confocal microscopy, reported that PR expression was not detectable in $CD4^+$ T cells (Arruvito *et al.*, 2008); whereas, others report detectable PR protein levels in whole PBMCs using western blot and immunofluorescence analysis (Asin *et al.*, 2008; Cabrera-Munoz *et al.*, 2012). The variability in reported SR expression levels within PBMCs can likely be attributed to several factors, including inter-donor variability, differences in methods of detection and antibodies used, and the use of either total or sorted PBMC populations in different studies.

Collectively, these results highlight that immune cell populations within PBMCs, relevant for HIV-1 acquisition and immune function, exhibit distinct GR and PR expression profiles. Notably, compared to the other immune cell populations, a significantly higher frequency of $CD4^+GR^+$ and $CD4^+PR^+$ T cells was detected (**Figure 4.5.2A**). When assessing MFI in PBMC subsets, GR expression appeared more prominent than PR in $CD3^+$, $CD4^+$ and $CD8^+$ T cells, as well as in $CD14^+$ monocytes, with $CD4^+$ T cells showing the highest levels. However, because different antibodies were used for GR and PR detection, and flow cytometry measures relative fluorescence rather than absolute protein abundance, these data cannot be interpreted as evidence of true differences in receptor abundance between GR and PR. Instead, the findings should be regarded as indicative of relative signal patterns within each receptor group, rather than direct quantitative comparisons across receptors. Nonetheless, these results provide valuable insights to support that the variation in progestin-induced HIV-1 infection and immunomodulatory effects observed in **Figures 3.2.5.1-3.2.6.1B and 4.2.1-4.4.1** may be attributed to differences in the apparent relative GR:PR expression ratios between PBMC donors.

Considering $CD4^+$ cells are primary target cells for HIV-1, these findings may be relevant for understanding GR involvement in HIV-1 susceptibility. Furthermore, while definitive conclusions cannot be drawn, the results in the present study imply that the GR, and less likely the PR, is involved in the progestin-induced effects on immune gene expression and HIV-1 infection in PBMCs *ex vivo* observed in **Figure 3.2.6.1 and Figure 4.4.1**. While *in vivo* relevance of these results remains

undetermined, the results in the present study offer valuable insights supporting a role for the GR in clinical findings that reported increased frequency of $CD4^+$ and $CD4^+CCR5^+$ T cells and expression of $CCR5^+CD4^+$ cells in PBMCs from women using DMPA-IM (Michel *et al.*, 2015; Sciaranghella *et al.*, 2015) and LNG-IUD (Sciaranghella *et al.*, 2015), compared to control subjects. Additionally, these results also support several *ex vivo* studies in PBMCs which reported on the GR-mediated increase in HIV-1 infection and immunosuppression of immunomodulatory genes induced by MPA using RU486 (Huijbregts *et al.*, 2013; Hapgood, Ray*, et al.*, 2014; Maritz *et al.*, 2018; Bick *et al.*, 2022). Considering the lack of comparable studies, it remains unclear whether the LNG- and ETG-induced increase in HIV-1 infection occurs through the same mechanisms.

While these findings are robust within the experimental framework, these results should be interpreted with caution given the relatively small number of donors (n=8) and fact that only a single measurement was taken for each donor. Nevertheless, these results provide proof of concept that individual donors can have different relative amounts of GR and/or PR in various immune cells, which may explain the inter-donor variability observed in studies conducted in PBMCs. This variability in receptor expression levels could influence progestin-induced effects on immune function and HIV-1 infection in these cell subtypes. Future studies using siRNAs targeting GR and PR selectively**,** could provide critical insights into which SR primarily mediates specific progestin responses.

6.4.2 *The GR and PR expression profile in FGT tissue compartments*

Building on the previous section, which reported the detection of both GR^+ and PR^+ immune cell populations within PBMC, the present study also examines the GR and PR expression profile in ectocervical tissue. In the present study, GR signals were detected in both the epithelial cell region and stromal cells (**Figure 4.6.4A-B**); whereas, PR signals were only detectable in the epithelial region (**Figure 4.6.4C**). Given the differences in overall study design and methods of detection, it is challenging to directly compare these results with previous findings. However, it is worth mentioning that the results in the present study are relatively consistent with a study in immunostained stromal fibroblast cultures established from the cervical tissues which reported undetectable levels of the PR; whereas, the ERα and GR were constitutively expressed in these cells (Ackerman *et al.*, 2016). Similarly, other *in vitro* and *ex vivo* studies have shown detectable levels of GR or PR protein in human cervical and vaginal cell lines, and ectocervical tissue explants using western blot analysis (Africander *et al.*, 2011; Govender *et al.*, 2014; Maritz *et al.*, 2018; Ray *et al.*, 2019). In contrast, a study by Khan *et al.* demonstrated the co-localization of the ER and PR in the endometrial stroma by performing ER and PR immunostaining in endometrial tissue (Khan *et al.*, 2005). Similarly, another study performed immunohistochemistry in cervical tissue sections and showed that the PR is expressed throughout the stroma but mostly in the basal epithelial cells of the ectocervix (Ackerman *et al.*, 2016). In addition, consistent with previous *ex vivo* studies, which found detectable levels of CD4 in vaginal and cervical tissue samples from women (Pudney, Quayle, and Anderson, 2005; Trifonova, Lieberman, and van

Baarle, 2014; Shanmugasundaram *et al.*, 2014; Joag *et al.*, 2016), the present study found detectable levels of CD4 in the epithelial cell region and stromal cells in ectocervical tissue (**Figure 4.6.4**). For the first time, the present study shows the co-expression $CD4^+$ cells with both GR and PR in ectocervical tissue (**Figure 4.6.4**). Interestingly, it appears as though there is a relatively larger proportion of $CD4^+$ cells co-expressing GR and relatively fewer PR-expressing $CD4^+$ cells in this tissue. These results are relatively consistent with the results in **Figure 4.5.2B,** which that showed $CD4^+$ T cells in PBMCs express relatively lower levels of PR compared to GR.

CD4 is expected to localise predominantly in the plasma membrane. Thus, the nuclear CD4 staining observed in **Figure 4.6.3** is not consistent with its expected location and known biological function. However, the presence of both membrane and nuclear CD4 signals suggest that these are $CD4^+$ cells. The apparent nuclear signal in this study is most likely attributable to technical factors such as antibody cross-reactivity, non-specific binding, or artefacts introduced during fixation and permeabilization. This interpretation is consistent with published reports highlighting the potential for altered staining patterns under certain fixation conditions (North, 2006; Cossarizza *et al.*, 2021). Thus, although membrane CD4 staining is also observed, the nuclear staining raises the possibility that the membrane staining may also be an artifact. Taken together, the identity of these cells as CD4 cells should be interpreted with caution. Future experiments should investigate the identity of CD4 cells with additional controls.

Overall, these results provide an initial qualitative assessment of GR, PR, and CD4 expression in cervical tissue. These findings indicate that ectocervical tissue compartments exhibit distinct GR and PR expression profiles, with GR appearing to be the main receptor expressed in $CD4^+$ cells found in the epithelial cell region. Given that CD4 cells are primary HIV-1 cell targets, the detection of $CD4^+$ cells in the epithelial cell region has significant implications for HIV-1 acquisition.

While *in vivo* relevance of these results remains undetermined, the results in the present study offer valuable insights supporting a role for the GR in clinical findings that reported increased frequency and cell numbers of $CD4^+$ and $CD4^+CCR5^+$ T cells and expression of $CCR5^+CD4^+$ cells in vaginal and cervical samples from women using DMPA-IM (Ildgruben, Sjoberg, and Hammarstrom, 2003; Chandra *et al.*, 2013; Byrne *et al.*, 2016; Thurman *et al.*, 2019; Li *et al.*, 2019; Lajoie *et al.*, 2019; Tasker *et al.*, 2020; Edfeldt *et al.*, 2022), LNG-implant and -IUD (Ildgruben, Sjoberg, and Hammarstrom, 2003; Shanmugasundaram *et al.*, 2016), as well as ETG-implant and -IVR users (Haddad *et al.*, 2020), compared to control subjects. The findings also support GR involvement in increasing HIV-1 infection and immunomodulatory regulation in cervical tissue explants treated with exogenous MPA (Ray *et al.*, 2019).

Taken together, the results from this chapter and previously reported findings in PBMCs *ex vivo* (Huijbregts *et al.*, 2013; Hapgood, Ray*, et al.*, 2014; Maritz *et al.*, 2018; Bick *et al.*, 2022), corroborate the finding in the present study that MPA and NES regulate immune gene expression and HIV-1 infection in a GR-dependent manner (**Figure 3.2.6.1 and Figure 4.4.1**). These data suggest that, unlike NET,

MPA and possibly NES, create an environment conducive for HIV-1 acquisition through increasing the frequency of $CD4^+$ T cells and enhancing CCR5 expression in $CD4^+$ T cells, as well as weakening host defences. The mechanisms by which ETG and LNG exert regulatory effects remain unclear. However, given that the PR was also detected in the tissue (albeit at low levels), and MPA, ETG and NES exhibit GC-like properties (**Section 3.2.2-3.2.6**), it is likely that when the relative GR levels are low, the effects of MPA, ETG and NES are potentially mediated by the PR in the FGT.

An additional consideration is that the variable progestin-mediated effects across different cell types may, in part, reflect differences in the expression of GR isoforms, particularly GRα and GRβ. The primary mediator of GC signalling is the GRα; whereas its splice variant, GRβ, does not bind GCs or progestins but functions predominantly as a dominant-negative inhibitor of GRα activity (Oakley and Cidlowski, 2013; Lockett, Inder, and Clifton, 2024). While historically thought to function mainly through heterodimerization with ligand-bound GRα and compete for DNA binding sites or cofactors, more recent work has shown that GRβ can also exert GRα-independent transcriptional activity to regulate genes involved in inflammation, migration, and cell survival (Ramos-Ramirez and Tliba, 2021; Nicolaides, 2022). Importantly, the GRα:GRβ ratio varies across immune lineages and inflamed tissues, with pro-inflammatory cytokines capable of upregulating GRβ and an elevated GRβ/GRα ratio is widely recognized as a mechanism contributing to GC resistance (Cain and Cidlowski, 2017; Nicolaides, 2022; Lockett, Inder, and Clifton, 2024). Isoform abundance was not quantified in the present study, however,

differences in the relative expression of GRα and GRβ may still influence the magnitude of progestin responses. The variation in the GRα:GRβ ratio between cell types could modulate the efficacy or potency of GRα-progestin complexes, contributing to the gene- and cell-specific progestin effects observed. As such, future work should include isoform-specific quantification and GRβ overexpression/knockdown in relevant cell types to better interpret GR-mediated progestin effects.

6.5 Progestins are metabolised to various degrees within and across different model systems

The *ex vivo* and *in vitro* metabolism of synthetic progestins has not been widely researched. The first aim in the third hypothesis was to determine whether the progestins are metabolised in COS-1, HeLa cells and PBMCs.

As published in Skosana *et. al.,* the data in the current study shows that in COS-1 cells, the progestins exhibit variable degrees of metabolism; the progestins are all metabolised to a similar degree in HeLa cells (Skosana *et al.*, 2019) (**Figure 5.2.1A-B**). Additionally, the present study shows for the first time, that the progestins also exhibit variable degrees of metabolism in PBMCs (**Figure 5.2.1C**). Further interrogation of this data also revealed that only NET and LNG were consistently metabolised to a similar degree across all three model systems, while MPA, LNG and NES were metabolised to various degrees across the three model systems (**Figure 5.2.2**).

These results suggest that the differences observed between *in vitro* and *ex vivo* progestin-induced effects on immune genes, soluble immune mediators, and HIV-1 infection in the present study may, in part, be due to variations in progestin metabolism. The results imply that progestins metabolised extensively in one model system may exhibit reduced potency or receptor affinity, while in systems with minimal metabolism, the effects could be more pronounced. However, this was not observed in the present study. For example, MPA, ETG, and NES were among the least metabolised progestins in PBMCs compared to COS-1 cells (**Figure 5.2.2**) yet exhibited lower potency in regulating GILZ gene expression in PBMCs compared to COS-1 cells (**Table 3.2.2 and Table 3.2.5**). This indicates that additional factors beyond metabolism influence progestin activity in PBMCs. As observed in COS-1 cells overexpressing GR (**Figure 3.2.2.2B and Table 3.2.2**), higher GR levels reduce progestin potency, shifting the dose–response curve to the left (Zhao *et al.*, 2003; Chow *et al.*, 2011). Moreover, the presence of competing SR, such as PR (**Figure 4.5.1**), may sequester progestins away from GR, lowering the progestin concentration available for GR activation (Hapgood, Africander*, et al.*, 2014; Hapgood, Kaushic, and Hel, 2018). Furthermore, if GR co-regulators are available at limiting concentrations, transcriptional activity may be modulated, resulting in reduced progestin potency and the dose–response curve shifts to the right (Simons Jr., 2010; Khan *et al.*, 2012). Therefore, the reduced progestin potency observed in PBMCs likely reflects a combination of lower endogenous GR expression, SR competition and limited cofactor availability and overall cell-specific regulatory mechanisms rather than metabolism alone.

The present study did not find a correlation between progestin metabolism and RBA for GR in COS-1 cells (**Figure 5.3.1A**), nor between progestin metabolism and progestin efficacy or potency on synthetic and endogenous GR-regulated genes in either COS-1 cells or PBMCs (**Figure 5.3.2**). These findings, suggest that the extent of progestin metabolism may not directly influence GR binding affinity, nor progestin efficacy and potency in these models. Various factors may contribute to the absence in correlation. While some differences in progestin activity were observed, it is likely the differences may have been too subtle for the present study design to detect statistically significant correlations. For example, the absence of significant correlations in the PBMC data may be due to the inherent inter-individual variability among PBMC donors, the limited sample size, and subsequent data variability. Moreover, cell-specific co-factors, post-translational modifications of the GR, or differences in cellular uptake of progestins between COS-1 cells and PBMCs may influence GR-mediated progestin responses. These results are not unexpected given the complexity of progestin-induced SR activity; further research is required to elucidate the relationship between progestin metabolism and GR-mediated transcriptional activity. However, these findings should be interpreted with caution as correlation analyses do not account for non-linear relationships between variables and may potentially fail to capture the true nature of the association.

Progestin metabolism varies significantly across different cell types, suggesting that this may be a key factor influencing local progestin concentration and downstream biological effects (Skosana *et al.*, 2019). Several mechanisms could contribute to

this variability. Firstly, the observed variability may stem from cell-specific differences in intracellular steroid metabolism. These include distinct expression patterns of steroidogenic enzymes, such as cytochrome P450s and hydroxysteroid dehydrogenases (HSDs), as well variations in the activity of specific interconversion pathways , such as those mediated by 11β-HSD1/2 and 17β-HSD isoforms (Bick *et al.*, 2021b; Cao *et al.*, 2023). These enzymes have been shown to modulate local exposure to bioactive steroid metabolites (Cao *et al.*, 2023). Additionally, there may be differences in the expression of uptake and efflux transporters, since cervicovaginal tissues have been shown to express transporter proteins at distinct levels, thereby influencing intracellular concentrations of progestins available for metabolism and receptor binding (Zhou *et al.*, 2014). Finally, there may be variability introduced by external factors since donor physiology and the *in vitro* culture conditions have been shown to alter the expression of both enzymes and transporters, further contributing to variable metabolic profiles in primary cells versus immortalised cell lines (Skosana *et al.*, 2019; Bick *et al.*, 2021b). Taken together, these factors suggest that the differences observed in the present study are more likely driven by cell-specific metabolic factors rather than by intrinsic properties of the progestin alone.

Overall, these results indicate the importance of considering cell-specific metabolism when interpreting dose-response results across different model systems. These data demonstrate the need to consider cell-specific metabolism when investigating biochemical and biological effects parameters of different progestins in different models systems via steroid receptors *in vitro* and *ex vivo*.

Addressing this gap in the literature could improve the interpretation of apparent discrepancies across studies and choice of model systems for experiments.

6.6 Study limitations

While the present study provides valuable insights, several experimental and technical limitations should be considered when interpreting the results

The present study relied on *in vitro* and *ex vivo* model systems, which while valuable, may not fully mimic the complexity of *in vivo* environments. However, *in vitro* and *ex vivo* studies provide a controlled environment to determine the direct cell- and/or tissue-specific SR-mediated effects of progestins on target genes and HIV-1 infection.

Another limitation of the present study is the use of COS-1 cells overexpressing exogenous GR to conduct whole cell binding assays and promoter-reporter assays. It has been shown that SR concentration can influence binding affinity, efficacy, as well as IC_{50}, and EC_{50} values (Tomlinson and Hnatowich, 1988; Zhao *et al.*, 2003; Africander, Verhoog, and Hapgood, 2011; Chow *et al.*, 2011; Caldwell *et al.*, 2012; Robertson *et al.*, 2013; Hapgood, Africander*, et al.*, 2014). In COS-1 cells overexpressing GR, increased SR availability may lead to apparent increases in binding affinity and efficacy. As a result, IC_{50} and EC_{50} values decrease and the dose–response curve shifts to the left. Consequently, GR-mediated progestin activity observed in this cell model may be overestimated due to these shifts in parameters. Nonetheless, these assays provide valuable insights into relative receptor activity and progestin interactions. The present study did not quantify endogenous GR levels in HeLa and PBMCs to assess relative GR expression compared to COS-1 cells.

While PBMCs are a well-established model system for investigating circulatory immune responses, inherent biological variations in immune responses and SR expression between donors, may contribute to the variability in observed responses. High inter-donor variability reduces the statistical power to detect true differences between treatment groups. Similarly, a small sample size can limit the ability to detect statistical significance.

While this study provides novel insights into GR and PR expression across PBMC cell subtypes using flow cytometry, several limitations must be acknowledged. The detection of PR expression in immune cell populations via flow cytometry may be influenced by antibody cross-reactivity, as the PR antibody may potentially bind to GR, and vice versa. While fluorescence-minus-one (FMO) controls were used to set gates and minimize non-specific signals, specific controls to directly assess antibody cross-reactivity in flow cytometry were not included. If PR expression is indeed present, it is likely to be lower than GR expression, However, without additional controls the specificity and expression levels of PR remain uncertain and requires further validation. Despite these challenges, this thesis provides a comprehensive analysis of GR and PR expression in PBMC immune cells using flow cytometry.

The present study provides novel insights into the co localisation of CD4 with GR and PR in different ectocervical tissue compartments; however, a key limitation is the potential cross-reactivity between the GR and PR antibodies in tissue staining for immunofluorescence . Cross-reactivity was assessed using western blot analysis and found to be confirmed negligible. While some degree of protein renaturation is

assumed, western blotting alters conformational epitopes and may influence antibody binding and detection. In contrast, like flow cytometry, immunofluorescence analysis detects native protein conformations under non-denaturing conditions. As a result, while it is unlikely, cross-reactivity cannot be completely ruled out and should be considered when interpreting the results in the present study. Furthermore, the present study did not include additional cell surface markers, thus, it remains unclear whether the observed CD4 signal originated from immune cells or epithelial cells, as some reports suggest that epithelial cells may also express CD4. Without additional markers or techniques to distinguish between these cell types, the cellular source of CD4 staining in the ectocervical tissue explants cannot be definitively identified.

A key limitation of correlation analyses is that they only assess linear relationships between variables. If the underlying relationship is non-linear, the correlation may appear weak or fail to capture the true nature of the association. This limitation should be acknowledged, as it can lead to an incomplete interpretation of the data. Additionally, while alternative methods for examining associations exist that can account for non-linear relationships, these approaches are beyond the scope of this thesis and the capabilities of the software (Prism) used in the analysis. This highlights the need for caution when interpreting correlation results and suggests that more advanced methods could be considered in future studies.

The *in vitro* metabolism studies in COS-1, HeLa, and PBMC models provide valuable insights into the gene expression experiments performed in this study.

However, it should be noted that these models would not be expected to express the same complement of cytochrome P450 enzymes expressed in human hepatocytes (Bulutoglu *et al.*, 2020), the main site of steroid metabolism in humans.

Chapter 7

Conclusions and future perspectives

7.1 Conclusions

The present study examined the GC actions of LNG, ETG and NES, in parallel with MPA and NET *in vitro* and *ex vivo*. The present study characterised progestin RBA for the GR in parallel within the same model system. For the first time, the present study shows that progestins exhibit distinct RBA for the GR (**Table 3.2.1**). MPA and ETG display similar IC_{50} as the synthetic GC–DEX, and higher affinity for the GR than NET, LNG, and NES in COS-1 cells. The results in the present indicate that MPA and ETG may elicit GC-like binding within serum concentrations ranges in DMPA-IM and ETG-implant, -IVR users (**Appendix A, Table B2**). The present study assesses progestin actions via the GR, reporting novel findings for LNG, ETG and NES responses on endogenous and synthetic GRE promoters across COS-1 and HeLa cell lines, and PBMCs. The present study shows that like MPA, NES displays potent agonist activity for the repression of GR-regulated genes compared to transactivation, suggesting that MPA and NES most likely exert immunosuppressive effects via mechanisms involving the GR. The progestins did not exhibit significant differences in the relative potencies in the cell lines; however, the progestins displayed selective relative potencies and gene-specific relative efficacies within PBMCs *ex vivo.* While the COS-1 and PBMC cell models may not mimic the *in vivo* environment, these systems provide a proof-of-concept for the relative progestin

binding characteristics and biological activity via the GR. These results support the first central hypothesis that the progestins exhibit differential affinity for the GR, and gene-specific immunoregulatory effects via the GR.

The present study also provides valuable insights into the complex interplay between the SR-mediated effects of progestins on the expression of select immune function genes and HIV-1 infection in PBMCs *ex vivo*. The present study shows that MPA and NES elicit distinct regulation of IFNγ mRNA and protein levels in PBMCs. While the study did not detect significant regulation of HIV-1 CD4 receptor and CCR5 co-receptor gene expression, the selective MPA-, and possibly NES-induced potential immunomodulatory effects contribute to the growing body of evidence to understand the GR-mediated actions of the progestins. The present study is the first to report the LNG-, ETG-, and NES-induced increase in HIV-1 infection in parallel to MPA and NET in PBMCs *ex vivo*. The exact mechanisms underlying these effects are not fully elucidated, however, the results in the present study suggest a GR-dependent mechanism for the MPA- and NES-induced increase in HIV-1 infection in PBMCs *ex vivo*, although PR involvement cannot be fully excluded. To further support the role of the GR, and possibly PR, in mediating the progestin-induced immunomodulatory effects, the present study shows that GR is predominantly expressed in systemic $CD4^+$ T cells and co-localised with CD4 in the GR-expressing epithelial cell region of the FGT. These results provide proof of concept that that distinct SR expression levels in PBMC cell subtypes and ectocervical tissue compartments may influence progestin-induced effects on select immune gene expression and HIV-1 infection. These results partially support the second central

hypothesis in the present study that MPA, ETG and NES differentially regulate the immune gene expression and secreted protein levels, and HIV-1 infection *ex vivo*, in a GR-dependent manner.

The present study shows that progestins are metabolised to varying degrees within and across different model systems. However, no significant correlation between the degree of metabolism and progestin RBA for the GR was detected, nor was there a correlation between the relative efficacy or potency of the progestin response via the GR in COS-1 cells. These results partially support the third central hypothesis that progestins are differentially metabolised within and across different model systems *in vitro* and *ex vivo*.

Overall, these findings provide valuable insights into the relevance of both SR expression and progestin actions when evaluating the safety and efficacy of contraceptives. The present study provides mechanistic insights into how MPA and NES, via GR-mediated pathways, may alter select immune gene expression and HIV-1 infection *in vitro*. In contrast, NET showed limited immunomodulatory effects under similar conditions. The immunological profiles of LNG and ETG appeared distinct from MPA and NES, however, further studies are needed to fully understand the underlying mechanisms and potential implications for HIV-1 susceptibility.

7.2 Future perspectives

Elucidating the comprehensive pharmacological profile of synthetic progestins is essential for optimising contraceptive methods and ensuring safety, particularly in populations at a high risk of HIV-1 acquisition. While the present study offers novel insights into the GR-mediated effects of several progestins, it also highlights significant areas for future investigation that are critical for bridging the gap between scientific research and informed public health practice.

To advance mechanistic understanding of progestin-mediated responses, future studies must address several methodological and biological considerations. The impact of progestin metabolism on SR activity is not fully understood and may vary between model systems. Since metabolism can reduce progestin levels and shift the dose-response curve, future studies should routinely quantify both parent compounds and metabolites, ideally in primary cells, to better approximate *in vivo* conditions. This approach will help clarify whether the observed SR-mediated responses reflect the intended exposure or are affected by progestin metabolic processing.

A significant area for future research involves examining how GR activity is influenced by the presence of specific coactivators and corepressors within the various cell types examined. Building on the gene-specific variability in GR responses observed in this thesis, future studies could evaluate cofactor expression

and recruitment in COS-1, HeLa, and PBMCs models to gain mechanistic insight into the pathways underlying differential progestin effects.

Future clinical research on the safety of contraceptive use and the association with HIV-1 acquisition should aim to address existing knowledge gaps through well-powered randomised trials. While such studies may be logistically challenging and costly, more robust study designs incorporating control groups will provide more reliable comparative data. It remains unclear whether progestins regulate immune gene expression and secreted protein levels as a potential mechanism to alter HIV-1 infection. To address the limitations of the present study, future research should increase the sample size for PBMCs and conduct more biological repeats to improve statistical power.

Given that RU486 is a GR/PR antagonist, it remains unclear whether the GR or PR mediates the progestin-induced responses. Future studies using siRNAs targeting GR and PR selectively could provide critical insights into which SR primarily mediates observed progestin-induced responses. Additionally, more biological repeats are required to validate the observed SR-CD4 expression profile. To improve the reliability of protein detection using flow cytometry and immunofluorescence future studies should minimise non-specific binding using antibodies that do not cross-react with different SR proteins. Complementary approaches, such as western blotting, could provide further validation by confirming the identity of the detected GR or PR proteins. A key improvement would be using

dual or triple staining with well-characterised antibodies targeting distinct epitopes to confirm the localisation and specificity of the target proteins.

At the clinical and public health level, the findings of this thesis may have implications for choice of contraceptive, particularly in regions with high HIV incidence, such as sub-Saharan Africa. The ECHO trial reported no substantial detectable difference in risk between DMPA-IM and LNG implant (Evidence for Contraceptive and Consortium, 2019). This finding appears consistent with the data in this thesis showing that both MPA and LNG increase HIV-1 infection *in vitro*. In contrast, the present study did not detect a significant increase in HIV-1 infection with NET *in vitro*, aligning with limited clinical observational data on NET-EN use, which reported no detectable increased risk of HIV-1 acquisition (Ralph, Gollub, and Jones, 2015; Noguchi *et al.*, 2015; Morrison *et al.*, 2015).

It is important to note that *in vitro* studies alone do not provide sufficient evidence to change public health policy or establish causal relationships regarding HIV risk. Nevertheless, these findings support consideration for expanding access to contraceptives containing NET, such as injectable NET-EN, which may offer a safer alternative to MPA-based methods for women at high risk of HIV-1 infection. Further clinical and epidemiological studies are needed to confirm the observations in this thesis and guide evidence-based policy decisions.

Consistent with observational studies, the present study suggests a possible increased risk of HIV-1 acquisition associated with DMPA-IM use compared to some

other progestin-only contraceptive methods; however, the World Health Organization (WHO), currently recommends that women should have unrestricted access to DMPA-IM and be fully informed about the potential risks, emphasizing the importance of individual choice and comprehensive HIV prevention strategies (World Health Organization, 2019).

Future studies can build on the foundation laid by this study to elucidate the complex interplay between GR and PR in the progestin-induced regulation of immune function and HIV-1 infection. This is critical for optimising contraceptive strategies that minimise adverse immunological outcomes and mitigate the risk of HIV-1 acquisition in vulnerable populations.

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Appendix A

Supplementary data for Chapter 2

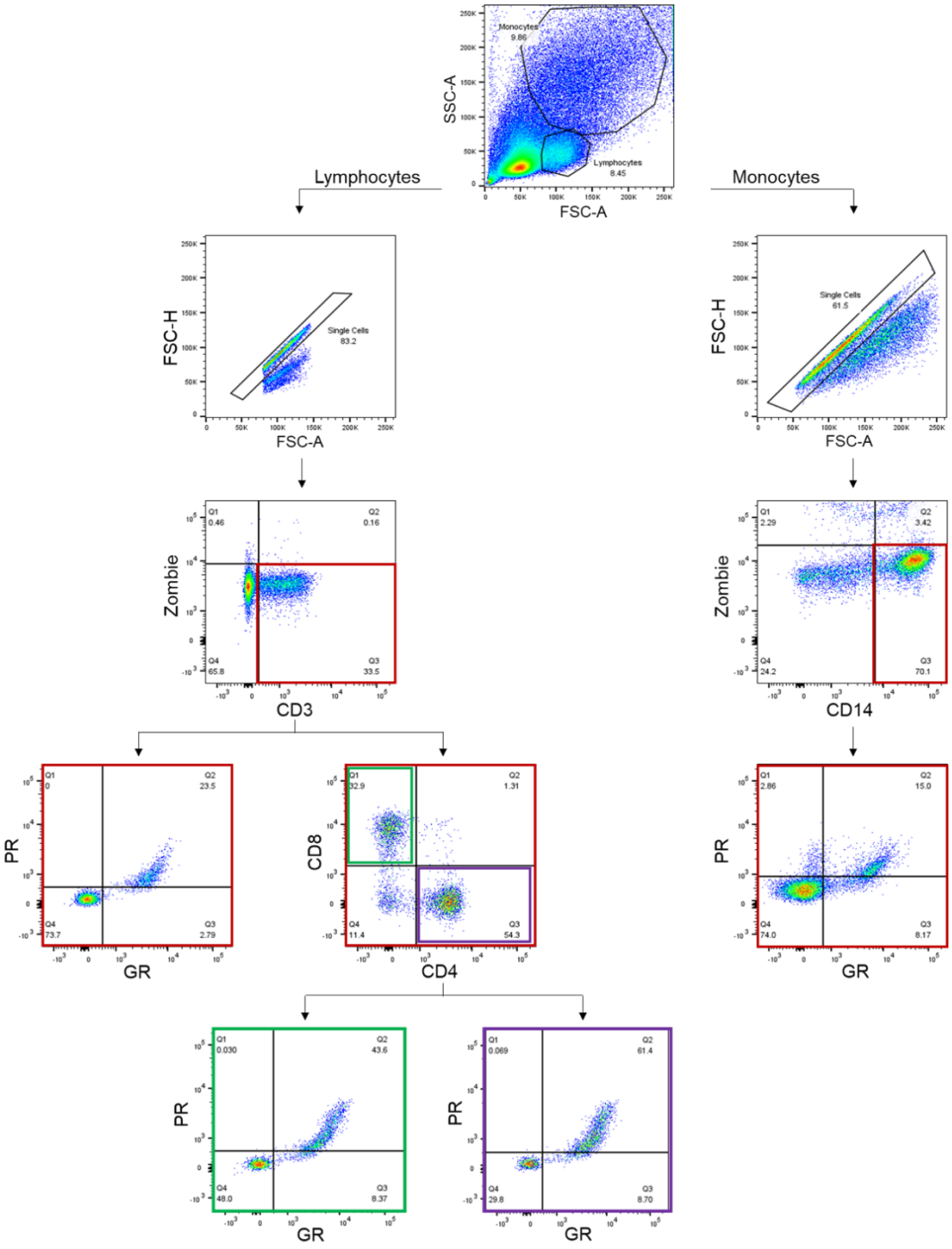


Figure A1: FACS gating strategy. Individual FMO controls were used to set the gates. The figure indicates scatter plots from a single representative PBMC donor.

Table A1: Summarized UHPSFC-MS/MS method validation data*

Ligand	LOD ng/mL (nM)	LLOQ ng/mL (nM)	Accuracy†			Precision‡			Mass transition: Qualifier ion	Mass transition: Quantifier ion	Retention time (min)
			1 ng/mL	10 ng/mL	100 ng/mL	1 ng/mL	10 ng/mL	100 ng/mL			
DEX	5 (12.7)	5 (12.7)	-	12.8	16.9	-	12.5	11.8	393.4>147	393.4>236.95	1.71
MPA	0.5 (1.3)	1 (2.6)	10.9	11.1	9.7	14.0	6.7	9.3	387.3>123.3	387.3>285	1.00
NET	0.5 (1.7)	1 (3.3)	14.3	11.5	9.8	14.1	4.4	9.7	299>231	299>109	1.46
LNG	0.01 (0.03)	5 (16.0)	-	4.7	11.2	-	7.4	9.7	313.1>109.4	313.1>131.1	1.30
ETG	1 (3.18)	5 (15.4)	-	13.1	8.0	-	10.9	8.7	352.2>147.1	352.2>109.1	1.28
NES	0.5 (1.3)	1 (2.7)	16.5	18.9	16.9	19.0	16.1	18.8	371.3>269	371.3>253.2	1.20

*Adapted from Skosana et al., 2019

†Defined as the % RSD from the analysis of independent replicate samples

‡Defined as the %RSD of the average calculated concentrations

Appendix B

Supplementary data for Chapters 3-5

Table B1: p-values associated with cytokine expression levels from PBMC supernatants treated with DEX and select progestins

A

	IL-5 p-values		
Ligand	**1 nM**	**10 nM**	**100 nM**
DEX	0.9666	0.0005	0.0352
MPA	0.8436	0.9818	0.4179
NET	0.9749	>0.9999	0.9486
LNG	0.9329	0.3959	0.7105
ETG	0.8521	0.9999	0.9163
NES	0.9687	0.9934	0.9501

B

	IL-13 p-values		
Ligand	**1 nM**	**10 nM**	**100 nM**
DEX	0.9999	0.0119	<0.0001
MPA	0.9921	0.5936	0.9981
NET	0.6013	0.1309	0.5947
LNG	0.9506	0.9769	0.8861
ETG	>0.9999	>0.9999	>0.9999
NES	>0.9999	>0.9999	0.9980

C

	IL-10 p-values		
Ligand	**1 nM**	**10 nM**	**100 nM**
DEX	0.9653	0.9989	0.9976
MPA	0.5586	0.8323	0.4582
NET	0.8203	>0.9999	0.6808
LNG	0.9090	0.9736	0.6982
ETG	0.7988	>0.9999	0.8761
NES	0.8164	0.9928	0.9570

D

	IL-1β p-values		
Ligand	**1 nM**	**10 nM**	**100 nM**
DEX	0.8018	0.9997	0.9994
MPA	0.9989	0.9980	0.9698
NET	0.9949	0.4293	0.2809
LNG	>0.9999	>0.9999	0.9106
ETG	>0.9999	0.7333	0.9730
NES	0.7191	0.1050	0.9954

E

	G-CSF p-values		
Ligand	**1 nM**	**10 nM**	**100 nM**
DEX	0.9971	0.9995	0.9954
MPA	>0.9999	0.2369	0.999
NET	0.9816	0.4689	0.9867
LNG	0.0887*	0.4789	0.9933
ETG	0.9954	0.5193	0.744
NES	>0.9999	0.4760	0.9381

F

	IFNγ p-values		
Ligand	**1 nM**	**10 nM**	**100 nM**
DEX	0.9915	0.7329	0.1456
MPA	0.4499	0.8524	0.0294*
NET	0.8472	>0.9999	0.9311
LNG	0.9305	0.6839	0.2501
ETG	0.1186	0.9646	0.9775
NES	0.3449	0.6133	0.9801

G

	TNFα p-values		
Ligand	**1 nM**	**10 nM**	**100 nM**
DEX	0.9345	0.0671*	0.0504*
MPA	0.9099	0.5009	0.9858
NET	0.8317	>0.9999	0.9641
LNG	0.9993	>0.9999	0.9988
ETG	0.9715	>0.9999	0.9096
NES	0.6642	0.9896	0.9900

H

	IL-1α p-values		
Ligand	**1 nM**	**10 nM**	**100 nM**
DEX	0.6775	0.9516	0.9998
MPA	0.9926	>0.9999	0.9020
NET	0.4840	0.4153	0.3953
LNG	>0.9999	0.9882	>0.9999
ETG	0.9915	0.9738	0.9998
NES	0.9909	0.9985	0.9847

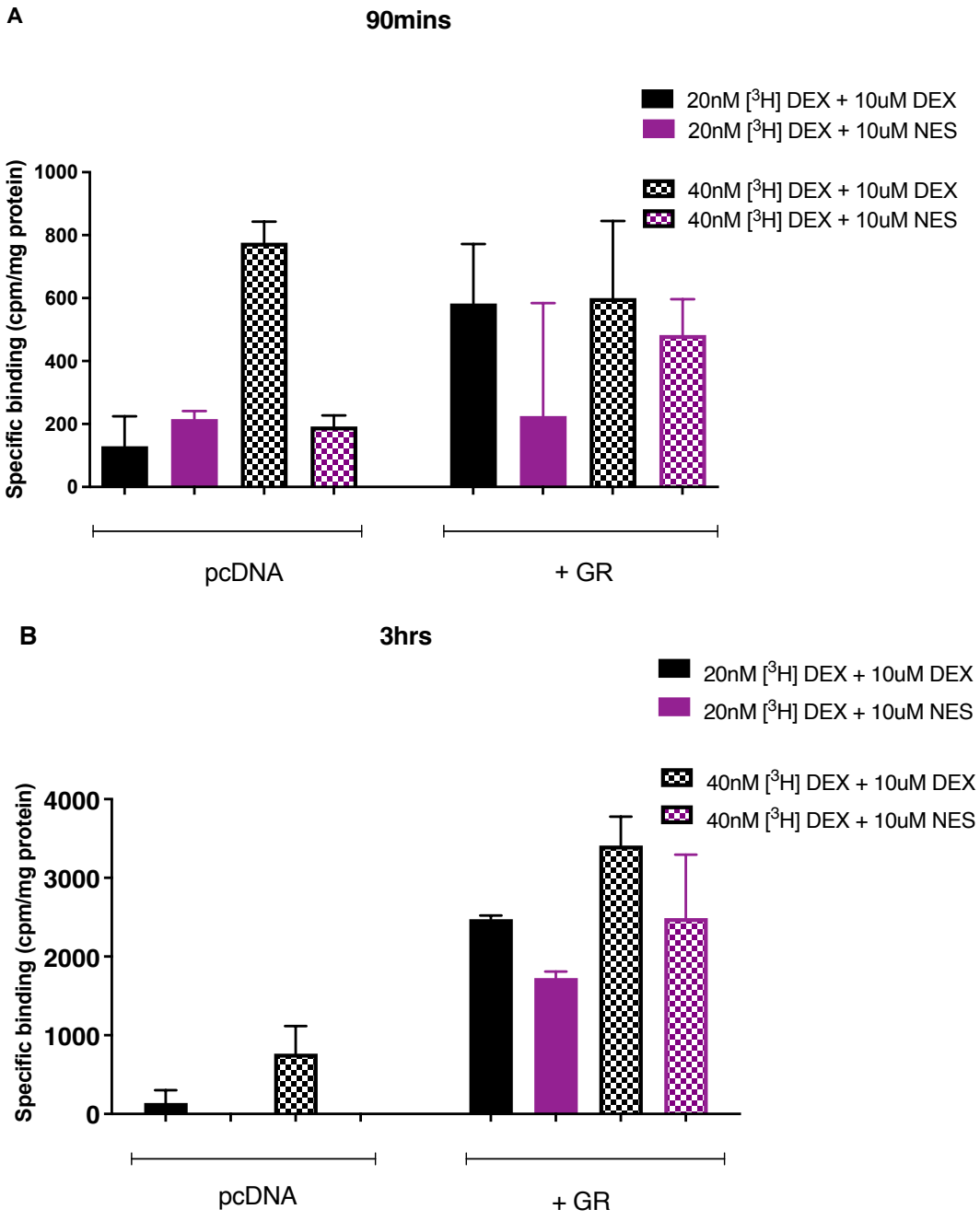
I

	IL-6 p-values		
Ligand	**1 nM**	**10 nM**	**100 nM**
DEX	0.7572	0.8566	0.8279
MPA	>0.9999	0.3497	0.9712
NET	0.9609	0.9918	0.9894
LNG	0.3906	0.7157	0.9957
ETG	>0.9999	>0.9999	0.9791
NES	0.9783	0.9991	0.9486

J

	IL-17α p-values		
Ligand	**1 nM**	**10 nM**	**100 nM**
DEX	0.9799	0.9590	0.2183
MPA	0.9896	>0.9999	0.8333
NET	0.1945	0.5850	0.3957
LNG	0.9420	0.9761	0.6729
ETG	0.9836	0.9986	0.8957
NES	0.9934	0.9926	0.9935

*p-values approaching significance


A
90mins
20nM [3H] DEX + 10uM DEX
20nM [3H] DEX + 10uM NES
40nM [3H] DEX + 10uM DEX
40nM [3H] DEX + 10uM NES
Specific binding (cpm/mg protein)
1000
800
600
400
200
0
pcDNA
+ GR
B
3hrs
20nM [3H] DEX + 10uM DEX
20nM [3H] DEX + 10uM NES
40nM [3H] DEX + 10uM DEX
40nM [3H] DEX + 10uM NES
Specific binding (cpm/mg protein)
4000
3000
2000
1000
0
pcDNA
+ GR

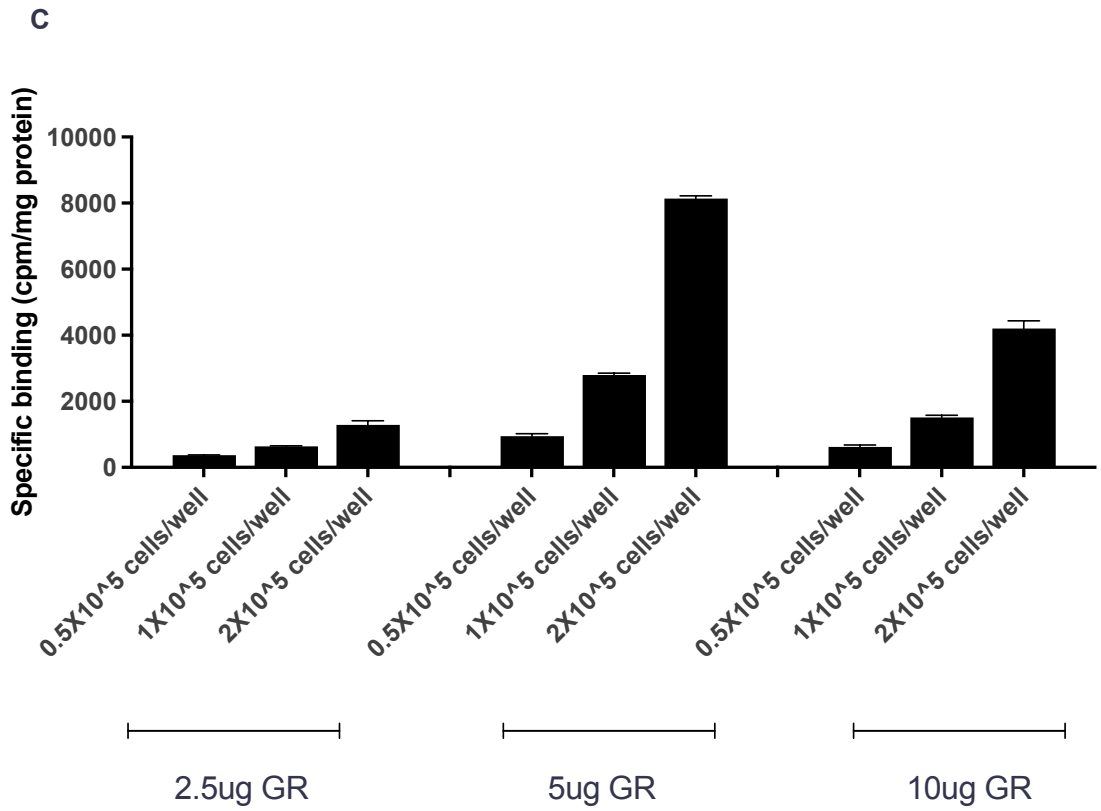


Figure B1: Optimal specific binding detected in COS-1 cells seeded at 2x10^5 cells/well expressing 5 μg of exogenous GR and treated with 20 nM [^{3}H]-DEX. COS-1 cells were seeded at 1x10^5 cells/well and transiently transfected with 10 μg pGR or pcDNA empty vector and treated for (A) 90 mins (B) 3 hours with 20 nM or 40 nM [^{3}H]-DEX in the absence or presence of 10μM competing unlabelled ligands. (C) COS-1 cells seeded at varying cell densities and transiently transfected with 2.5 μg or 5 μg or 10 μg of pGR and treated for 3 hours with 20 nM [^{3}H]-DEX in the absence or presence of 10μM unlabelled DEX. Specific binding was calculated as the difference between total binding ([3H] DEX only) and nonspecific binding ([3H] DEX + 10 μM unlabelled DEX). The mean ± SEM is shown from 2 independent experiments, where each condition was performed in triplicate.

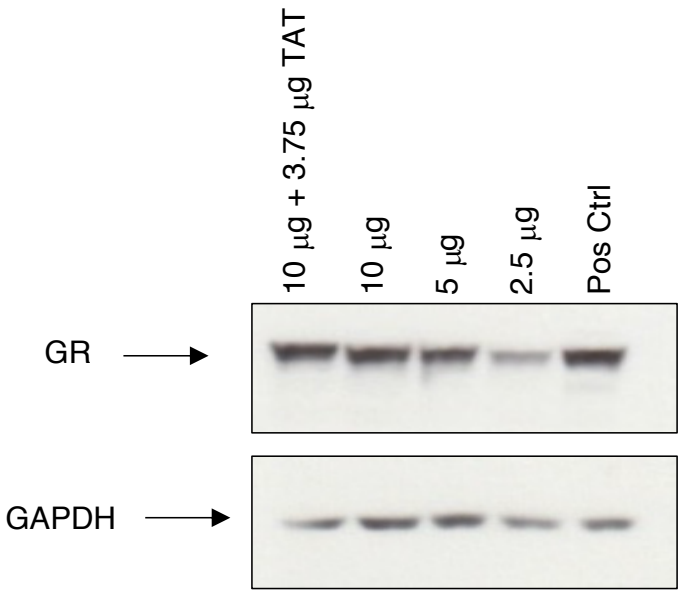


Figure B2: Representative western blot of GR at 95 kDa with GAPDH (37 kDa) as loading control. COS-1 cells were transiently transfected with 2.5 *µ*g or 5 *µ*g or 10 *µ*g pGR or 10 *µ*g pGR + 3.75 *µ*g TAT-GRE or pcDNA empty vector. Total protein lysates from COS-1 cells were analysed for GR protein levels by western blotting.

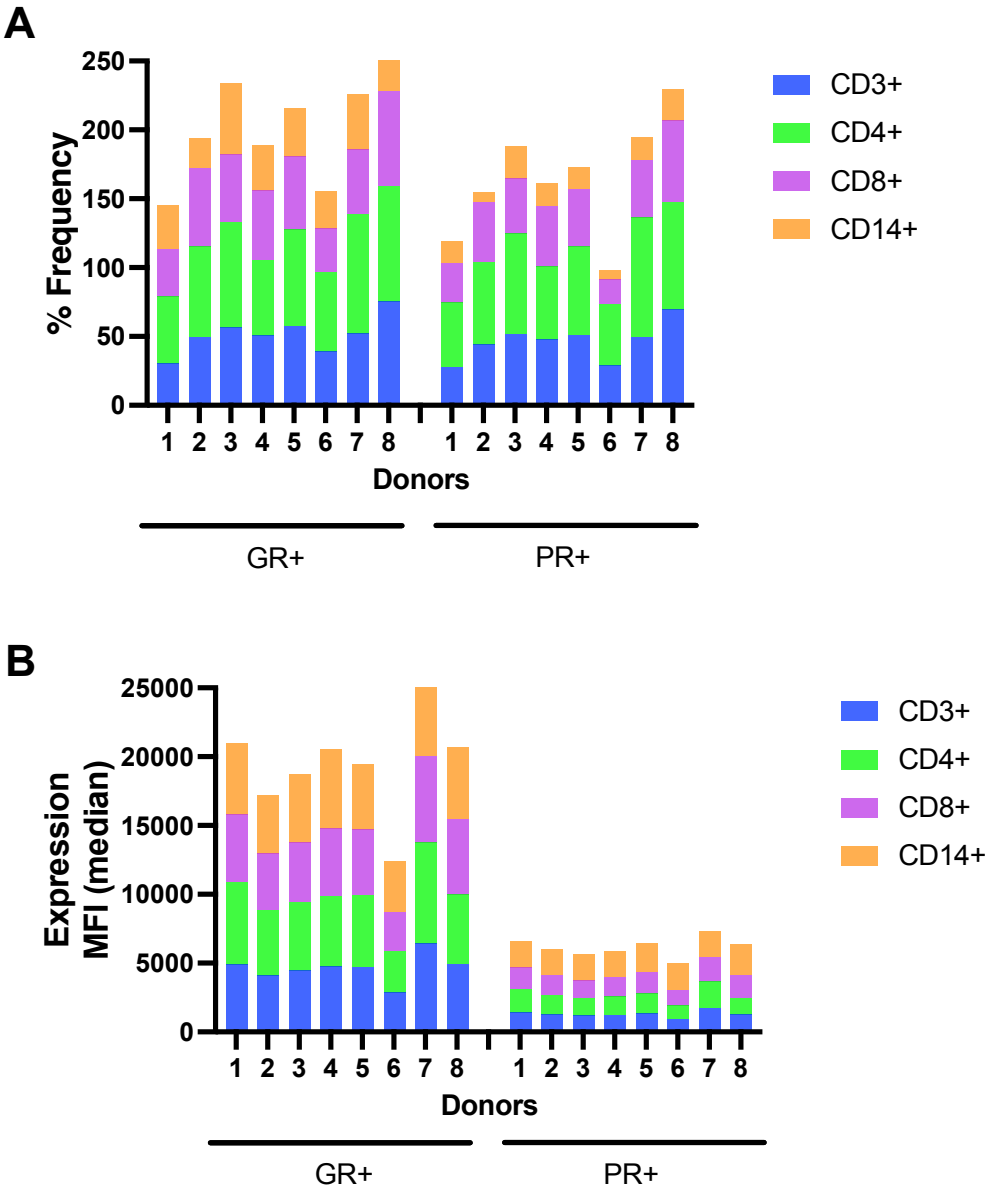


Figure B3: The frequency and MFI of GR and PR varies between donors across PBMC cell subtypes: PBMCs were thawed and rest overnight, then the levels of GR or PR were determined in CD3⁺(blue), CD4⁺ (green) and CD8⁺ T cells (purple) as well as CD14⁺ monocytes (orange) using flow cytometry. Results shown as (A) % frequency or (B) MFI of GR or PR expressing CD3⁺, CD4⁺ and CD8⁺ T cells, as well as and CD14⁺ monocytes in eight donors.

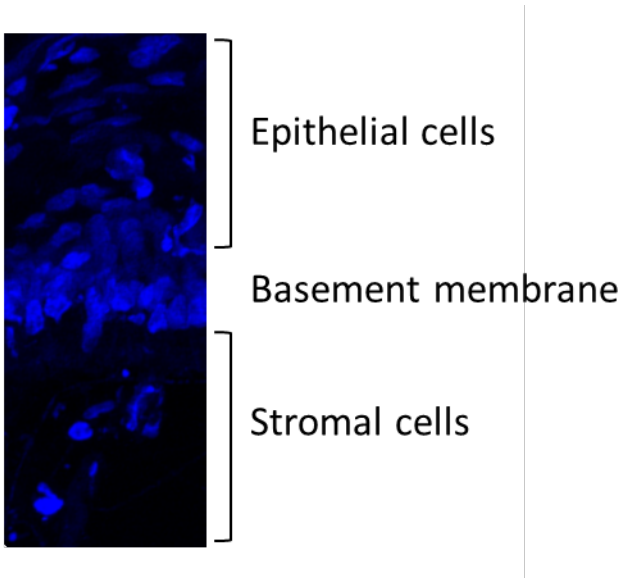


Figure B4. Annotation of epithelial and stromal cells separated by the basement membrane. Hoechst stain (**Blue**) nuclear stain of ectocervical tissue.

Table B2: Table showing the potencies of the ligands that fall within published serum concentration ranges.

Ligand	COS-1		PBMC		Serum Concentration (nM)
	GRE	GILZ	GILZ	IL-6	
MPA	41.7 nM	56.3 nM	OOR	9.2 nM	2.6-99.6[a]
NET	ND	ND	51.2 nM*	4.66 nM†	2.4-117[b]
LNG	ND	ND	ND	29.5 nM	0.3-616.4[c]
ETG	OOR	8.45 nM	OOR	OOR	0.28-19.1[d]
NES	18.5 nM	OOR	OOR	5.49 nM	0.18-27.3[e]

[a]Oral 10mg/ Intramuscular injection 150mg
[b]Intramuscular injection 200mg
[c]Oral 0.1-1.5mg (+ EE)/ Implant 150mg/ IUD 52mg
[d]Oral (0.150 DSG + 0.030 EE)/ Vaginal ring 11.7mg + 2.7mg EE)/ Implant 68mg
[e]Subcutaneous capsule 0.04mg/ Vaginal ring 0.15-200mg (+EE)
*The EC_{50} values for NET should be interpreted with caution as very weak agonist activity was observed
†The EC_{50} values for NET should be interpreted with caution as Prism 9 deemed this curve unstable due to variable data.
ND, not determined.
OOR, out of range – EC_{50} falls outside of the serum concentration range

Appendix C

Publications and statements of work conducted by the present author:

Part of the data shown in Chapters 3 (**Figures** 3.2.1.1, 3.2.1.2, 3.2.2.1, 3.2.2.2, 3.2.4.1, 3.2.5.1 and 3.2.6.1; **Tables** 3.2.1, 3.2.2 and 3.2.5; Appendix B, **Figure** B2 and **Table** B2) and 5 (**Figure** 5.2.1A and B) are contained in the following publications:

Maleshigo Komane, Chanel Avenant, Renate Louw-du Toit, Donita J. Africander and Janet P. Hapgood (2022). **Differential off-target glucocorticoid activity of progestins used in endocrine therapy.** *Steroids.* 182. DOI: 10.1016/j.steroids.2022.108998.

Statement by the present author: All binding and transactivation data presented in COS-1 cells and PBMCs, the present author conducted all cell culture maintenance and experimental procedures, including whole cell binding and transactivation assays, as well as western blotting. The present author performed all statistical analyses and preparation of associated figures. Furthermore, the present author wrote the initial draft and contributed to incorporating valuable feedback and proof-reading subsequent drafts.

Salndave B. Skosana, John G. Woodland, Meghan C. Cartwright, Kim Enfield, **Maleshigo Komane**, Renate Louw-du Toit, Zephany van der Spuy, Chanel Avenant, Donita J. Africander, Karl. H. Storbeck, Janet P. Hapgood (2019). **Differential metabolism of clinically relevant progestogens in cell lines and**

tissue: Implications for biological mechanisms. *J Steroid Biochem.* 189. DOI:10.1016/j.jsbmb.2019.02.010.

Statement by the present author: All data presented in which COS-1 and HeLa cells were used were generated by the present author, including all cell culture maintenance, as well as conducting the MTBE steroid extraction and UHPSFC-MS/MS experimental procedure. The present author performed all statistical analyses for data generated in COS-1 and HeLa cells and prepared all associated figures. The present author contributed intellectually to the discussion, interpretation of the results and providing valuable feedback.

Parts of the work in this thesis were presented by the present author at the following meetings:

- HIV Research for Prevention (HIVR4P), Madrid, Spain, October 2018
- 17th International Congress on Hormonal Steroids and Hormones & Cancer, Cape Town, South Africa, November 2018

Appendix D

Research Articles

HHS Public Access

Author manuscript

J Steroid Biochem Mol Biol. Author manuscript; available in PMC 2020 May 01.

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Differential metabolism of clinically-relevant progestogens in cell lines and tissue: Implications for biological mechanisms

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Abstract

Steroid hormones regulate a variety of physiological processes, including reproductive function, and are widely used in hormonal therapy. Synthetic progestogens, or progestins, were designed to mimic progesterone (P4) for use in contraception and hormonal replacement therapy in women. Medroxyprogesterone acetate (MPA) and norethisterone (NET) are the most widely used injectable contraceptives in the developing world, while other progestins such as levonorgestrel (LNG), etonogestrel (ETG) and nestorone (NES) are used in or being developed for other forms of contraception. As concerns remain about the most appropriate choice of progestin and dosage, and the associated side-effects, the mechanisms and biological effects of progestins are frequently investigated in various *in vitro* mammalian cell line and tissue models. However, whether progestogens are differentially metabolised in different cell types *in vivo* or *in vitro* is unknown. For nine mammalian cell lines commonly used to investigate progestogen mechanisms of action, we developed and validated an ultra-high performance supercritical fluid chromatography-tandem mass spectrometry (UHPSFC-MS/MS) protocol for simultaneously quantifying the metabolism of the above-mentioned steroids. We show for the first time that, while 50–100% of P_4 was metabolised within 24 hours in all cell lines, the metabolism of the progestins is progestin-and cell line-specific. We also show that MPA and NET are significantly metabolised in human cervical tissue, but to a lesser extent than P4. Taken together, our findings suggest that differential progestogen metabolism may play a role in cell-specific therapeutic and side-effects. Relative affinities for binding to steroid receptors as well as potencies, efficacies and biocharacters for transcriptional activity of progestins, relative to P4, are most frequently determined using some of

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Declarations of interest: None

the cell lines investigated. Our results, however, suggest that differential metabolism of progestins and P_4 may confound these results. In particular, metabolism may under-estimate the receptor-mediated intrinsic *in vitro* binding and dose-response values and predicted endogenous physiological effects of P_4.

Keywords

contraceptives; metabolism; progesterone; progestins; steroids; UHPSFC-MS/MS

1. Introduction

Choice of hormonal contraception and hormone replacement therapy (HRT) in women is an important public health issue, especially regarding possible side-effects relevant to cancer, metabolic disorders, cardiovascular complications, bone mineral density and susceptibility to infectious diseases [1,2,3]. Synthetic steroids are commonly utilised in contraceptive treatments and HRT. These synthetic steroids, known as progestins or synthetic progestogens, are intended to mimic the actions of the endogenous hormone P_4 [1–3] and are classified into two groups. The first class of progestins, which include medroxyprogesterone acetate (MPA) and nestorone (NES), is structurally related to P4, while the second class is structurally related to testosterone (T), and includes norethisterone (NET), etonogestrel (ETG) and levonorgestrel (LNG) [2,3]. Injectable progestins, which are especially popular in the developing world as contraceptives due to their discreet nature, include MPA and NET, the latter administered in its enanthate form (NET-EN) [2,3]. Other progestins such as LNG and ETG are widely used in combined oral contraceptives and implants and together with NES, are currently being investigated for use intravaginally or in multipurpose prevention technologies [3].

Progestin research relies extensively on model systems using well-established laboratory cell lines [2–6] or *in vitro* experiments with primary cells, tissue or tissue extracts [7–12]. In such experiments, specific concentrations of the steroids are used and these concentrations are assumed to remain constant over the incubation period. Differences in activity between steroids is thought to be due to their different biocharacters, and metabolism is not taken into account. Differential metabolism may confound the results of concentration-dependent experiments such as dose-response analyses and binding studies [2–4]. It is well established that progestins act intracellularly via binding to and activating the progesterone receptor (PR) [2,3], which is a ligand-activated transcription factor. Evidence is emerging that some of the side-effects of progestins may occur by off-target effects via binding to and activating steroid receptors other than the PR [3,5]. However, very little is known about the metabolism of progestins, in particular whether this is cell-specific, which metabolites are produced, what the role is of metabolites and whether metabolism may confound interpretation of the results when investigating relative biological activities.

The aim of this work was therefore to investigate the metabolism of P_4 and selected progestins in nine commonly used laboratory cell lines, and to validate select findings in endocervical tissue. To this end, we developed and validated an ultra-high-performance

supercritical fluid chromatography-tandem mass spectrometry (UHPSFC-MS/MS) method for the separation and quantification of these progestogens in the nanomolar range, as detected in the serum of women. We included the synthetic glucocorticoid dexamethasone (DEX) in our panel of steroids, since the activity of progestins is often investigated in parallel with DEX, given the established glucocorticoid activity of MPA [10, 13–14]. Results showed that P_4 was substantially metabolised in all cells lines and the endocervical tissue after 24 hours, while cell line-and steroid-specific metabolism were observed for the different progestins.

2. Materials and Methods

2.1 Steroids and solvents

LNG was obtained from the United Stated Pharmacopoeia (USP, Rockville, MD, USA) and Sigma Aldrich (South Africa). P4, MPA, NES, DEX, NET, ETG, T, UHPLC-grade methanol, absolute ethanol, formic acid and methyl *tert*-butyl ether (MTBE) were all purchased from Sigma-Aldrich (South Africa).

2.2 Cell lines and endocervical tissue

Human embryonic kidney cells (HEK293T), human epithelial cervical cancer cells (HeLa), human endocervical cells (END-1), human bone osteosarcoma epithelial cells (U2OS) and monkey kidney fibroblast cells (COS-1) were purchased from American Type Culture Collection (ATCC, USA). Human cervical cells (TZM-bl) were procured from the NIH AIDS Reagent Program, Division of AIDSNIAID, NIH, from Dr John C. Kappes, Dr Xiaoyun Wu and Tranzyme Inc. (ARP, NIH, USA). The human MDA-MB-231 breast cancer cell line was originally acquired from ATCC, but were received from Prof Adrienne Edkins at Rhodes University, South Africa, while Prof Ana Soto at Tufts University, Boston, USA provided the human MCF-7 BUS breast cancer cells. The human T47D breast cancer cell line was donated by Prof Iqbal Parker at the University of Cape Town, South Africa.

Endocervical tissue was obtained after informed consent from HIV-1 negative, post-menopausal women undergoing hysterectomies for benign reasons. Ethical permission was obtained from the Human Research Ethics Committee (University of Cape Town) for the duration of this study (HREC 258/2017). Fresh tissue was supplied from two sites in the Western Cape, South Africa; namely, Groote Schuur and Tygerberg Hospitals.

2.3 Cell line culture

HEK293T, HeLa, U2OS, TZM-bl, T47D and COS-1 were all cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, South Africa) supplemented with 1 mM sodium pyruvate (Sigma-Aldrich, South Africa), 44 mM sodium bicarbonate (Sigma-Aldrich, South Africa), 10% (v/v) fetal bovine serum (FBS) (Thermo Scientific, South Africa), 100 IU/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich, South Africa). Culture medium for MDA-MB-231 cells was as described above, with the addition of 2 mM L-glutamine (Sigma-Aldrich, South Africa). Culture medium for MCF-7 BUS cells was as described above, except that 5% heat inactivated FBS was used. END-1 cells were maintained in keratinocyte serum-free (KSF) medium (Sigma-Aldrich, South Africa)

supplemented with the provided keratinocyte growth supplement, 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco Invitrogen). Cells were maintained at 37°C in a water-jacketed incubator (90% humidity and 5% CO2). All cells were routinely tested and found to be mycoplasma-free.

2.4 Cervical tissue experiments

Cervical tissue was processed as previously described by Fletcher *et al.* (i.e. between one to three hours post-operation) [15]. Excess underlying stromal tissue was removed from the epithelial layer of the endocervical tissue. The epithelial layer was then diced into 3 mm^3 explant pieces that were randomly placed into separate wells of 96-well round-bottomed plates. Non-polarised explants were cultured in 200 μL Roswell Park Memorial Institute medium (RPMI) (Lonza, Switzerland) supplemented with 10% (v/v) charcoal stripped FBS (Thermo Scientific, USA), 2 mM L-glutamine (Sigma-Aldrich, South Africa), 10 μg/mL Fungizone (Sigma-Aldrich, South Africa), 10 U/mL interleukin-2, 100 IU/mL penicillin and 100 mg/mL streptomycin (Sigma-Aldrich, South Africa). Cervical tissue explants were incubated in quadruplicate with steroids in RPMI and incubated at 37°C in a water-jacketed incubator (90% humidity and 5% CO2) for 24 hours.

2.5 Cell line and tissue incubations with steroids

Cells were seeded at 5×10^4 cells per well (T47D, MCF-7 BUS, MDA-MB-231) or 1×10^5 cells per well (END-1, U2OS, TZM-bl, HEK293T, COS-1 and HeLa) in full phenol red-containing media in a 24-well Greiner Bio-One CELLSTAR tissue culture plate. Tissue was processed and plated as described above. Following a 24-hour incubation period T47D, MCF-7 BUS and MDA-MB-231 cell media was replaced with phenol red-free media. For analysis of extent of metabolism, the cells and no-cell controls were washed with pre-warmed media then treated with 100 nM steroid or vehicle (0.1% v/v ethanol) in serum-free media. The U2OS, TZM-bl, HEK293T, COS-1, END-1 and HeLa cells were treated in phenol red-containing DMEM, while T47D, MCF-7 BUS and MDA-MB-231 cells were treated in phenol red-free DMEM. Upon treatment, 500 μL of the steroid-and vehicle-containing media was aliquoted into a glass tube and stored at −20°C; this served as the T0 control. After 24 hours, 500 μL aliquots of media were removed from the cells (or no-cell control) and transferred into clean glass tubes and stored at −20°C prior to extraction

2.6 Preparation of standards and samples

Individual stock solutions of the seven steroids (P4, MPA, NES, NET, LNG, ETG and DEX) plus internal standard T were prepared in absolute ethanol (1 mg/mL) and stored at −20°C until use. These individual stock solutions were later used to prepare two standard master mixes (1 000 ng/mL and 1 ng/mL) containing all of the above-mentioned steroids in ethanol. These standard master mixes were subsequently used to prepare standards (1 mL, 0.01–100 ng/mL) by the addition of the appropriate volume of the standard master mix to either (i) DMEM containing 1% penicillin-streptomycin and 10% FBS (“supplemented DMEM”), (ii) DMEM without penicillin-streptomycin or FBS (“unsupplemented DMEM”), (iii) KSF without penicillin-streptomycin or FBS, (iv) RPMI 1640 without penicillin-streptomycin or FBS or (v) 50% methanol (no matrix). Samples used for method validation (1 mL) were prepared by spiking the matrix with the appropriate volume of the master mixes. 100 μL of

internal standard prepared in distilled water to a final concentration of 1 ng/mL was added to all samples and standards.

2.7 Steroid extractions

Samples and standards were extracted using a 1:3 ratio of sample to MTBE (v/v). The samples were shaken at 1 000 rpm for 15 minutes before being placed at −80°C for an hour to allow the aqueous phase to freeze. The MTBE layer containing steroids was transferred to a pyrolyzed glass test tube and the MTBE was evaporated at room temperature in a fume hood overnight, or under a stream of nitrogen gas. Samples were subsequently reconstituted in 150 μL 50% methanol and stored at −20°C prior to analysis.

2.8 Instruments and chromatographic conditions for UHPSFC-MS/MS

Steroids were separated using an Acquity Ultra High Performance Convergence Chromatography (UPC^2) system (Waters Corporation, Milford, USA) with an Acquity UPC^2 Ethylene Bridged Hybrid (BEH) column (3 mm x 100 mm, 1.7 μm particle size). The mobile phase consisted of liquid CO2 (Mobile phase A) and methanol [Mobile phase B (MPB)]. A 2.5-minute gradient inlet method was used to separate the steroids using a constant flow rate of 1.9 mL/min according to the following protocol: 4% MPB from 0–1 min; 10% MPB from 1–1.5 min; 25% MPB from 1.5–2.5 min and back to 4% MPB at 2.5 min for re-equilibration. The column temperature and automated back pressure regulator were set to 60°C and 1700 pounds-force per square inch (psi), respectively. The injection volume was 2.0 μL. Quantitative mass spectrometric detection was carried out using a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, USA). A make-up pump fed 1% formic acid in methanol into the mixer preceding the MS line at a constant flow rate of 0.2 mL/min. All steroids were analysed in multiple reaction monitoring (MRM) mode using an electrospray probe in the positive ionisation mode (ESI+). The following settings were used: capillary voltage of 3.8 kV, desolvation temperature 350°C, desolvation gas 900 L/h and cone gas 150 L/h. MRM transitions are included in Supporting Table 1. Data collection and analysis were performed using MassLynx 4.1 (Waters Corporation).

2.9 UHPSFC-MS/MS method validation

Standard curves were generated for each steroid metabolite using standards prepared in either of the four matrices listed above or 50% methanol (no matrix), and included the following concentrations: 0, 0.01, 0.1, 0.25, 0.5, 1.0, 5.0, 10, 25, 50 and 100 ng/mL. The limit of detection (LOD) for each steroid was defined as the lowest concentration at which a signal-to-noise (S/N) ratio greater than three was measured for the quantifier ion. The lower limit of quantification (LLOQ) for each steroid was defined as the lowest concentration for each steroid at which: a S/N ratio greater than ten was measured for the quantifier ion; a S/N ratio greater than three was measured for the qualifier ion; an acceptable precision [% relative standard deviation (% RSD) <20] could be measured. The upper limit of quantification (ULOQ) was defined as the maximum concentration at which the % RSD values did not exceed 20. Precision was defined as the % RSD from the average calculated concentrations following the repeated injection (n=6) of a simple sample. Accuracy was defined as the % RSD from the analysis of independent replicate samples (n=6).

2.10 Statistical analysis

Results were analysed using GraphPad Prism 7 from GraphPad Software, Inc. (La Jolla California, USA). Data are expressed as mean ± SEM. To evaluate whether the metabolism of a steroid within a cell line/tissue was statistically significant, a paired *t*-test was performed to compare results in the absence and presence of cells. Statistical significance is denoted by the relevant p-value. Multiple paired *t*-tests were used to compare the metabolism of the seven steroids within a cell line to each other, and to compare the metabolism of a specific steroid across different cell lines. (ANOVA was not used since these experiments were not all performed in parallel.) Where statistical significance was determined, it is denoted by *, **, ***, or **** to indicate $p<0.05$, $p<0.01$, $p<0.001$, or $p<0.0001$, respectively.

3. Results

3.1 Validation and performance of UHPSFC-MS/MS method

A UHPSFC-MS/MS method was developed for the quantification of six clinically relevant, commercially-available progestogens and DEX. Their chemical structures are depicted in Figure 1. Molecular ion species, MRM mass transitions and retention time for each steroid are given in Supporting Table 1. Comprehensive method validation was performed, the results of which are listed in Table 1.

As most of the cell lines were treated with unsupplemented DMEM, accuracy and precision were determined only for this medium at a minimum of three concentrations within the calibration range of each steroid as shown in Table 1, i.e. 1, 10 and 100 ng/mL. Acceptable % RSDs were obtained for all concentrations for both accuracy and precision, which were less than 20 at concentrations of 1, 10 and 100 ng/mL. Accuracy at low concentrations ranged from 11–18% at 1 ng/mL, 11–18.9% at 10 ng/mL and 8–17% at 100 ng/mL. Precision at low concentrations ranged from 14–19% at 1 ng/mL, 4–16% at 10 ng/mL and 9–19% at 100 ng/mL. LLOQs ranged from 0.01 ng/mL for P_4 to 5.00 ng/mL for LNG, ETG and DEX, allowing for the quantification of steroids at levels at the low nanomolar range. For each of unsupplemented DMEM, KSF and RMPI media, the ULOQ was 100 ng/mL (the highest concentration measured), while ULOQ in supplemented DMEM was 50 ng/mL.

3.2 Effects of adsorption and hydrophobicity

Following the development and validation of the UHPSFC-MS/MS method we first considered the potential effects of adsorption of the steroids to the cell culture plates, before measuring the metabolism of the steroids. To do this we assessed the differences in steroid concentration between the T0 and media from no-cell control plate incubations for all the steroids in the different experiments (Supporting Figure 1). There was 0%−40% adsorption of the steroids to the cell culture plates across experiments (Figure 2 **and** Supporting Figures 1). We further investigated whether there was a correlation between hydrophobicity and adsorption of steroids. Results showed a positive correlation suggesting that the adsorption of steroids to the cell culture plates increases with increasing hydrophobicity (Figure 2). We investigated whether retention of the steroids occurred within the cell pellets. We found that this was negligible (Supporting figure 2) and hence was not taken into account when

calculating percentage metabolism. Based on these results metabolism (Section 3.3–3.5) was calculated relative to a no-cell control parallel incubation in cell culture plates and hence independent of adsorption to the cell culture plates.

3.3 Differential metabolism of steroids between cell lines

Next, we incubated nine common laboratory cell lines, along with a parallel plate without any cells ("no-cell control"), with 100 nM of each steroid for 24 hours. We subsequently measured the concentration of steroid present in the cell and no-cell supernatants. The percentage metabolism of each individual steroid in a cell line was calculated as the difference between the steroid remaining in the absence (no-cell) and presence of cells. Where percentage metabolism was less than zero, the percentage is represented as zero. It should be noted that steroid incubations were performed in the absence of serum for 24 hours as is frequently done for experiments in cell lines incubated with steroids [16–18]. It is possible that the presence of serum may affect metabolism.

When comparing metabolism effects, it was noted that the error bars were in general much greater for some cells than for others, as well as between some progestins for a particular cell line. Assessment of no cell samples indicate that this reflects variations in technical error during experiments. Only differences that were found to be statistically significant are highlighted and discussed below. It should, however, be noted that there may be other differences that are significant, but beyond the statistical power of the experiments. The most striking result was that P_4 was extensively metabolised by all the cell lines, although the extent of metabolism varied from 50–97%, showing some cell line-specific effects (Figures 3 **and** 4). Although the metabolism of the progestins and DEX was also cell line-specific, it ranged from 0–50%, with the rank order for most to least metabolised steroid different for each cell line. These did not appear to be related to the anatomical source or type of cell line, as shown in Figure 4. However, some trends were apparent (Figures 3 **and** 4). Of the cervical cell lines, HeLa exhibited a higher percentage of significant metabolism for most progestogens than observed in TZM-bl and END-1 cells, except for NET and ETG where END-1 cells exhibited more metabolism. The T47D cell line exhibited the highest significant metabolism within the breast cancer cell lines, except for NES where MCF-7 BUS showed greater significant metabolism. END-1, T47D, MCF-7 BUS and COS-1 cells were the most metabolically diverse cells in the panel, with all four of these cell lines displaying significant metabolism of P_4 and ETG; and three of these four cell lines showing metabolism of DEX (Figure 3). Interestingly, COS-1 cells displayed a similar high degree of significant metabolism for both P_4 (60%) and DEX (53%). These cell lines were followed by U2OS cells, which significantly metabolised three steroids. TZM-bl and MDA-MB-231 cells had significant metabolism of P_4 and one other steroid, namely, MPA and ETG respectively. HeLa and HEK293T cells displayed the lowest metabolic activity of progestogens with significant metabolism observed only for P_4 (Figure 3). Taken together, there was no significant difference in the overall metabolism of the progestins structurally related to P_4 (MPA and NES) than those structurally related to T (NET, LNG and ETG) (Figure 4), with an average of four times more metabolism observed for P_4 than the other steroids.

3.4 Differential metabolism of steroids within cell lines

Upon comparison of the metabolism of each steroid within each cell line, it was observed that apart from P4, all steroids were metabolised in a cell-specific manner (Figure 3 **and** Supporting Figure 3). T47D, END-1 and HeLa cells had the highest percentage metabolism of P_4 with over 90% metabolism in all three cell lines. HEK293T and COS-1 cells displayed the least metabolism of P_4 with only 50% and 60% metabolism, respectively. P_4 demonstrated the greatest differences in metabolism compared to other steroids within each cell line (Figure 4 **and** Supporting Figure 3).

ETG was the second most metabolised steroid, with significant metabolism in five cell lines ranging between 14% and 43% (Figure 3 **and** 4). HeLa cells showed a high percentage of metabolism of ETG with 28% metabolism; this, however, was not significant (Figure 4). MPA and DEX were significantly metabolised in 3 cell lines each. Significant metabolism of MPA was observed in U2OS, TZM-bl and T47D cells, ranging between 19% and 55% (Figure 3). While, significant metabolism of DEX was observed in END-1, MCF-7 BUS and COS-1 cells with metabolism ranging from 8% and 52%. As shown in Figure 4, metabolism of DEX in HeLa, T47D and U2OS cells, although not significant, ranged from 21% to 37%. TZM-bl, HEK293T and MDA-MB-231 cells exhibited less than 10% metabolism of DEX. NET and NES were significantly metabolised only in two cell lines each (Figure 3). NET was significantly metabolised only in U2OS and MCF-7 BUS cells (Figure 3). NES was metabolised in END-1 and MCF-7 BUS cell lines by 9% and 40%, respectively (Figure 4). There was 41% metabolism of NES in COS-1 cells, and between 0% and 20% in the remaining cell lines; however, these effects were not statistically significant (Supporting Figure 3). LNG was significantly metabolised only in COS-1 cells with 23% metabolism which was comparable to the metabolism in T47D and HeLa cells which exhibited 22% and 20% metabolism, respectively. However, these latter effects were not statistically significant. The metabolism of LNG in the remaining six cell lines was less than 10% (Supporting Figure 3).

3.5 Metabolism in endocervical tissue

To determine if the metabolism observed in cell lines would be similar in a more physiologically-relevant system, we investigated the metabolism of three progestogens in post-menopausal endocervical tissue explants following a 24-hour incubation. P_4 was investigated due to the high rate of metabolism in all cell lines, whilst MPA and NET were chosen as representative of progestins structurally related to P_4 and T, respectively.

P_4 was completely metabolised in the endocervical tissue explants after 24 hours (Figure 5). The results for P_4, but not MPA and NET, are comparable to those observed in the three cervical cell lines (Figure 3). There was between approximately 87% and 96% metabolism of P_4 in the cervical cell lines, which is similar to the 100% metabolism in tissue (Figure 5). As depicted in Figure 5, 38% of MPA was metabolised in tissue. This is different from the pattern observed in cervical cell lines where TZM-bl cells exhibited significant, but less, metabolism of MPA, (Figure 3) while no significant metabolism was observed in the other cervical cell lines. While NET was not significantly metabolised in the cervical cell lines, endocervical tissue metabolised 43% of available NET.

4. Discussion

Previous research into progestogen metabolism has been limited and has typically focused on measuring the serum or urine concentrations of progestogens and/or their metabolites in a clinical setting [19–26]. In this work we investigated, for the first time, the cell-and steroid-specific metabolism of a range of clinically-relevant steroids using *in vitro* cell line models. These cell line models are widely used to investigate the biocharacter and mechanisms of action of these steroids, which themselves are commonly used in hormonal therapy and steroid receptor-based studies. We developed and validated a UHPSFC-MS/MS method of measuring the concentration of seven steroids in cell culture media, which allowed for the determination of the metabolism of these steroids by these cell lines. Our experimental design corrected for adsorption (up to 40%) of the steroids to the cell culture plates, where we found a positive correlation between adsorption and increasing hydrophobicity. The extent of metabolism could be measured from analysing the medium (supernatant) alone, since we found no significant retention of parent steroid in the cell pellets.

We found that individual progestins and DEX are differentially metabolised within the same cell line, and amongst different cell lines. For example, over 24 hours, some progestins are not significantly metabolised in a particular cell type (<20%), while others display a high degree of metabolism (20–50%) in the same cell type. A particular progestin can be metabolised to a vastly different degree in different cell lines (e.g. MPA metabolised by 55% in T47D and less than 10% metabolism in HEK293T and MDA-MB-231 cells). We detected no correlation between extent of metabolism and whether progestins were structurally related to P_4 or T. Taken together, these results may have important physiological and pharmacological implications. Progestins used in hormonal contraception and HRT are known to exert several side-effects, such as effects on bone mineral density, metabolism, cardiovascular effects and reproductive cancers [2–4, 6]. Progestins also exert their contraceptive effects at several levels at different target tissues. If these *in vitro* effects of metabolism are translated *in vivo*, this suggests that different progestins may exert very different side-effects and may be more or less efficacious for contraception due not only to their inherent biocharacters, potencies and efficacies, but also due to differential metabolism in different target cells and tissues. This metabolism could both selectively lower their effective concentrations at the target cells in a cell-specific manner and may, also result in different metabolites with different off-target effects. The tissue findings are particularly interesting since they suggest that doses and types of progestins used for intravaginal delivery need to be carefully considered, taking into account that the progestins may be significantly metabolised in the tissues. The results also suggest that different cell types contain different steroid metabolising enzymes which discriminate between the progestins, despite some of their structures being similar. Although, the identification of the enzymes that metabolise DEX and the progestins in our models was beyond the scope of the present study, it would be an interesting avenue to explore in future studies.

Our results with P_4 showing the rapid and substantial metabolism of this steroid in all the cells lines and in the cervical tissue, are particularly interesting. These results suggest that enzymes that metabolise P_4 are widely expressed in most cell types, including the female genital tract, bones and breast tissue, and that its rapid turnover may be a mechanism

required physiologically to fine-control PR responses to endogenous P_4. Our results are consistent with those of Arici et al. who reported 90% metabolism of P_4 in isolated primary endometrial stromal and gland cells after 24 hours [27]. However, it should be noted that only one time point was assessed and the temporal dynamics of P_4 metabolism may differ between cell lines. Moreover, the metabolism of P_4 appears to be independent of PR expression since a similar extent of metabolism was seen in all three breast cancer cell lines even though T47D and MCF-7 BUS cells are PR-positive whilst MDA-MB-231 cells are PR-negative [28]. Several researchers have reported that 20α-(*S*)-hydroxyprogesterone is a major metabolite of P_4 via the actions of AKR1C1 [28–30]. Whether AKRIC1 or its isozymes are involved in metabolism of P_4 in our model systems remains to be investigated.

Wiebe and Lewis found that breast cancer cell lines express higher levels of SRD5A1 and lower levels of AKR1C enzymes [31]. Therefore, breast cancer cells have a higher conversion of P_4 to 5α-pregnane metabolites as opposed to other systemic cells and tissues. This has major implications, as 5α-pregnanes modify the growth of tumour cells within breast tissue. This highlights the importance of examining the metabolism of steroids, as some metabolites are active and may be a confounding factor in receptor-based studies comparing P_4 -activity to other progestins [28, 32–33]. HEK293T (human embryonic kidney cells) and COS-1 cells (monkey kidney cells) had the lowest metabolism of P_4, which may mean that kidneys have lower turnover of P_4.

An important implication of our findings is that the detected differential and rapid metabolism of P_4, progestins and DEX may confound the interpretation of results when investigating mechanisms of action and biological responses via steroid receptors using *in vitro* and preclinical models. This would be particularly relevant to the determination of relative binding affinities and potencies (EC_{50} values), which are highly relevant to drug efficacy, specificity and design. We have previously proposed that the determination of progestogen binding affinities and potencies via a specific receptor are dependent on a number of factors, including metabolism of the progestogen [4]. Several researchers have investigated binding affinities of one or more progestogens and/or DEX in COS-1 cells or cytosols prepared from MCF-7 cells [16–18, 34–35] or in cytosols prepared from tissue [11, 12]. Given that COS-1 cells show high metabolism of P_4 and DEX relative to progestins such as MPA and NET, which show no metabolism in these cells, the reported relative binding affinities may be underestimated for P_4 and DEX [16–18]. If metabolising activity is retained in cytosols, our results suggest that relative binding affinities for NET, but not LNG or ETG, from MCF-7 cytosols [34–35] may also be underestimated. Similarly, potencies ($EC50_s$) and/or efficacies (maximal activities) have been reported for transcriptional activity using one or more progestogens and/or DEX in either COS-1, T47D, MCF-7 BUS, END-1, or HEK293 cell lines investigated in this study [11, 16–18, 36–40], or in primary cell models [8–9]. The reported potencies and efficacies may also be underestimated, particularly for P_4, compared to some progestins, and to different degrees, depending on the cell model. For example, the relative potency of P_4 via the androgen receptor (AR) and PR in HEK293T cells may be greater than that reported relative to LNG or NES [39], while the potency of P_4 and DEX may be greater than that reported for other progestins in COS-1 cells for a particular receptor such as the PR, androgen, glucocorticoid and mineralocorticoid receptors [16–18, 39]. Potentially further complicating the interpretation, are different metabolites of

these steroids produced in different cells that may also confound the results. Clearly, further investigation into the steroid-and cell-specific effects of metabolism of these clinically-relevant compounds, and the biological activities of their metabolites, is urgently required.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

DEX	dexamethasone
ETG	etonogestrel
LNG	levonorgestrel
MPA	medroxyprogesterone acetate
NES	nestorone
NET	norethisterone
P_4	progesterone
PR	progesterone receptor
T	testosterone

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Highlights

- Progestins are differentially metabolised in a cell line-specific manner.
- Progesterone is rapidly metabolised by 24 hours in all cell lines investigated.
- MPA and NET are significantly metabolised in human cervical tissue *ex vivo.*
- Metabolism may under-estimate effects determined for progesterone *in vitro.*
- Differential metabolism may affect cell-specific therapeutic and side-effects.

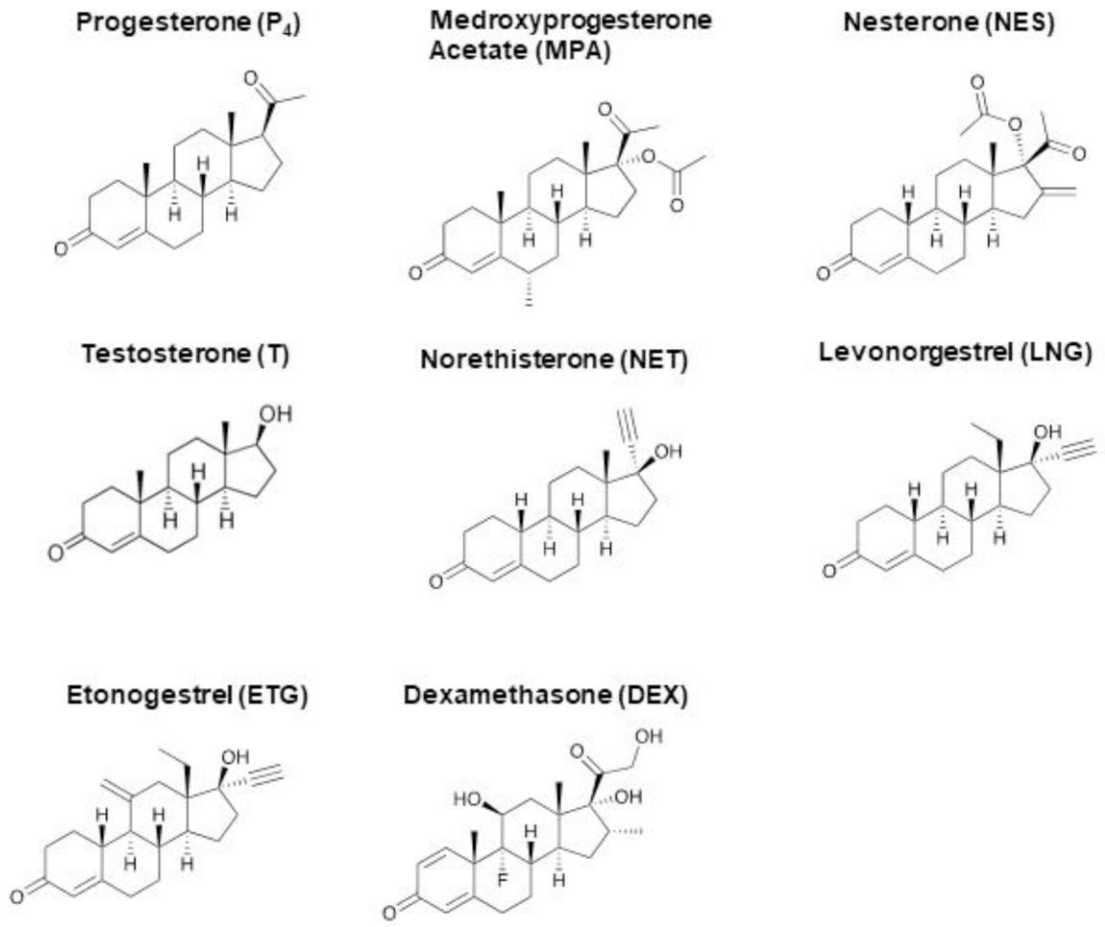

Figure 1. The chemical structures of the seven commercially-available steroids described in this work, and T.

MPA and NES are structurally related to P4; while NET, LNG and ETG are structurally related to T. DEX is the synthetic glucocorticoid often investigated in parallel with progestins.

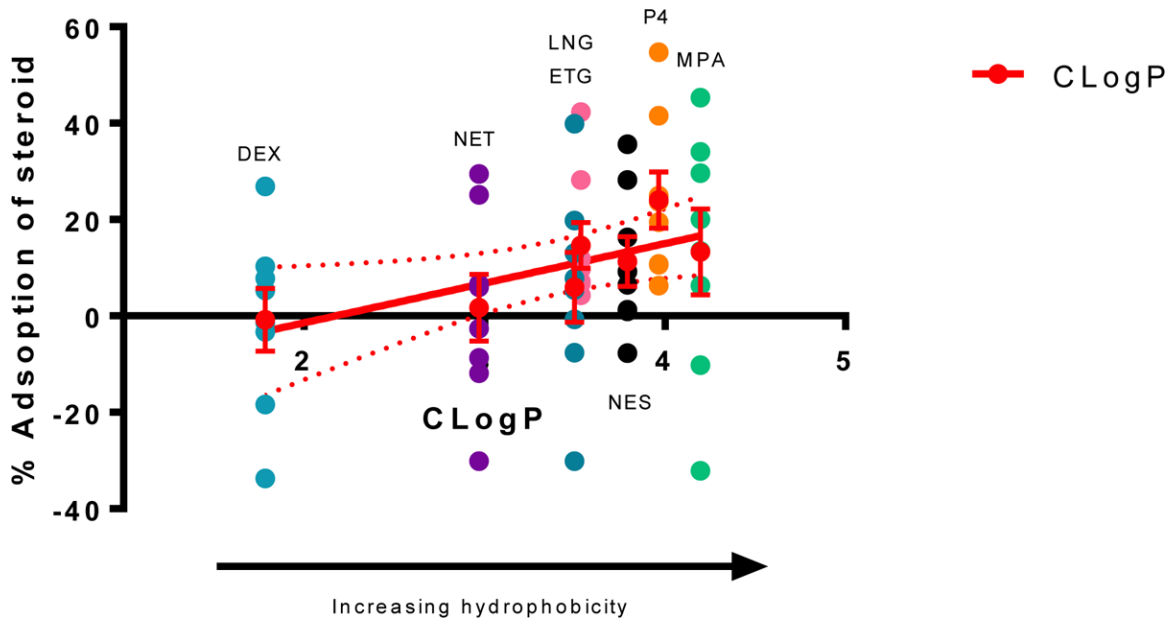

Figure 2. Predicted steroid hydrophobicity correlates with adsorption.

Regression analysis was performed to determine whether there is a correlation between the adsorption and hydrophobicity of the seven clinically-relevant steroids. The percentage adsorption of each steroid to the tissue culture plates was determined from the difference between the time-zero (T_0) measurement and its corresponding no-cell control. CLogP values were predicted using ChemDraw Professional Version 16.0.1.4 (PerkinElmer Informatics, Inc.). Non-linear regression using GraphPad Prism Version 7 (GraphPad Software, Inc.) revealed a correlation with $r^2 = 0.5394$.

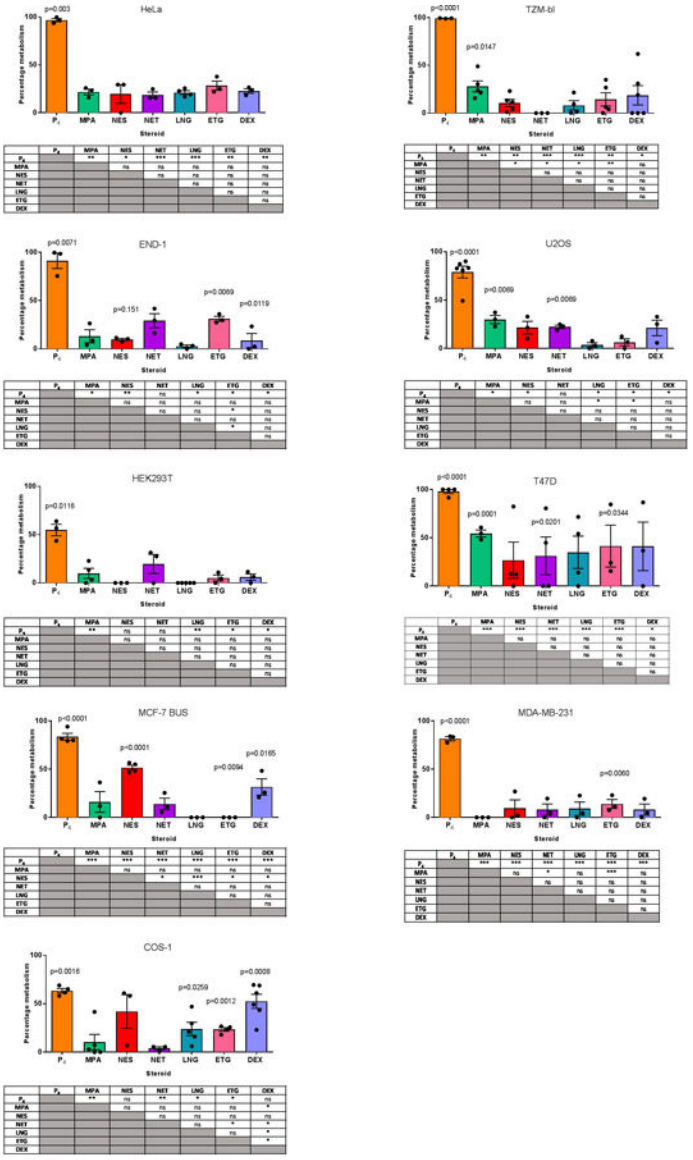

Figure 3. Differential metabolism of seven clinically-relevant steroids following incubation at 100 nM for 24 hours in nine different cell lines.

Medium containing the steroids (no cells) was added to a 12-well plate as a negative control for metabolism. Steroids were extracted and analysed by UHPSFC-MS/MS. The amount of steroid present in the medium after incubation with the cells was expressed as a % relative to the amount of progestin in the negative control for metabolism, which was set as 100%. These data show the mean ± SEM of a minimum of three independent biological repeats. Statistical analysis via paired *t*-tests was performed on each steroid and statistically significant differences relative to its no cell control are indicated by the p-value above bar. To quantify whether the relative metabolism of two steroids within a cell line was significantly different, multiple *t*-tests were performed between steroids. Significant differences in tables below the histograms are indicated by asterisks where *, **, ***, **** represent $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively.

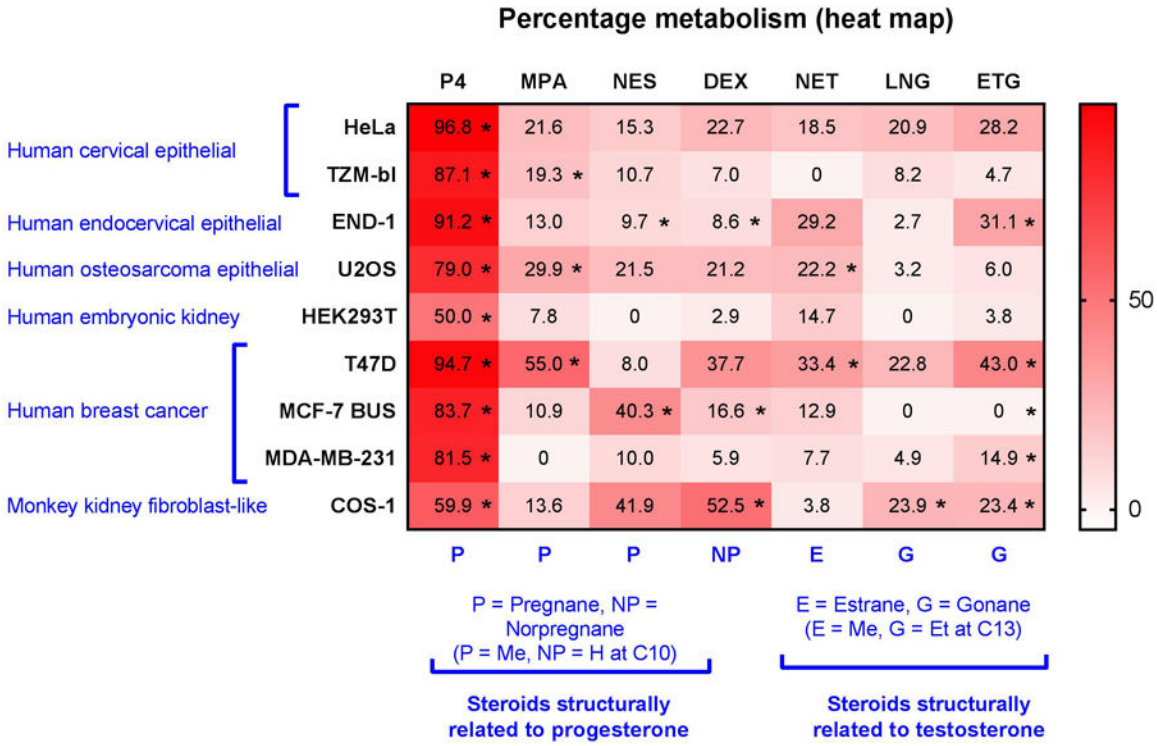

Figure 4. Heat map summarising differential metabolism of seven clinically-relevant steroids.

Data are from Figure 3 and cell lines and steroids are grouped according to anatomical and structural similarities, respectively. Pregnanes (P) have a methyl group at C10 position, while norpregnanes (NP) have a hydrogen group at C10; estranes (E) have a methyl group at C13, while gonanes (G) have an ethyl group at C13. Data is represented as % metabolism relative to the no-cell control which was set to 100%. Statistical significance is indicated by *s.

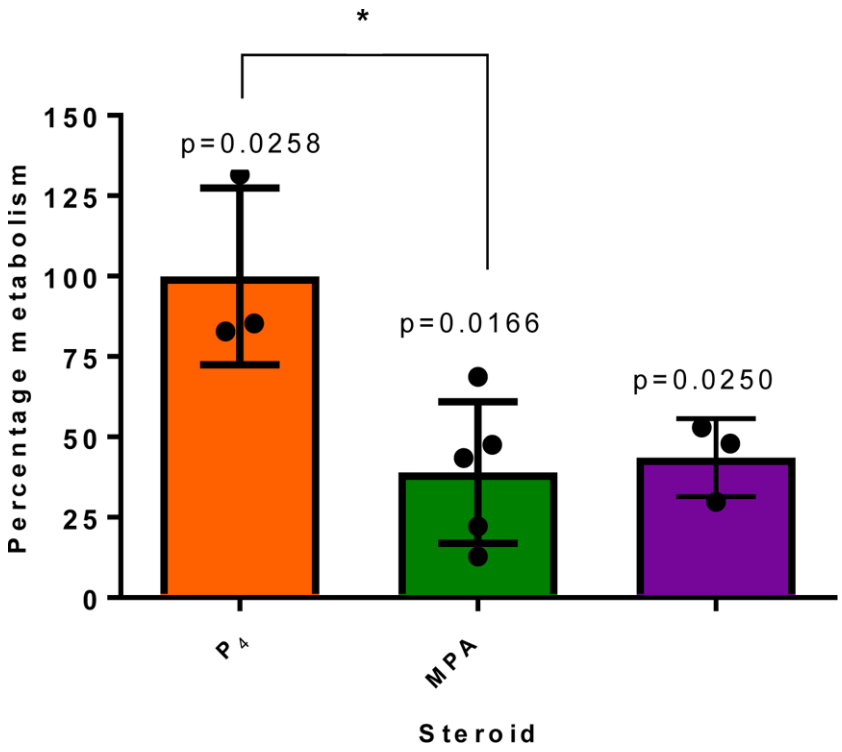

Figure 5. Differential metabolism of three clinically-relevant progestogens in post-menopausal endocervical tissue.

Tissue explants were incubated with 100 nM progestogens for 24 hours. The supernatant was removed and extracted before quantification via UHPSFC-MS/MS, as described in methods. Results were normalised to the progestogen concentration detected in a corresponding no-tissue control experiment to account for adsorption loss. The data show the mean ± SEM of three to five independent biological repeats. Statistical analysis via paired *t*-test*s* was performed comparing the progestogen concentration in the supernatant of the tissue condition to the no-tissue control, significant results are indicated by the p-value above the bar. To determine whether the relative metabolism of the progestogens was significantly different, an unpaired *t*-test with Welch's correction was performed. Statistical significance indicated with * represents $p<0.05$.

Table 1.

Comprehensive method validation data: r^2, LOD (ng/mL and nM), LLOQ (ng/mL and nM), accuracy (% RSD, n=6) and precision (% RSD, n=6). LOD, LLOQ, accuracy and precision are shown for unsupplemented DMEM only. (−) indicates that the concentration is below the LLOQ for that steroid and is therefore not included.

Steroid	r^2 (Unsupplemented DMEM)	LOD, ng/mL (nM)	LLOQ, ng/mL (nM)	Accuracy % RSD, 1 ng/mL	Accuracy % RSD, 10 ng/mL	Accuracy % RSD, 100 ng/mL	Precision % RSD, 1 ng/mL	Precision % RSD, 10 ng/mL	Precision % RSD, 100 ng/mL
P_4	0.9928	0.01 (0.03)	0.01 (0.03)	18.2	12.6	8.3	16.9	9.5	9.5
MPA	0.9939	0.5 (1.3)	1 (2.6)	10.9	11.1	9.7	14.0	6.7	9.3
NES	0.9955	0.5 (1.3)	1 (2.7)	16.5	18.9	16.9	19.0	16.1	18.8
NET	0.9951	0.5 (1.7)	1 (3.3)	14.3	11.5	9.8	14.1	4.4	9.7
LNG	0.9739	0.01 (0.03)	5 (16.0)	-	4.7	11.2	-	7.4	9.7
ETG	0.9960	1 (3.18)	5 (15.4)	-	13.1	8.0	-	10.9	8.7
DEX	0.9951	5 (12.7)	5 (12.7)	-	12.8	16.9	-	12.5	11.8

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Differential off-target glucocorticoid activity of progestins used in endocrine therapy

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ABSTRACT

The glucocorticoid receptor (GR) regulates transcription of genes involved in multiple processes. Medroxyprogesterone acetate (MPA), widely used in the injectable contraceptive Depo-MPA (DMPA), has off-target effects via the GR, which may result in side-effects in endocrine therapy. However, very little is known about the GR activity of other progestins used in endocrine therapy. This study compared GR activities for several progestins, using whole cell binding, dose–response, and GR phosphorylation assays, in both a cell line model and peripheral blood mononuclear cells (PBMCs). MPA, etonogestrel (ETG) and nestorone (NES) exhibit greater relative binding affinities for the GR than levonorgestrel (LNG) and norethisterone/norethindrone (NET) and are partial GR agonists for transactivation but agonists for transrepression on synthetic promoters in COS-1 cells. MPA is a potent agonist for endogenous GR-regulated GILZ and IL6 genes in PBMCs. While ETG and NES also display agonist activity on IL6, they have little effect on GILZ. In contrast, LNG and NET exhibit little to no activity in transactivation models, while both exhibit some transrepressive activity but are generally less potent and/or efficacious than MPA. Antagonist and phosphorylation assays confirmed that MPA and NES act via the GR on endogenous genes in PBMCs. Our results suggest GR-mediated dose-dependent and gene-specific transcriptional side-effects are likely to occur at physiologically relevant concentrations in vivo for MPA, may possibly occur selectively for ETG and NES, but are unlikely to occur for LNG and NET. This suggests that these progestins will exhibit differential side-effects in endocrine therapy via the GR.

1. Introduction

Synthetic progestogens or progestins are widely used in women for hormonal contraception, hormone replacement therapy (HRT) and treatment of endometriosis [1–3]. Progestins exert their therapeutic actions by mimicking the actions of progesterone via binding to the progesterone receptor (PR) [2]. However, use of many progestins has been associated with a number of side-effects, including increased risk of breast cancer, cardiovascular disease, weight gain, loss of bone density and possibly increased susceptibility to infections such as HIV-1 [1,4–12]. While some side-effects of progestins may be mediated via the PR which is activated by progestins, others may be mediated via other steroid receptors (SRs) [12–15]. Particular concerns have been raised regarding side-effects of the progestin medroxyprogesterone acetate (MPA), used in HRT and in the contraceptive DMPA [4,5,12,16–19]. We and others have proposed and provided plausible *in vitro* evidence that at least some of the side-effects of DMPA may be due to its glucocorticoid (GC)-like effects, mediated via the glucocorticoid receptor (GR) [2,12,20–25].

GCs regulate a wide range of physiological functions via the ubiquitous GR, including development, homeostasis, metabolism, immune function, reproduction and the stress response [26–30]. Exogenous GCs are widely used to treat acute and chronic and inflammatory diseases due to their potent anti-inflammatory and immunosuppressive properties. Long-term exposure to GCs is associated with multiple adverse health outcomes including weight gain, elevated glucose levels, hypertension, glucose intolerance, insulin resistance, osteoporosis, and psychological effects leading to depression and mood changes [28,29] and could lead to Cushing's syndrome, a pathological condition characterized by muscle wasting, fat accumulation, and susceptibility to

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infections [31]. Neither the use of DMPA nor any other progestin used in endocrine therapy has been associated with broad systemic immunosuppression or the degree of symptoms found in Cushings syndrome patients. However, several of the reported side-effects of DMPA, such as select changes in immune function, weight gain and increased susceptibility to HIV-1, are consistent with select GC activity, although the clinical data are not consistent [11,12,32–41]. The extent to which these side-effects are due to MPA acting via the GR is unknown, and very difficult to determine clinically. In general, only some, but not all possible side-effects that would be consistent with select and weak GR activity have been investigated in clinical studies for widely-used progestins [1,7,42–44] A key outstanding question is whether other progestins used in endocrine therapy exert any side-effects via the GR.

Long-acting progestin-only injectable contraceptives like DMPA and the two-monthly intramuscular injectable, Nuristerate, containing norethisterone enanthate (NET-EN) are widely used in the developing world and are highly effective, discreet, and reversible contraceptive methods [1,2,12]. Etonogestrel (ETG), levonorgestrel (LNG) and nestorone (NES) are increasingly being used in contraceptives as long-term alternatives to injectables [45]. ETG is used in combined oral contraception, administered as subdermal implants and used in intravaginal rings [46], while LNG is administered in intrauterine devices, subdermal implants, vaginal tablets or gels, as well as for progestin-only or combined oral contraception[12,46,47]. NES is used in contraception in vaginal rings in combination with ethinyl estradiol (EE), in subcutaneous capsules, or in implants [12,46].

ETG has been associated with mild insulin resistance and could affect bone density and increase body weight [42,48–51], while NET has been associated with an increase in blood glucose levels and an unfavourable lipid profile, but is not associated with increased HIV-1 acquisition relative to no contraception or infrequent condom use [11,52,53]. In contrast, LNG and NES have been associated with relatively few side-effects [42,44,54,55] for those that have been investigated. However, it is not possible to exclude the possibility that some select gene- and/or cell-specific and biologically significant GR-mediated side-effects occur for any of these contraceptives, based on the available clinical data.

Given the difficulty in establishing whether the GR is involved in side-effects of progestins used in endocrine therapy clinically, another approach is to establish the potential for such side-effects as proof-of-concept, by determining the relative affinity, potency and efficacy of progestins for regulation of gene expression via the GR *in vitro*. The GR is a ligand-activated transcription factor that exert their biological responses by binding to target gene promotors to either increase (transactivation) or decrease (transrepression) transcription [12].

We and collaborators have previously focussed on determination of the relative binding affinity and transcriptional activity of MPA and NET via other SRs, including the GR [13–15,21,56–58]. While side-effects of progestins that may be mediated via the PR are likely to be similar [59], others mediated via the GR may differ between progestins. We have established that MPA, but not NET, exhibits relatively potent and efficacious GC-activity via the GR [2,13,21,60]. However, very little information is available about the relative activity via the GR for LNG, ETG and NES, compared to each other and to MPA and NET. Limited data from animal models suggest that ETG, LNG and NES bind with low affinity to the GR [2,61,62]. *In vivo* bioassays in rats found that, like NET, both LNG and NES exhibit no GR agonist activity. In contrast, ETG was shown to have weak GR agonist activity in a cell line transactivation reporter assay [61] and in *in vivo* bioassays in rats [12,63]. However, most of these data are likely to be confounded by multiple factors including comparisons between non-parallel investigations, species-specific effects, and the presence of competing SRs.

Given the potential for the ubiquitous GR to regulate multiple genes and the resulting potential for side-effects of progestins via the GR, more comparative data on the activity of progestins via the GR are urgently required. This may be particularly relevant to HIV-1 acquisition, since both *in vitro* and clinical data suggest that DMPA, but not NET-EN, may increase HIV-1 infection [12]. For the first time, this study directly compared the relative binding affinities (RBAs) and transcriptional activities for both transactivation and transrepression by the GR, using dose–response analysis, for MPA, NET, LNG, ETG and NES in parallel, in a cell line on synthetic promoters and in a primary cell model on endogenous genes, as well as the ability of these progestins to result in phosphorylation and activation the GR.

2. Experimental

2.1. Cell culture and materials

The COS-1 (RRID:CVCL_0223) monkey kidney cell line was purchased from ATCC, USA, maintained as previously described [64], and was mycoplasma negative for the duration of the study. Sources of reagents were as follows: Dexamethasone (DEX), MPA, NET, LNG, ETG, NES, RU486 (mifepristone) and phorbol 12-myristate13-acetate (PMA) (Sigma-Aldrich, RSA); [^{3}H]-DEX (78 Ci/mmol) (AEC Amersham, RSA); The human GR expression plasmid, pcDNA3.1-GR (pGR) (D.W. Ray, University of Manchester, UK) (Ray et al., 1999); empty vector pcDNA3.1 (Invitrogen, USA); pTAT-GRE-E1b-luciferase plasmid (pGRE) (G. Jenster, Erasmus University of Rotterdam, The Netherlands) [65]; 5x Nuclear Factor kB-luciferase (pNFκB) and 7x Activator Protein-1-luciferase (pAP-1) promoter-reporter plasmids (Stratagene, Houston, USA); antibodies to GR (H-300, sc-8992, RRID: 2155784) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (0411; sc-47724, RRID: 627678) and secondary anti-rabbit (sc-2313, RRID: 641181) (Santa Cruz Biotechnology, USA); anti-P-S226 or anti-P-S211 specific antibody (Dr. M.J. Garabedian, New York University, USA) [66,67].

2.2. Binding assays

Although multiple methods are available for the determination of binding affinities [68,69], we conducted competitive whole cell binding assays as described [21,70] with minor modifications. COS-1 cells were transiently transfected with either 5 μg of pGR or the pcDNA3.1 empty vector, using X-tremeGene9 (Roche, RSA) as per manufacturer's instructions. Counts per minute were measured and normalised to total protein content per well as determined by the Bradford assay [71]. Total binding ([^{3}H] DEX in the absence of unlabelled competitor) was set at 100%. Specific bound [^{3}H] DEX was calculated as the difference between total and nonspecific binding ([^{3}H] DEX plus 10 μM unlabelled DEX). RBAs (%) were calculated as follows: [IC_{50} value of DEX (M) / IC_{50} value for each ligand (M)] × 100, with DEX IC_{50} value set as 100%.

2.3. Reporter assays

Reporter assays were performed essentially as previously described [13,14,21] with a few modifications. For transactivation assays, COS-1 cells were seeded into 10 cm dishes (Griener, Germany) at a density of 1.5×10^6 cells and transiently transfected with 10 μg pGR or pcDNA3.1 empty vector and 3.75 μg pGRE using X-tremeGene9. Twenty-four hours later, the transfected cells were plated into 96-well plates at a density of 1×10^4 cells/well. The next day cells were stimulated and treated for 24 h with varying concentrations of the ligands. For transrepression assays, COS-1 cells were seeded into 10 cm dishes at a density of 2×10^6 cells. After 24 h, the cells were transiently transfected with 3.75 μg pGR and 7.5 μg pAP-1 or pNFκB using X-tremeGene9. The next day, the transfected cells were plated into 96-well plates at a density of 1×10^4 cells/well and treated for 24 h with 10 ng/mL PMA in the absence and presence of varying concentrations of the test compounds. Luciferase activity was measured in relative light units and normalised to total protein content per well as determined by the Bradford assay [71].

2.4. PBMC isolation and stimulation

PBMCs were isolated, cultured and stimulated as previously described [22]. Briefly, isolated PBMCs were washed, counted, and cultured in RPMI at 2×10^6 cells/ml. For endogenous gene analysis 4×10^6 PBMCs were seeded in round-bottom tubes and stimulated with respective ligands, as indicated in the figure legends, for 48 h. Thereafter the PBMCs were centrifuged at 1 200xg for 5 min and the supernatant was discarded. Pelleted cells were used for RNA isolation. For western blot analysis, 1×10^6 PBMCs were seeded in round-bottom tubes and stimulated with 100 nM ligand or vehicle (ctrl 0.1% v/v EtOH), for 30 min. Thereafter the PBMCs were centrifuged at 1 200xg for 5 min, the supernatant was discarded and pelleted cells were used for western blotting.

2.5. Real time quantitative PCR (qPCR)

RNA was isolated from PBMCs stimulated with ligands as previously described [22,72], using Tri-reagent (Sigma Aldrich, RSA) as per the manufacturer's instructions. RNA (250 ng) was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosciences, ThermoFisherScientific). qPCR was performed using FastStart PCR Master kit containing SYBR green (Sigma Aldrich, RSA) and Rotorgene, RG-3004 (Corbett Research, RSA). Primer sets were as follows: Glucocorticoid-induced leucine zipper (GILZ) (cat#QIA249900-QT00091035, Qiagen, RSA); Interleukin 6 (IL6) (forward) 5′-TCTCCACAAGCGCCTTCG-3′ and (reverse) 5′-CTCAGGGCTGAGATGCCG-3′ and GAPDH (forward) 5′TGAACGGGAAGCTCACTGG-3′ and (reverse) 5′CCACCACCCTGTTGCTGTA-3′ [73,74]. The relative expression of GILZ and IL6 was calculated using the Pfaffl method and normalized to relative GAPDH expression levels [75].

2.6. Western blotting

Whole cell lysates were prepared using a N-[Tris(hydroxymethyl)-methyl]-3-aminopropanesulfonic acid (TAPS) buffer (0.1 M TAPS, pH 9.5) on ice as described. Western blot analysis was performed as previously described [76]. Briefly, equal amounts of cell lysate were loaded onto 8% SDS-polyacrylamide gels and separated by electrophoresis at 75 V for 20 min then 120 V for 1 h in 1X running buffer (25 mM TRIS-HCl, 250 mM glycine and 0.1% (*v/v*) SDS pH 8.4) using a Bio-Rad Mini Protean II electrophoresis system (Bio-Rad, South Africa). Proteins were then blotted onto Hybond-ECL nitrocellulose membrane (AEC-Amersham, South Africa) for 1 h at 180 mA in cold 1X transfer buffer (25 mM TRIS, 200 mM glycine, 20% (*v/v*) methanol). Membranes were subsequently blocked for 1 h at room temperature by shaking in 4% (*w/v*) ECL advance blocking powder (AEC-Amersham, South Africa) in 1X TRIS-buffered saline (50 mM TRIS, 150 mM NaCl; TBS) containing 0.1% (*v/v*) Tween (TBS-Tween; TBST). Primary antibodies were diluted in 4% ECL-TBST and incubated on membranes overnight with shaking at 4 °C. Membranes were then washed three times in 1X TBST for 5 min and incubated with secondary antibodies diluted in 5% (w/v) skim milk powder in 1X TBST for 1 h at room temperature with shaking. After three 5-minute washes in 1X TBST, membranes were placed in 1X TBS prior to a 1-minute incubation with Pierce ECL-chemiluminescent western blotting substrate (Thermo Scientific, USA). Proteins were visualized by autoradiography using Amersham Hyperfilm™ MP high performance autoradiography film (AEC-Amersham, South Africa) and quantified using ImageJ. For the phosphorylation assays, membranes were first incubated using P-S226- or P-S211-specific antibodies, with their respective secondary antibodies. After developing and autoradiography, the membranes were stripped as described in [77] and re-probed for total GR using the H-300 GR antibody.

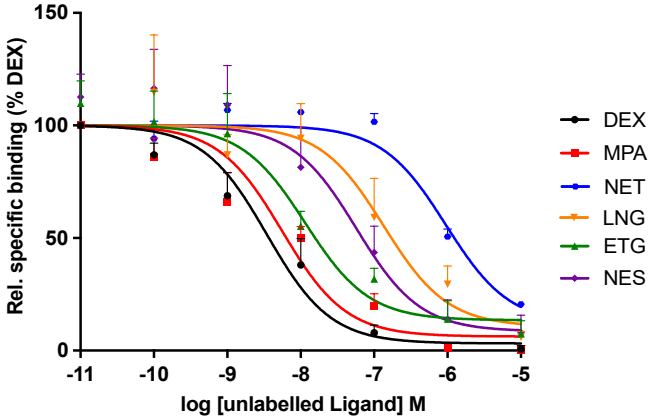


Fig. 1. MPA and ETG display similar binding affinities to DEX, but higher than that of NET, LNG and NES for the human GR. COS-1 cells were transiently transfected with pGR and treated for 3 h with 20 nM [^{3}H]-DEX in the absence or presence of increasing concentrations of competing unlabelled ligands. Counts per minute (cpm) were normalized to the protein concentration (mg/mL). The mean is shown from six independent experiments, where each condition was performed in triplicate. The relative specific binding of [^{3}H]-DEX only was expressed as 100% and the binding of the unlabelled competitors expressed as a percentage relative to this.

2.7. Data analysis

Statistical and data analysis were performed using GraphPad Prism™ software version 9. All data were first tested for normality, before parametric or non-parametric tests were performed. Competitive binding data were analysed using non-linear regression and one site – fit logIC_{50} options and the Newman-Keuls post-test was used for statistical analysis. Dose-response analysis of the luciferase reporter assays, as well as endogenous genes was plotted as described. Non-linear regression and sigmoidal dose–response were used for which the slope was set to +1 for transactivation and −1 for transrepression and one-way ANOVA and the Tukey (compares all pairs of columns) post-test were used for statistical analysis of the efficacies and potencies. For dose–response analysis, all curves are shown as "best-fit" curves that are not ambiguous, unless stated as "unstable" in the respective figure legends. These "unstable" curves should be interpreted with caution, as a wide range of values would essentially lead to the same curve. For 100 nM ligand responses on endogenous genes, a non-parametric Kruskal-Wallis ANOVA with Dunn's multiple comparisons test was performed when comparing all samples to vehicle (control). A Mann-Whitney *t*-test was performed when comparing between different ligands. GR phosphorylation data was analysed using a one-way ANOVA, with a Dunnett's multiple comparisons post-test, comparing each ligand to control. Statistically significant differences are indicated by different letters or symbols (* or

Table 1
Table showing RBA and IC_{50} values, of the ligands via the GR.*.

Ligand	RBA (%) ± SEM	IC_{50} (M) ± SEM
DEX	100.0 ± 0.00[a]	$3.46 \pm 1.0 \times 10^{-9}$[a]
MPA	62.0 ± 0.50[b]	$5.58 \pm 2.0 \times 10^{-9}$[a]
NET	0.360 ± 0.30[c]	$9.50 \pm 2.9 \times 10^{-7}$[d]
LNG	2.56 ± 0.10[d]	$1.35 \pm 9.1 \times 10^{-7}$[c]
ETG	30.1 ± 0.20[e]	$1.15 \pm 5.2 \times 10^{-8}$[a]
NES	6.03 ± 0.30[f]	$5.73 \pm 3.6 \times 10^{-8}$[b]

* Data shown in Fig. 1, were analyzed to obtain the RBA ± SEM and IC_{50} ± SEM values. RBAs were calculated for each ligand for individual experiments from the IC_{50} (M) values (obtained using GraphPad Prism™ software version 9) and expressed as a percentage relative to DEX (100%) for that experiment. The mean RBA ± SEM values were then calculated for each ligand.

[a,b,c,d,e,f] Statistical significance between treatment groups was determined using one-way ANOVAs with Newman-Keuls post-test.

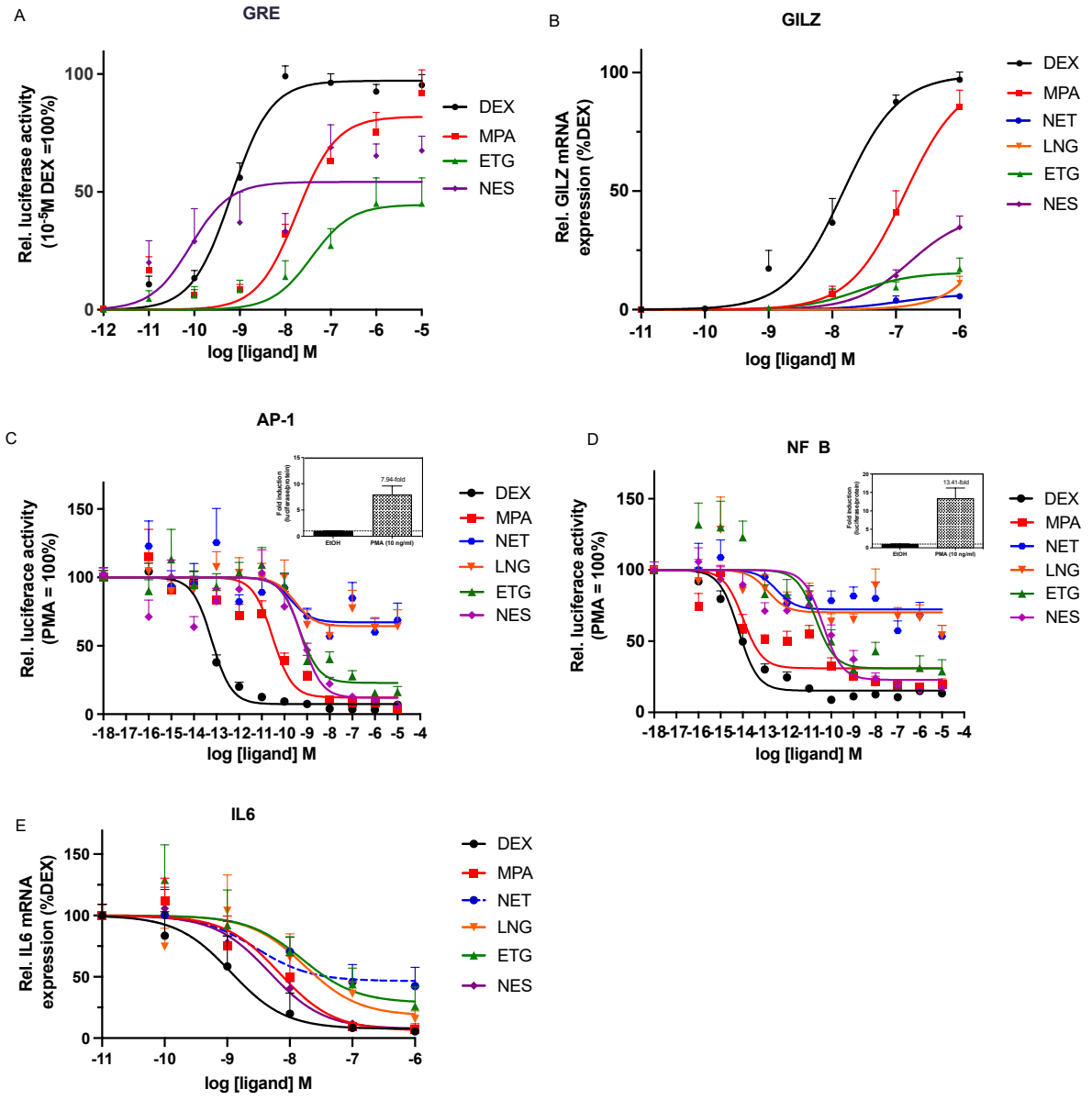


Fig. 2. ETG and NES are partial agonists for transactivation on the endogenous GILZ gene and GRE reporter gene via the GR and agonists for transrepression via the GR on both AP-1 and NFκB *cis* elements whereas all progestins, except NET, are agonists for transrepression of the IL6 gene. COS-1 cells transfected with pGR and pGRE (A), pGR and pAP-1 (C) or pGR and pNFκB (D) were treated with increasing concentrations of ligands in the absence (A) or presence (C and D) of 10 ng/mL PMA for 24 h. Luciferase activity was measured and normalised to protein concentration determined using the Bradford method. Results in (A) are expressed as % DEX where the DEX maximal response was set to 100% and all other responses were set relative to DEX, while results in (C and D) were normalised to PMA set as 100%. Treatment with PMA resulted in a ~7.94-fold and ~13.41-fold induction (2C and 2D inserts, respectively). PBMCs were isolated and treated with increasing concentrations of ligands for 48 h (B and E). RNA was harvested, cDNA was synthesized and then subjected to real time qPCR using primer sets specific for GILZ or IL6 and GAPDH. Levels of GILZ (B) or IL6 (E) mRNA were normalised to GAPDH levels for each condition. Pooled results from six (B) or five (E) PBMC donors and at least three independent experiments performed in triplicate for the promoter-reporter assay are shown. Results are expressed as % DEX where the DEX maximal response was set to 100% and all other responses were set relative to DEX.

#), as indicated in the figure legends. All data, except for the representative western blot, were expressed as means, where the errors bars represent the standard error of the mean (SEM) with n values given in each figure legend.

3. Results

3.1. MPA and ETG bind to the GR with relatively high affinity.

Competitive whole cell binding assays were conducted in COS-1 cells exogenously expressing human GR to investigate the RBA of the selected progestins for the GR (Fig. 1). RBAs expressed as a % DEX (reference agonist (100%)) revealed that all the progestins exhibited significantly different RBAs with the rank order DEX > MPA > ETG > NES > LNG > NET (Table 1). No statistically significant differences were observed between the IC_{50} values for the synthetic GR agonist DEX versus MPA and ETG, while NES, LNG and NET have significantly lower IC_{50} values. However, NES, LNG and NET exhibited significantly different affinities when compared to each other. Experiments in the absence of expressed human GR showed no binding to the endogenous GR (Supplementary Fig. 1).

Table 2
Table showing relative agonist efficacies and potencies of the ligands for transactivation via the GR on GRE and GILZ gene*.

	Transactivation			
	COS-1		PBMC	
	GRE		GILZ	
Ligand	MAX (%) ± SEM	EC_{50} (M) ± SEM	MAX (%) ± SEM	EC_{50} (M) ± SEM
DEX	100 ± 2.17[a]	1.07 ± 0.55x10^{-9a}	100 ± 4.87[a]	1.91 ± 2.51x10^{-8a}
MPA	84.36 ± 3.99[b]	4.17 ± 1.63x10^{-8a}	98.47 ± 7.09[a]	2.5 ± 0.66x10^{-7a}
NET	ND	ND	6.50 ± 2.34[b]	5.12 ± 9.91x10$^{-8\#a}$
LNG	ND	ND	ND	ND
ETG	45.88 ± 4.97[c]	3.19 ± 1.21x10^{-8a}	15.85 ± 2.29[b]	2.04 ± 3.19x10^{-7a}
NES	55.81 ± 4.85[c]	1.85 ± 0.77x10^{-8a}	40.70 ± 4.40[c]	1.47 ± 1.01x10^{-7a}

#The EC_{50} values for NET should be interpreted with caution as very weak agonist activity was observed.
ND-not determined.
* Data shown in Fig. 2A and B, were analyzed to obtain the MAX (%) ± SEM and EC_{50} (M) ± SEM values for each ligand for the GR.
[a,b,c,d] Statistical significance between treatment groups was determined using one-way ANOVAs with Tukey post-test.

Table 3
Table showing relative agonist efficacies and potencies of the ligands for transrepression via the GR on AP-1 or NFκB *cis*-elements and the IL6 gene*.

	Transrepression					
	COS-1				PBMC	
	AP-1		NFκB		IL6	
Ligand	MAX (%) ± SEM	EC_{50} (M) ± SEM	MAX (%) ± SEM	EC_{50} (M) ± SEM	MAX (%) ± SEM	EC_{50} (M) ± SEM
DEX	100 ± 0.0[a]	7.24 ± 1.57 × 10^{-14a}	100 ± 0.0[a]	1.08 ± 0.38 × 10^{-14a}	100 ± 9.16[a]	7.63 ± 6.31x10^{-9a}
MPA	96.94 ± 4.60[a]	2.49 ± 0.64 × 10^{-11b}	84.46 ± 4.92[a]	2.84 ± 1.88 × 10$^{-14a, c}$	101.62 ± 11.50[a]	0.92 ± 1.45x10^{-8a}
NET	33.86 ± 9.97[b]	3.58 ± 2.85 × 10$^{-9\ \#c}$	32.63 ± 9.86[b]	8.35 ± 5.65 × 10$^{-13\#\ a,b,c}$	57.95 ± 9.55$^{\$a}$	4.66 ± 4.32x10$^{-9\$a}$
LNG	39.60 ± 4.31[b]	3.32 ± 2.89 × 10$^{-10\ \#b,c}$	38.04 ± 9.70[b]	5.00 ± 3.00 × 10$^{-13\#\ a,b,c}$	88.53 ± 14.82[a]	2.95 ± 6.27x10^{-8a}
ETG	79.60 ± 7.27[a]	2.90 ± 0.99 × 10$^{-10b,c}$	81.65 ± 7.32[a]	6.04 ± 3.49 × 10^{-11b}	77.19 ± 17.00[a]	2.44 ± 0.10x10^{-8a}
NES	92.03 ± 4.39[a]	5.41 ± 3.06 × 10$^{-10b,c}$	85.66 ± 8.58[a]	3.49 ± 2.05 × 10$^{-11b,c}$	100.13 ± 7.92[a]	5.49 ± 5.82x10^{-9a}

#The EC_{50} values for NET and LNG should be interpreted with caution as very weak agonist activity was observed.
$The MAX and EC_{50} values for NET should be interpreted with caution as Prism 9 deemed this curve unstable due to variable data.
* Data shown in Fig. 2C–E, were analyzed to obtain the MAX (%) ± SEM and EC_{50} (M) ± SEM, values for each ligand for the GR.
[a,b,c] Statistical significance between treatment groups was determined using one-way ANOVAs with Tukey post-test.

3.2. MPA, ETG and NES are partial GR agonists with variable potency, while NET and LNG exhibit little to no activity for transactivation.

We investigated the relative agonist efficacies (maximal responses) and potencies (EC_{50} values) for transactivation (Fig. 2, Table 2) of a synthetic GRE-containing promoter-reporter construct (pGRE) and the endogenous GILZ gene of the progestins via the GR. Incubation with 10 μM progestin confirmed that LNG and NET exhibited no significant activity on the synthetic GRE promoter via the GR above the background (Supplementary Fig. 2) and they were thus excluded from further promoter-reporter dose–response analysis. The results show that MPA, ETG and NES are partial GR agonists for transactivation on the promoter-reporter construct (Fig. 2A). Relative to the reference agonist DEX, MPA is significantly more efficacious than NES and ETG, while NES and ETG display statistically similar efficacies. While no significant differences in potency were detected between MPA, ETG and NES, MPA appears to be less potent than DEX on the promoter-reporter construct.

Similar results were obtained on the endogenous GILZ gene in human PBMCs (Table 2). ETG and NES have a lower efficacy and are weaker agonists than DEX, with MPA displaying statistically similar efficacy as DEX (Fig. 2B). ETG is significantly less efficacious than NES, with NET appearing to be the least efficacious ligand (Fig. 2B). Non-linear regression found the LNG curve to be unstable and thus the potency and efficacy were not obtained. Stimulation with 100 nM MPA or NES significantly increased mRNA levels of GILZ (Supplementary Fig. 3A). Co-treatment with the GR/PR antagonist RU486 prevented this upregulation of GILZ (Supplementary Fig. 3A).

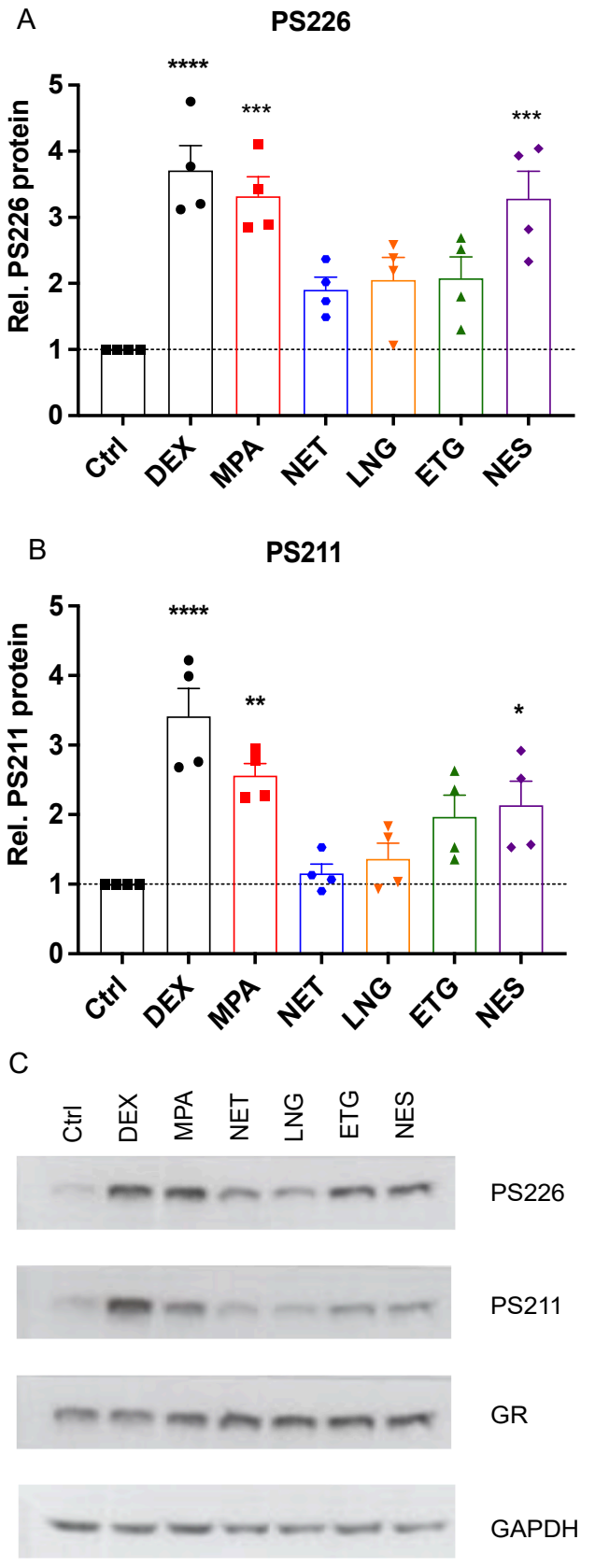
3.3. MPA, ETG and NES are agonists, while NET and LNG display weak partial GR agonist activity for transrepression of promoter-reporter constructs and LNG is a weak agonist on the endogenous IL6 promoter.

The relative agonist efficacies and potencies of the progestins for transrepression via the GR on AP-1 (Fig. 2C) and NFκB-containing (Fig. 2D) promoter-reporter constructs were investigated in COS-1 cells (Table 3). Treatment with 10 ng/mL PMA resulted in a ~7.94-fold and ~13.41-fold induction, respectively (Fig. 2C and D). Like DEX, MPA, ETG and NES are GR agonists via AP-1 (Fig. 2C) and NFκB (Fig. 2D), while both NET and LNG are weak partial GR agonists. Although all progestins investigated are less potent than DEX via the AP-1 promoter, MPA is more potent than NET, while significant differences were not detected between potencies of LNG, ETG and NES. However, on the NFκB promoter (Fig. 2D), no significant difference was observed in potency for DEX versus MPA, NET and LNG via the GR. No significant differences in potencies were observed between NET, LNG, ETG and NES via both the AP-1 and NFκB promoter-reporter constructs, while MPA is significantly more potent than ETG via the NFκB promoter-reporter construct.

Broadly consistent with the reporter gene results, Fig. 2E shows that all the progestins, except NET, are agonists for transrepression of the endogenous IL6 gene in PBMCs (Table 3). The result for NET was inconclusive due to large error in the data and the resulting instability of the curve. Although no significant differences were detected, the progestins appear less potent than DEX, with NES appearing to display higher potency than MPA and ETG. Stimulation with 100 nM MPA or NES significantly decreased the mRNA levels of IL6 (Supplementary Fig. 3B). Co-treatment with the GR/PR antagonist RU486 prevented this repression of IL6 (Supplementary Fig. 3B).

3.4. MPA and NES, but not NET, LNG or ETG, ligand-selectively induce GR phosphorylation.

Selective regulation of the GR may exhibit varying therapeutic effects depending on the molecular mechanism involved in the regulation of inflammation [78]. Ligand-selective GR phosphorylation at S226 and S211 has previously been shown to be indicative of transcriptionally active GR [76,77]. Having shown that the GR is most likely required for the transcriptional regulation of GILZ and IL6 by MPA and NES in PBMCs, next we wanted to determine if the progestins could phosphorylate the GR in PBMCs. We found that stimulation with MPA and



(caption on next column)

Fig. 3. Like the glucocorticoid DEX, MPA and NES, but not NET, LNG or ETG induce GR phosphorylation at S226 and S211. PBMCs were isolated and treated with 100 nM ligand or vehicle (ctrl; 0.1% v/v EtOH) for 30 min before being harvested. The levels of PS226, PS211, total GR and GAPDH were quantified after western blotting as described in [77]. The amount of PS226 (A) or PS211 (B) was normalised to total GR levels and is represented as fold differences relative to ctrl being set to 1. Figures show pooled results from four PBMC donors. (C) A representative Western blot is shown, indicating signals for PS226, PS211, total GR and GAPDH.

NES, but not NET, LNG or ETG, results in GR phosphorylation at S226 and S211, similar to the effect of DEX, albeit to a slightly lower level for S211 (Fig. 3).

4. Discussion

We report for the first time the RBAs, potencies, and efficacies for transactivation and transrepression for this panel of progestins determined in parallel on synthetic and endogenous genes. The synthetic promoters include AP-1 and NFκB *cis*-elements involved in inhibition of immune function [2]. Upregulation and downregulation of the GILZ and IL6 endogenous genes, respectively, are associated with inhibition of inflammation and immune function [12]. GILZ modulates important inflammatory signaling pathways through its interaction with transcription factors [79,80]. Consequently, GILZ expression protects against damage caused by neuroinflammation, allergy and heart disease, amongst other conditions, as it plays a role in the anti-inflammatory activities of GCs [79,81]. Our results show that while the progestins all bind to the GR at high concentrations, MPA, ETG and NES exhibit much greater affinity for the GR than LNG and NET. Relative to DEX (100%), our RBA results for LNG (3%) and NES (6%) are consistent with animal model binding data [2,61,62], unlike our finding of substantial GR binding for ETG (30%). LNG and NET exhibit no or relatively very little agonist activity on synthetic or endogenous promoters transactivated by the GR, consistent with other reporter gene and rat data for LNG [82,83]. Despite a low RBA, NES exhibited potent and substantial partial agonist activity on synthetic and endogenous promoters transactivated by the GR, unlike the lack of activity observed in rat bioassays, possibly due to inactivation of NES in the bioassay [82]. Consistent with our binding data, ETG exhibited potent and substantial partial agonist activity on synthetic and endogenous promoters transactivated by the GR, unlike other reports using reporter genes [61] or rat bioassays [82]. ETG and NES are agonists for transrepression of synthetic and endogenous genes by the GR but partial agonists for transactivation. Both LNG and NET exhibit substantial activity at high concentrations for transrepression of synthetic and endogenous genes by the GR, although they are much less efficacious than MPA at peak serum concentrations used in contraception (Supplementary Table 1) [46]. We show for the first time that MPA is the most and NET the least efficacious ligand compared to NES, ETG and LNG for both transactivation and transrepression of synthetic promoters via the GR.

Taken together, our novel results in PBMCs are consistent with previously reported data showing MPA, unlike NET, is a potent and efficacious GR ligand for transactivation and transrepression [21,60]. We now also show for the first time that relative to DEX and MPA, NES and ETG both bind to the GR with a RBA greater than LNG and NET and exhibit significant GR activity with variable potencies and efficacies for transactivation and transrepression, in a promoter- and context-specific manner.

Several lines of evidence support our conclusion that the biological activities measured in the COS-1 cells and in PBMCs occur via the GR. Our binding and synthetic promoter assays, performed in the absence and presence of expressed GR, as well as our comparison with results for the GR agonist DEX, show that the ligands bind to the GR, which is required for transcriptional regulation. The results obtained on the synthetic promoters compared to the endogenous genes in PBMCs for

both transactivation and transrepression are remarkably similar, with only a few small differences. Additionally, as for DEX, the GR/PR antagonist RU486 inhibited MPA- and NES-induced transcriptional regulation of GILZ and IL6 in PBMCs. Both MPA and NES were shown to ligand-selectively phosphorylate the GR at Serine 226 (S226) and Serine 211 (S211) in a manner similar to DEX, a property only observed with GR agonists or partial agonists. Taken together with the observation that the GR is the only SR detected in PBMCs by western blotting under our conditions [72], our results strongly suggest that the responses in PBMCs are due to the GR.

Our findings suggest that binding of MPA to the GR is more likely to be physiologically relevant than for the other progestins, based on whether their IC_{50} or EC_{50} values fall within the ranges of serum concentrations for contraceptive users (Supplementary Table 1). However, since potencies and efficacies can increase when GR levels are increased [84], it is possible that both ETG and NES could exert physiologically relevant biological effects in select environments. Our findings suggest that MPA, ETG and NES, but less so for LNG and NET, have the potential to exert side-effects on multiple GR-mediated functions. While the effects of high levels of chronic exposure to potent GCs are relatively obvious, the effects of lower levels of potent GCs or less potent or less efficacious GR agonists are less predictable. It is emerging that the mechanisms of GR regulation are very complex, exhibit differential sensitivity to GC concentrations and are cell-, gene-, locus- and signal-specific [21,26,27,30,85]. Thus, genes vary in their sensitivity to GR ligand concentrations, depending on multiple factors, including GR expression levels. Our findings are consistent with this complexity and further show that progestin activity via the GR for transrepression of transcription is more potent than for transactivation. These data provide a plausible potential biological mechanism for some of the observed differential GC-like side-effects of progestins. Moreover, awareness is raised of the need to use the lowest possible concentrations of progestins in endocrine therapy required for therapeutic efficacy and for more robust and directed clinical studies to investigate potential side-effects on specific physiological processes that may be affected by the GR.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability

Some or all data generated or analysed during this study are included in this published article or in the data repositories listed in References.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.steroids.2022.108998.

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