Drug transporter expression and genetic polymorphisms in HIV endemic settings

Presented by

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Preface

Biological factors: drug transporter proteins, single nucleotide polymorphisms in drug transporter genes, and genital inflammation have all been noted as key modulators of antiretrovirals used for HIV treatment or prevention. The purpose of this study was to determine if there is an association between these biological factors, circulating tenofovir and varied drug transporter mRNA levels in the female genital tract and blood of South African women taking oral PrEP- Truvada®. This study is essential especially in South Africa since Truvada® is the standard of care modality for HIV prevention. Findings from this study could be used in PrEP dosage and customisation for South African women, to ensure efficacy.

Declaration I

I, Miss Nomusa Margaret Zondo, declare that:

1. The work described in this thesis was carried out at the Centre for the AIDS Programme of Research in South Africa (CAPRISA) laboratory.

2. The work presented in this thesis has not been previously submitted for any degree or examination to the University of KwaZulu-Natal or any other tertiary institution.

3. The research reported in this thesis, except where otherwise indicated, is my original research. This dissertation does not contain other people's data, pictures, graphs, or other information, unless specifically acknowledged as being sourced from other people. All sources have been detailed in the thesis and in the reference section.

4. I contributed to the study conceptualization, conducted all experiments, data acquisition, statistical analyses, and data interpretation. I further declare that I wrote and complied this thesis.

5. I declare the following contributions:

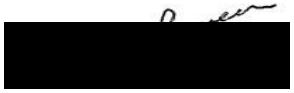
   Professor Derseree Archary and Dr Parveen Sobia: Conceptualized and designed the study, provided research funding, statistical data analyses, data interpretation and writing and editing of the papers and thesis.

   Professor Veron Ramsuran: Helped in study design, statistical data analyses, data interpretation and writing and editing of thesis.

   Lara Lewis: Helped in statistical data analyses, data interpretation and writing and editing of thesis.

Nomusa Margaret Zondo *PhD Candidate* Date:16 March 2023

Professor Derseree Archary *Supervisor* Date:16 March 2023



Dr Parveen Sobia *Co-Supervisor* Date:16 March 2023

Declaration II: Publications and presentations emanating from this project

Publications

Zondo NM, Sobia P, Sivro A, Ngcapu S, Ramsuran V, Archary D **(2022).** Pharmacogenomics of drug transporters for antiretroviral long-acting pre-exposure prophylaxis for HIV. *Frontiers in Genetics*. 13:940661. *https://doi: 10.3389/fgene.2022.940661.*

Oral Presentations

1. **Nomusa M. Zondo**, Parveen Sobia, Aida Sivro, Sinaye Ngcapu, Leila E Mansoor, Lara Lewis, Veron Ramsuran, Derseree Archary. "***The impact of single nucleotide polymorphisms (SNPs) in ABC drug transporter genes on circulating tenofovir in healthy South African women exposed to pre-exposure prophylaxis (PrEP)***". University of KwaZulu-Natal College of Health Science 2022 Research Day Symposium: 2nd Best Oral Presentation; 30th of November 2022; University of KwaZulu-Natal- Nelson Mandela Medical School, Durban, South Africa.

Poster Presentations

1. **Nomusa M. Zondo**, Parveen Sobia, Aida Sivro, Sinaye Ngcapu, Leila E Mansoor, Lara Lewis, Veron Ramsuran, Derseree Archary. "***The impact of single nucleotide polymorphisms (SNPs) in ABC drug transporter genes on circulating tenofovir in healthy South African women exposed to pre-exposure prophylaxis (PrEP)***". 11th Infectious Diseases in Africa (IDA) 2022 Symposium; 5th to the 10th of September 2022; Stellenbosch, Cape Town, South Africa.

2. **Nomusa M. Zondo**, Parveen Sobia, Aida Sivro, Sinaye Ngcapu, Leila E Mansoor, Lara Lewis, Veron Ramsuran, Derseree Archary. "***SNPs alter gene expression and tenofovir in African women on PrEP***". 14th International Congress of Human genetics (ICHG 2023); 22nd to the 26th of February 2023, Cape Town International Convention Centre (CTICC); Cape Town, South Africa.

Dedication

I would like to dedicate my thesis to my daughter Elihle Sinakhokonke Sibisi. Thank you for giving my life more purpose. All that I do is for you, I hope one day you have the opportunity to read this and be inspired to never give up and always strive to do your best. I would also like to dedicate my thesis to my parents (Sipho Elliot and Zandile Terressa Zondo), my siblings (Dem, Thabile and Mthobisi Zondo), my life partner and best friend Sithabiso Sibisi, my best friend Sinegugu Shude and my late friend and brother Lucky Marufu. Thank you for all your sacrifices, unwavering support, for loving and always encouraging me though my studies and life. I will always appreciate and love you.

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words throughout my studies. Thank you for always assisting and always supporting me during difficult times. Thank you for teaching me and always pushing me to give my best. To Professor Veron Ramsuran, thank you for giving me the opportunity to collaborate with you and your lab for my PhD studies. Thank you for all your assistance and guidance throughout my studies. Thank you for your patience and teachings.

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Abbreviations

3TC	Lamivudine
5PL	Five- parameter
ABC	ATP-binding cassette
ADME	Absorption, Distribution, Metabolism and Excretion
AIDS	Acquired immunodeficiency syndrome
ARVs	Antiretrovirals
ATV	Atazanavir
BCRP	Breast cancer resistance protein
BV	Bacterial vaginosis
CAB	Cabotegravir
CNTs	Concentrative nucleoside transporters
CNVs	Copy-number variations
CYP2B6	Cytochrome P450 2B6
DMET	Drug metabolizing enzymes and transporter
DMPA	Depot-medroxyprogesterone acetate
EFV	Efavirenz
ENTs	Equillibrative transporters
FACTS-001	Follow on African Consortium for Tenofovir Studies
FGT	Female genital tract
FTC	Emtricitabine
FTC-TP	Emtricitabine- triphosphate
HAART	Highly active antiretroviral therapy
hCMEC/D3	Human brain cell lines
HEK293	Human embryonic kidney cells
HCs	Hormonal contraceptives
HIV-1	Human Immunodeficiency Virus type 1
HSV-2	Herpes simplex virus type 2
IFN-γ	Interferon gamma
IL-1α	Interleukin 1-alpha
IL-1β	Interleukin 1- beta
IL-6	Interleukin 6
IL-8	Interleukin 8
InDels	Small insertions and deletions
IP-10	Interferon gamma-induced protein 10
iPrEX	Pre-exposure Prophylaxis Initiative trial
IPERGAY	On-Demand Antiretroviral Pre-exposure Prophylaxis for HIV Infection
IRF3	Interferon regulatory factor 3
KZN	KwaZulu-Natal province
LPV	Lopinavir
LPS	Lipopolysaccharide
LXR	Liver X receptors
MalDA	Malaria Drug Accelerator
MATE	Multidrug and toxin extrusion proteins
MCP-1	Monocyte chemoattractant protein1
MDCK II	Madin-Darby canine kidney
MIP-1α	Macrophage inflammatory protein-1 alpha

MIP-1β	Macrophage inflammatory protein-1 beta
MTN	Microbicide Trial Network
MRP	Multi-drug resistance protein
MSM	Men who have sex with men
MTX	Methotrexate
N-9	Nonoxynol-9
NET-EN	Norethindrone enanthate
NF-kb	Nuclear factor *kappa B*
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleotide reverse transcriptase inhibitors
OAT	Organic anion transporters
OATPs	Organic anion-transporting polypeptides
OCTs	Organic cation transporters
PBMCs	Peripheral blood mononuclear cells
P-gp	P-glycoprotein
PIs	Protease inhibitors
PMPA	(R)-9-(2-phosphonylmethoxypropyl) adenine
PMSG	Pregnant mare's serum gonadotropin
PrEP	Pre-exposure prophylaxis
PROUD	Pragmatic open-label randomised trial
Raw 264.7	Mouse macrophage cell lines
RTV	Ritonavir
SLC	Solute Carrier proteins
SIV	Simian immunodeficiency virus
SNPs	Single nucleotide polymorphisms
SSA	sub-Saharan African
STIs	Sexually transmitted infections
TAF	Tenofovir alafenamide
TDF	Tenofovir disoproxil fumarate
TFV-DP	Tenofovir-Diphosphate
TFV	Tenofovir
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor alpha
TRIF	Toll/interleukin-1-domain-containing adapter-inducing interferon β
VOICE	Vaginal and Oral Interventions to Control the Epidemic
ZDV	Zidovudine

Abstract

Pre-exposure prophylaxis (PrEP) in the form of oral Truvada® remains the standard of care for HIV prevention in South Africa. Despite the availability of PrEP, HIV infections continue in young women significantly more than in men. Clinical trials testing antiretrovirals containing tenofovir as topical or oral PrEP formulations in African women, produced inconsistent patterns of efficacies against HIV. Effectiveness of oral and topical PrEP is dependent on adequate drug delivery and availability to cells and tissues targeted by HIV. Our study, therefore, focused on how different biological factors: drug transporter expression, single nucleotide polymorphisms (SNPs) in drug transporter genes and genital inflammation modulate PrEP disposition in African women. We characterized drug transporter mRNA expression in two compartments, the female genital tract (FGT) and blood, at baseline, 3 and 6 months in 45 women taking oral PrEP-Truvada®. Additionally, the impact of SNPs in 393 women and genital inflammation in 45 women on circulating tenofovir and drug transporter mRNA expression were determined. SNPs in drug transporter genes: *ABCB1* 3435G>A; *ABCC1* 198217T>C; *ABCC2* 1249G>A; *ABCC4* 3463T>C; *ABCC4* 4131A>C and *ABCC4* 4976A>G were evaluated using real-time PCR. mRNA expression of efflux P-gp, MRP-2, MRP-4, MATE-1 and influx OAT-1 and OAT-3 drug transporters was evaluated using quantitative real-time PCR. Genital inflammation was measured in cervicovaginal specimens using a 28-cytokine multiplexed platform. Results showed that *ABCC4* 4976A>G and *ABCC4* 3463T>C SNPs alter circulating tenofovir differently. While the *ABCC4* 4976A>G SNP significantly increased the mRNA expression of the *ABCC4* gene ($p=0.0132$), there was inverse association with circulating tenofovir ($p=0.018$). In contrast, although the *ABCC4* 3463T>C SNP did not significantly impact mRNA expression of the *ABCC4* gene, it was significantly and directly associated with circulating tenofovir ($p<0.05$). Correlation analyses showed moderately significant associations between the mRNA expression of the influx drug transporter OAT-1 in the FGT and blood pre- and post- PrEP exposure ($r_s<1$, $p<0.05$). In contrast efflux drug transporters P-gp, MATE-1, MRP-2 and MRP-4 showed significance after PrEP initiation (3 and 6 months) ($r_s<1$, $p<0.05$). For pro-inflammatory cytokines, linear mixed models showed negatively correlated trends between IL-1β and MCP-1 and influx drug transporter OAT-1 and OAT-3 ($p<0.1$), while IL-1Rα and TNF-α showed these correlations with efflux drug transporters MRP-2 and MRP-4 ($p<0.1$). Collectively our results suggested that PrEP disposition can be modified through a convergence of host genetics and different biological factors: drug transporter expression, SNPs in drug transporter genes and inflammation. Findings from such studies may be used to better understand PrEP pharmacokinetics and aid in the implementation of optimal PrEP dosages. This

will ultimately inform on effective and safe PrEP for HIV prevention especially in vulnerable and at-risk African women.

1. CHAPTER ONE: LITERATURE REVIEW

1.1 Introduction

Acquired immunodeficiency syndrome (AIDS) was recognised as a new disease in 1981, subsequently; in 1983 the retrovirus human immunodeficiency virus type 1 (HIV-1) was identified as the causative agent of this disease (Barré-Sinoussi *et al.* 1983). By 2003, HIV had infected over 60 million people globally (Sharp and Hahn 2011) and currently, over 38.4 million people are reported to be living with HIV (UNAIDS 2022b). At the end of 2021, an estimated 1.5 million new HIV global infections were reported, of which 51% occurred in sub-Saharan African (SSA) countries (UNAIDS 2022b).

Among SSA countries, South Africa is the most severely affected by the HIV epidemic; with an ~ 7.5 million people living with HIV (UNAIDS 2022a). In 2021, an estimated 210,000 new HIV infections were reported in South Africa, of which ~130,000 were women aged 15 and above, while only 70,000 were men aged 15 and above (UNAIDS 2022a). This data underscored the disproportionate risk of women in acquiring HIV. Many of these infections were reported in the KwaZulu-Natal province (KZN), which is one of the provinces with the highest HIV incidence and prevalence rates (UNAIDS 2022a).These 2021 statistics are in line with previous studies conducted in KZN which also showed high HIV prevalence in young women when compared to their male counterparts. This study showed that HIV prevalence was highest in young women aged 15 to 25 years, while for men, HIV prevalence was highest in men aged 25 to 40 years old (Figure 1.1A) (Abdool Karim *et al.* 2009). An HIV transmission study was also conducted in KZN to understand why adolescent girls and young women continue to be vulnerable to HIV infections at a younger age. This study suggested that men (aged 25 to 40 years) are the primary source of HIV acquisition for adolescent girls and young women (aged 15 to 25 years) Figure 1.1B (purple arrows). Once these adolescent girls and young women are infected, when they grow older over time, they infect men of a similar age. This typically causes HIV transmission for people above 25 years to be from women to men Figure 1.1B (orange arrows). The HIV transmission cycle is then perpetuated when these now HIV infected older men infect the newer group of adolescent girls and young women (Figure 1.1B) (de Oliveira *et al.* 2017). Collectively these studies provide evidence as to why women are at a significantly higher risk of acquiring HIV at a much younger age and are twice as likely to acquire HIV when compared to their male counterparts (Abdool Karim *et al.* 2009, de Oliveira *et al.* 2017, Karim *et al.* 2022).

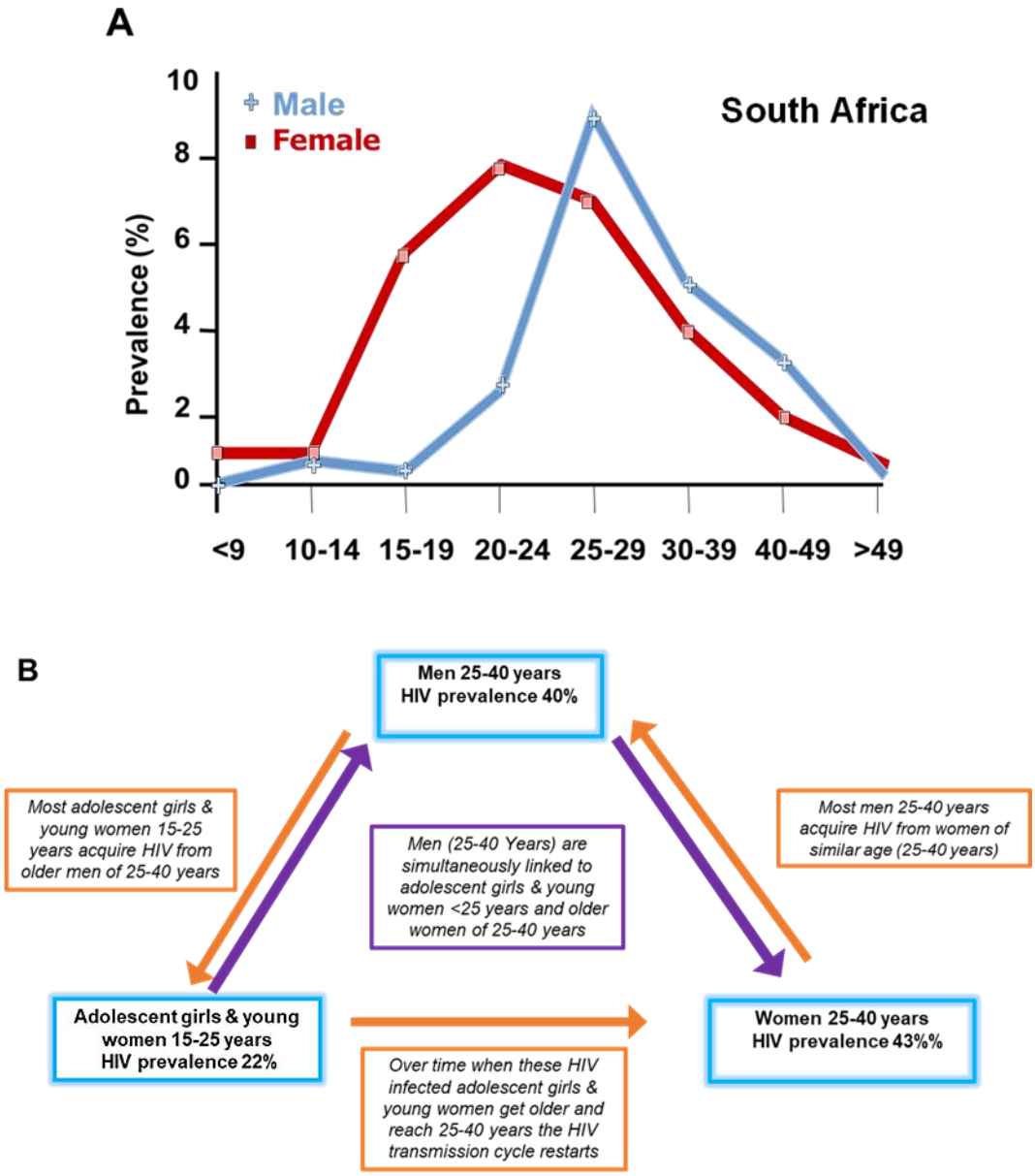

Figure 1.1: HIV prevalence according to disparities in age and gender in KZN South Africa.
A) Disparities in HIV infections are highest for women between the ages of 15 and 25 years while for men it is between the ages of 25 to 40 years old; suggesting higher HIV infection rates among young women which remains the current and dominant pattern. **B)** Heterosexual HIV transmission cycle between older men aged 25 to 40 years and adolescent girls and young women aged 15 to 25 years indicating the intergenerational sexual partnering patterns that fuels the epidemic in a continuous cycle. Images adapted from **A)** (Abdool Karim et al. 2009) and **B)** (de Oliveira et al. 2017).

The use and coverage of ARVs has, however, had a positive impact in many SSA countries including South Africa. However, despite the advent and use of ARVs, HIV's continuous and ongoing spread in South Africa and Africa remains a major concern (UNAIDS 2022a).

The increasing challenge of providing ARVs to a rapidly growing HIV population prompts the need for new interventions to decrease HIV incidence rates (Nicol *et al.* 2018). Previously tested HIV prevention methods have included the use of ARVs as oral, topical gels or long-acting pre-exposure prophylaxis (PrEP) formulations in uninfected individuals (Abdool Karim *et al.* 2010, Janes *et al.* 2018, Nicol *et al.* 2018). Clinical trials using oral and topical PrEP regimens in high risk heterosexual HIV-serodiscordant couples (Baeten *et al.* 2012, Thigpen *et al.* 2012) and men who have sex with men (MSM) (Grant *et al.* 2010, Molina *et al.* 2015, McCormack *et al.* 2016) reported high levels of protection against HIV acquisition ranging from 44% to 86% (Grant *et al.* 2010, Thigpen *et al.* 2012, McCormack and Dunn 2015, Molina *et al.* 2015). However, clinical trials using the same PrEP regimens that focused primarily on at-risk African women, produced inconsistent levels of protection against HIV, ranging from -49% to 39%, with a majority leading to trial termination (Abdool Karim *et al.* 2010, Van Damme *et al.* 2012, Marrazzo *et al.* 2015, Delany-Moretlwe *et al.* 2018).

The main contributory factor for these low efficacies was identified as low to no adherence to PrEP. However, underlying biological factors beyond adherence have been proposed to play an integral role in low PrEP efficacies too (Hu *et al.* 2015, Nicol *et al.* 2018). These include drug transporters, which are transmembrane proteins that are expressed in various cells and tissues of the body. Various ARVs used as PrEP have been identified as substrates of different drug transporters (Hu *et al.* 2015, Taneva *et al.* 2016, Reznicek *et al.* 2017). Therefore, drug transporter expression levels and functionality are considered essential for optimal PrEP delivery and for maintaining optimal drug concentrations in cells and tissues targeted by HIV (Hu *et al.* 2015, Nicol *et al.* 2018). In addition, there are also host biological factors such as inflammation (Saib and Delavenne 2021) and genetic polymorphisms (Shenfield 2004, Arruda *et al.* 2016) affecting drug transporter disposition, which subsequently affects drug efficacy (Shenfield 2004, Arruda *et al.* 2016, Saib and Delavenne 2021). These findings underscore drug transporters as critical determinants of drug pharmacokinetics. However, there is limited data on drug transporter expression profiles and host factors affecting drug transporter expression and function in anatomical compartments such as the female genital tract (FGT), the predominant site for HIV

infection in women during heterosexual intercourse (Hu *et al.* 2015, Nicol *et al.* 2018). This warrants the need for further studies that will evaluate biological factors affecting PrEP pharmacokinetics [absorption, distribution, metabolism and excretion (ADME)] to better understand inconsistencies in PrEP effectiveness observed in clinical trials with at-risk African women.

1.2 Biological, behavioural, and socio-economic factors that increase women's' susceptibility to HIV

Despite noticeable reductions in HIV infections and increases in ARVs accessibility, there are several biological, behavioural and social factors that contribute to higher HIV prevalence rates in women (Ramjee and Daniels 2013, Abdool Karim *et al.* 2020). Socio-economic factors that drive high HIV incidence rates in women include sexual abuse, lack of education, lack of food security and the lack of proper social services such as education on HIV and insufficient provision of health services; especially in highly affected regions (Abdool Karim *et al.* 2012, Ramjee and Daniels 2013, Nicol *et al.* 2018, Durevall *et al.* 2019).

Behavioural factors also play an integral role in high rates of HIV acquisition in young women. These include early age of sexual debut (Mabaso *et al.* 2018), multiple concurrent sex partners, intergenerational sexual partnering with older men and transactional sexual encounters (Maartens *et al.* 2014, de Oliveira *et al.* 2017, Mabaso *et al.* 2018). Other factors include low marriage rates (Alcaide *et al.* 2014), intravaginal practices, and low to no condom use due to the inability to negotiate safe sexual practices with their male partners (Ramjee and Daniels 2013, de Oliveira *et al.* 2017, Mabaso *et al.* 2018). Additionally, the use of injectable drugs and alcohol have also been associated with increased HIV transmission through shared needles and high-risk sexual behaviour, respectively (Maartens *et al.* 2014). Together, these factors suggest that the economic and social disempowerment of young women especially in a developing country such as South Africa contributes largely to high HIV prevalence rates within this population.

Apart from behavioural and socio-economic factors that fuel HIV infections, biological factors also drive higher rates of HIV infections in women. The greater mucosal surface area of the FGT makes this surface highly susceptible through increased opportunities for target CD4+ T cells to become infected with HIV and other sexually transmitted infections (STIs) during sexual intercourse (Ramjee and Daniels 2013). Other biological factors that increase women's

susceptibility to HIV include bacterial vaginosis (BV) (Heffron *et al.* 2017, Klatt *et al.* 2017), vaginal micro-abrasions (Stanley 2009), cervical ectopy (Critchlow *et al.* 1995) and genital inflammation (Masson *et al.* 2015, McKinnon *et al.* 2018). Additionally, the use of long-acting injectable progestin hormonal contraceptives [(particularly depot-medroxyprogesterone acetate (DMPA)] has also been associated with increased women's susceptibility to HIV, however, this remains a topic of ongoing debate, with some studies showing an increased HIV risk (Heffron *et al.* 2012, Hapgood 2020) while, others showed no differences (Myer *et al.* 2007, Shen *et al.* 2017).

1.3 PrEP in HIV prevention

Since the main route of HIV infection in women is through sexual intercourse, many prevention strategies are aimed at protecting the FGT (Celum 2011, Nicol *et al.* 2018). As a result, many different modalities have been tested, which include a vaginal ring containing dapivirine (Baeten *et al.* 2016), various microbicide gel formulations such as Carraguard (Skoler-Karpoff *et al.* 2008) and PRO2000 vaginal gels (McCormack *et al.* 2010) and spermicide gel formulations such as nonoxynol-9 (N-9) (Wilkinson *et al.* 2002). Besides topical gels and rings, implants containing tenofovir alafenamide (TAF) (Gunawardana *et al.* 2015) and long-acting injectable formulations containing for example cabotegravir (CAB) (Landovitz *et al.* 2018) are currently being tested for HIV prevention in PrEP clinical trials. Truvada® which is the co-formulated tenofovir disoproxil fumarate (TDF)/TFV and emtricitabine (FTC)) is a licensed oral PrEP drug that is also used for HIV prevention (Alvarez *et al.* 2011).

The concept of using TFV as PrEP in preventing HIV infections was initially investigated in macaques with simian immunodeficiency virus (SIV) (Person and Hick 2012). During the 1990s, studies on TFV (a nucleotide reverse transcriptase inhibitor of HIV) previously known as (R)-9-(2-phosphonylmethoxypropyl)adenine (PMPA) demonstrated evidence of complete protection against SIV infections (Tsai *et al.* 1995, Person and Hick 2012). The success observed in animal models was, however not fully translated in human HIV prevention clinical trials (Person and Hick 2012). One of the major factors that attributed to these inconsistencies was low to no adherence, which limited PrEP exposure to tissues and cells targeted by HIV in areas such as the FGT (Rohan and Sassi 2009, Hu *et al.* 2015).

1.4 PrEP clinical trials: Efficacy in African women

Significant breakthroughs in using PrEP to prevent HIV infections have been observed in PrEP trials focused-on high-risk HIV-serodiscordant heterosexual couples and MSM (Baeten *et al.* 2012, Thigpen *et al.* 2012) (Table 1.1). The Partners PrEP trial, performed in HIV-serodiscordant heterosexual couples in Kenya and Uganda showed significant HIV reductions of 75% and 67% respectively, with oral TDF-FTC and with TDF alone (Baeten *et al.* 2012). The TDF2 trial which evaluated the effectiveness of TDF-FTC drugs in sexually active HIV negative heterosexual adults from Botswana; showed that TDF-FTC prevented new HIV infections, by demonstrating a 62% reduction in HIV incidence (Thigpen *et al.* 2012). In the Pre-exposure Prophylaxis Initiative trial (iPrEX) in MSM from South America, the United States of America, South Africa and Thailand, a daily single oral dose of Truvada® demonstrated a 44% reduction in HIV incidence (Grant *et al.* 2010). Similarly, in other MSM European studies testing Truvada® the Pragmatic open-label randomised trial of pre-exposure prophylaxis (PROUD) (McCormack and Dunn 2015) and the On-Demand Antiretroviral Pre-exposure Prophylaxis for HIV Infection (IPERGAY) in men who have sex with men (MSM) (Molina *et al.* 2015), both showed an 86% reduction in HIV incidence (McCormack and Dunn 2015, Molina *et al.* 2015). Results from these studies further supported the effectiveness of PrEP among MSM who are at risk of acquiring HIV (McCormack and Dunn 2015, Molina *et al.* 2015). Currently, in African women, the CAPRISA 004 trial remains the only trial that showed an overall 39% efficacy with a topical gel containing ARV (Table 1.1) (Abdool Karim *et al.* 2010). Furthermore, when the data was stratified according to degree of adherence, women with high adherence had a corresponding reduction in HIV incidence by 54%, while women with low adherence had only a 28% reduction in HIV incidence (Abdool Karim *et al.* 2010).

Table 1.1: PrEP clinical trials demonstrating various efficacies in high-risk populations from different regions

Clinical Trials	Study Population (regions)	PrEP Drugs	PrEP efficacy - reduction in HIV incidence %	References
CAPRISA 004	African women (South Africa)	1% TFV gel	39**%**	(Abdool Karim *et al.* 2010)
Partners PrEP	Heterosexual couples (Kenya and Uganda)	Oral TDF-FTC	75%	(Baeten *et al.* 2012)
		Oral TDF alone	67%	
TDF2	Heterosexual couples (Botswana)	Oral TDF-FTC	62%	(Thigpen *et al.* 2012)
iPrEX	MSM (South America, the United States of America, South Africa, and Thailand)	Oral TDF-FTC	44%	(Grant *et al.* 2010)
PROUD	MSM (England)	Oral TDF-FTC	86%	(McCormack and Dunn 2015)
IPERGAY	MSM (France and Canada)	Oral TDF-FTC	86%	(Molina *et al.* 2015)

Other PrEP clinical trial studies that focused primarily on at-risk African women demonstrated inconsistent levels of protection, showing HIV incidence of -49% to 14.5% (Table 1.2). These include the FEM-PrEP trial (Van Damme *et al.* 2012) which evaluated, daily Truvada® in women from high-risk areas in South Africa, Kenya and Tanzania. This trial was, however, terminated following low efficacy largely attributed to lack of adherence (Corneli *et al.* 2016) and low drug concentrations in the FGT (Abdool Karim *et al.* 2011). The Vaginal and Oral Interventions to Control the Epidemic (VOICE) Microbicide Trial Network (MTN 003) trial was also conducted in women from high HIV prevalence areas in South Africa, Uganda and Zimbabwe (Marrazzo *et al.* 2015). Women were randomised to either oral TDF, oral TDF-FTC, TFV gel, or respective oral or vaginal placebos. Similar to the FEM-PrEP study, results from this trial showed no efficacy (Marrazzo *et al.* 2015). The moderate success of the topical CAPRISA 004 1% TFV gel trial led to the Follow on African Consortium for Tenofovir Studies (FACTS-001) (Delany-Moretlwe *et al.* 2018). The study was conducted at nine community-based clinical trial sites where it assessed the safety and efficacy of the precoitally applied 1% TFV gel in high-risk South African women. Here too, the FACTS-001 trial showed no significant reduction in HIV incidence between the active arm and the control (Delany-Moretlwe *et al.* 2018).

Table 1.2: PrEP clinical trials demonstrating low PrEP efficacies in high-risk populations from different regions

Clinical Trials	Study Population (regions)	PrEP Drugs	PrEP efficacy - reduction in HIV incidence %	References
FEM-PrEP	African women (South Africa, Kenya and Tanzania)	Oral TDF-FTC	4.7%	(Van Damme *et al.* 2012)
VOICE	African women (South Africa, Uganda and Zimbabwe)	Oral TDF-FTC	-4.4%	(Marrazzo *et al.* 2015)
		Oral TDF alone	-49%	
		1% TFV gel	14.5%	
FACTS-001	African women (South Africa)	1% TFV gel	6.52%	(Delany-Moretlwe *et al.* 2018)

A potential explanation for the disparities in efficacy observed in these PrEP clinical trials could be due to differential drug penetration levels in the rectal compared to the vaginal mucosal tissues (Patterson *et al.* 2011, Janes *et al.* 2018). Data from Cottrell *et al.* (2015), observed that similar levels of adherence of two doses per week of Truvada® reduced HIV incidence by 90% in the MSM population of the iPrEX study, whereas in heterosexual women populations of the FEM-PrEP and VOICE studies low to no protection was observed (Cottrell *et al.* 2015). Additionally, the complex composition of the vagina's microbiome and inflammation may affect PrEP disposition in women (Patterson *et al.* 2011, Klatt *et al.* 2017, Janes *et al.* 2018, McKinnon *et al.* 2018). These findings urge the need to better understand the mechanisms of drug availability and metabolism within the area of vulnerability, the FGT (Rohan and Sassi 2009, Hu *et al.* 2015).

1.5 Compartmental heterogeneity in PrEP drug disposition

The FGT is a highly active and diverse immune environment, with a wide range of heterogeneous immune cells such as macrophages, dendritic cells, Langerhans cells, natural-killer cells, B and T-cells, making it highly susceptible to HIV infections (Hu *et al.* 2015, Nicol *et al.* 2018). The increased vulnerability of the FGT to HIV infections is also largely ascribed to the presence of a single columnar epithelium cell layer in the endocervix as opposed to the multi-layered squamous epithelium of the ectocervix (Nicol *et al.* 2018). These cell layers are vulnerable to micro-abrasions caused by friction during heterosexual intercourse allowing for easy access of HIV (Nicol *et al.* 2018). A previous study by Shen *et al.* (2014) also observed that even with intact epithelium, vaginal myeloid dendritic cells expressing HIV receptors can facilitate the capture and

dissemination of HIV into the deeper mucosal tissue layers (Shen *et al.* 2014). The vulnerability of the FGT prompts the need for HIV prevention interventions that provide sufficient protection within all compartments exposed to the virus (Nicol *et al.* 2018).

Interventions such as PrEP should therefore provide optimal ARV drug concentrations that are sufficient in preventing HIV viral entry, transcription, and replication in HIV targeted cells (Cottrell *et al.* 2015). Drug exposure of PrEP in the FGT has previously shown variability in drug delivery and availability (Cottrell *et al.* 2015). To understand this variability; a 14-day open-labelled study by Patterson *et al.* (2011), demonstrated varying concentration levels of TFV, FTC and their respective active metabolites TFV-DP and FTC triphosphate (FTC-TP) in rectal, vaginal and cervical tissues, following a single dose of Truvada®. In rectal tissues, TFV and TFV-DP were detected throughout the 14-day period and concentrations were 100-fold higher when compared to cervical and vaginal tissues; while FTC concentrations were 10 to 15- fold higher in vaginal and cervical tissue when compared to rectal tissue. The active metabolite FTC-TP was, however, only detected for two days in all tissues (Patterson *et al.* 2011).

A prior study also compared ARVs drug exposure in cervicovaginal fluid and blood. In the FGT, nucleoside reverse transcriptase inhibitor (NRTIs) lamivudine (3TC), zidovudine (ZDV), FTC and TFV exhibited high drug concentrations relative to the blood. However, non-nucleoside reverse transcriptase inhibitor (NNRTIs) efavirenz (EFV) and protease inhibitors (PIs) lopinavir (LPV) and atazanavir (ATV) exhibited low concentrations in the FGT when compared to the blood (Dumond *et al.* 2007). These results indicate the heterogeneity of drug disposition and their respective metabolites within sub-compartments of the same anatomical surface or different compartments (Patterson *et al.* 2011). These studies suggested that certain ARVs may or may not be good PrEP candidates according to their ability to penetrate certain areas (Dumond *et al.* 2007). In addition, these findings indicated that concentration thresholds for TFV that correlate with high levels of protection against HIV differ significantly for men compared to women. This suggests that women require consistent use of oral PrEP to confer similar protection (Patterson *et al.* 2011, Louissaint *et al.* 2013, Cottrell *et al.* 2016, Sheth *et al.* 2016). This underscores the importance of understanding factors affecting drug pharmacokinetics in tissues that are highly susceptible to HIV infections.

These variations show differential drug penetration levels, giving an insight into the varying levels of protection against HIV observed in some PrEP trials (Cottrell *et al.* 2015). These discrepancies

may be due to the interplay between these PrEP drugs and various membrane-bound proteins that mediate drug transport and availability (Cottrell *et al.* 2015). For example ARVs such as TFV, FTC and ZDV have been previously shown to be substrates of drug transporters P-glycoprotein (P-gp), multi-drug resistance protein-1 (MRP-1) and organic anion transporters-1 (OAT-1), respectively (Kis *et al.* 2010, Hu *et al.* 2015). These data indicated that intracellular and extracellular ARV drug levels can be predominantly regulated by certain drug transporters (Kis *et al.* 2010, Hu *et al.* 2015). Therefore, understanding the distribution and biological characteristics of drug transporters may help further define their roles in affecting PrEP efficacy.

1.6 Drug transporters involved in PrEP pharmacokinetics

Drug transporters are types of transmembrane proteins that are ubiquitously expressed in the human body in areas such as the gastrointestinal tract, in epithelial cells in the FGT, blood (white blood cells), lungs, blood-brain barrier, endothelial cells, and liver cells (Zhou *et al.* 2013, Arruda *et al.* 2016). Drug transporters comprise of two superfamilies: the ATP-binding cassette (ABC) and Solute Carrier (SLC) proteins (Hu *et al.* 2015). The ABC proteins are a large family of efflux pumps that bind ATP and utilize its hydrolysis energy to transport molecules across and out of the cell membrane. This family comprises seven subfamilies of which three are the most relevant in the efflux of ARVs (Petzinger and Geyer 2006, Hu *et al.* 2015). These include (Figure 1.2):

i) the *ABCB* subfamily that comprises P-glycoprotein (P-gp),
ii) the *ABCC* subfamily that comprises multidrug and toxin extrusion proteins (MATE) and MRPs, and,
iii) the *ABCG* subfamily that comprises breast cancer resistance protein (BCRP) (Petzinger and Geyer 2006, Hu *et al.* 2015, Nicol *et al.* 2018).

The SLC proteins influx or uptake molecules across and into the cell membrane via ATP energy dependant carriers or through an electrochemical gradient (Lin *et al.* 2015). Subfamilies that are the most relevant in the uptake or influx of ARVs include (Figure 1.2):

i) the *SLC22* subfamily that comprises OAT and organic cation transporters (OCTs),
ii) the *SLC28* subfamily that comprises concentrative nucleoside transporters (CNTs),
iii) the *SLC29* subfamily that comprises equillibrative transporters (ENTs) and

iv) the *SLCO* subfamily that comprises organic anion-transporting polypeptides (OATPs) (Petzinger and Geyer 2006, Hu *et al.* 2015, Nicol *et al.* 2018).

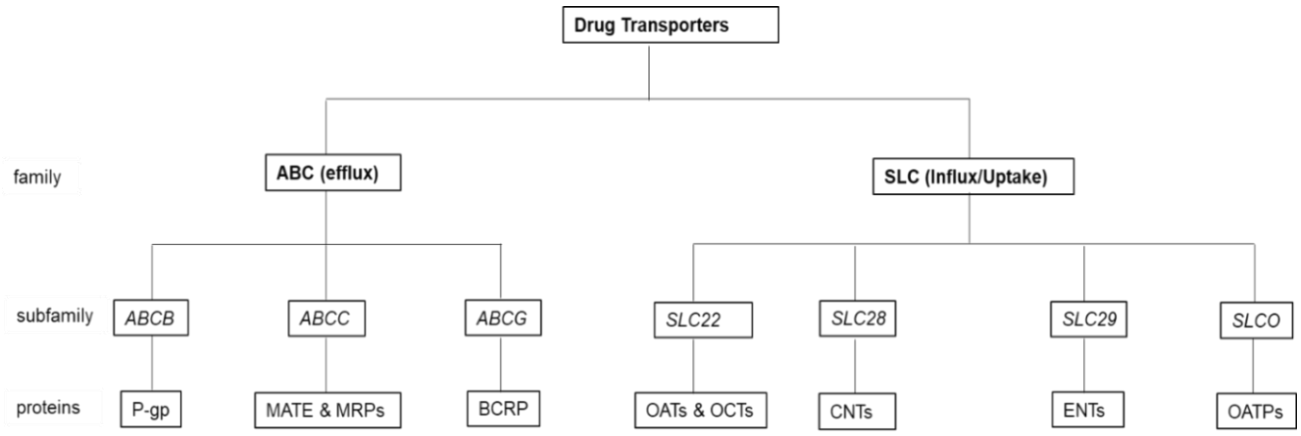

Figure 1.2: Drug transporters most relevant in the efflux and influx of ARVs.
In the ABC (efflux) family there are three-drug transporter subfamilies along with their respective proteins that are the most relevant in the efflux of ARVs drugs, while in the SLC (influx/uptake) family, there are four drug transporter subfamilies along with their respective proteins that are the most relevant in the uptake or influx of ARVs.

Most of these drug transporters are localised on polarized cells, and regulate substrate distribution on the apical or basolateral surfaces of cells, contributing to the pharmacokinetics of several ARVs (Sissung *et al.* 2012). In previous studies, TFV and FTC have been shown as substrates of these ABC and SLC drug transporters (Hu *et al.* 2015, Nicol *et al.* 2018). This data indicated that the delivery and absorption of these drugs in cells is facilitated by drug transporters, establishing their emerging role as critical determinants in drug pharmacokinetics. This interaction has been noted especially in HIV target cells such as the immune cells of the FGT that include macrophages, vaginal epithelial cells, T cells, and dendritic cells expressing CD4 receptors (Hu *et al.* 2015, Taneva *et al.* 2016), and also in peripheral blood mononuclear cells (PBMCs) (Turriziani *et al.* 2008) and epithelial cells of the intestinal and renal system (Cihlar *et al.* 2007, Reznicek *et al.* 2017)

1.6.1 Role of drug transporters in the FGT:

The extracellular accumulation of TFV and FTC in cells overexpressing certain drug transporters has been demonstrated in various studies focused on the FGT. Findings from these studies suggested that the delivery of effective PrEP drug concentrations to cells and tissues in the FGT is highly associated with the mRNA expression level and functionality of drug transporters (Grammen *et al.* 2014, Nicol *et al.* 2014, Hijazi *et al.* 2015, Taneva *et al.* 2016). Zhou *et al.* (2013) showed varying drug transporter expression levels in the FGT (vaginal and ectocervix tissues) and liver. mRNA expression was defined as ≤2% (undetectable), 2-10% (low expression), 10-50% (moderate expression) and 50-100% (high expression) (Zhou *et al.* 2013). In the FGT, high mRNA expression of drug transporters (MRP-1, MRP-4, P-gp, BCRP, ENT-1 and OCT-2) was observed, while moderate to low mRNA expression of MRP-2 and influx drug transporters OAT-1 and OAT-3 relative to the liver (Zhou *et al.* 2013). Similarly, a study by Taneva *et al.* (2016) also showed significantly low expression of uptake drug transporters OAT-1 and OAT-3 in vaginal epithelial cells and T-cells, which accounted for the poor permeability of TFV across the cell membranes and into the cells. Additionally, this study showed that the *in-vitro* transfection of T cells with the drug transporter OAT-1 increased TFV uptake, resulting in high intracellular drug accumulation (Taneva *et al.* 2016). These studies indicated that there is variability in drug transporter expression levels within different tissues. Therefore, analysing the expression levels of drug transporters in the FGT could aid in better understanding their role in the pharmacokinetics of drugs (Zhou *et al.* 2013, Taneva *et al.* 2016).

Nicol *et al.* (2014) showed high mRNA expression levels of efflux drug transporters P-gp and MRP-2 in vaginal tissues compared to colorectal tissue, while MRP-4 was only highly expressed in colorectal tissues. In contrast, uptake drug transporters OAT-1, OAT-3 and OATP-1B1 exhibited extremely low to no expression in colorectal and vaginal tissues, respectively (Nicol *et al.* 2014). Additionally, immunohistochemistry that informed on the localisation of these drug transporters revealed high protein expression of P-gp and MRP-2 in vaginal epithelial cells compared to colorectal epithelial cells, while low to no protein expression of OAT-1 was observed in colorectal epithelial and vaginal cells, respectively (Nicol *et al.* 2014). Differences in protein localisation and expression suggested an increased expression of efflux drug transporters in vaginal tissues compared to colorectal tissues (Nicol *et al.* 2014). These data show that more drug is pumped out of cells in the vagina, while an increased expression

of uptake drug transporters in colorectal tissues promoted an uptake of drugs (Nicol *et al.* 2014). These findings highlighted that inter-tissue variability in drug transporter expression may contribute to the greater intracellular accumulation of ARVs such as TFV and maraviroc in colorectal tissues compared to vaginal tissues (Nicol *et al.* 2014). High expression levels of efflux drug transporters P-gp, MRP-2 and BCRP in vaginal and endocervical tissues was also reported by Grammen *et al.* (2014). The study further established in intestinal cell lines-Caco-2 and vaginal epithelial cell lines-SiHa using specific drug transporter inhibitors, that ARV drugs darunavir, maraviroc and saquinavir are substrates of efflux drug transporters P-gp and MRP-2, which are likely to contribute to lower intracellular levels of these respective drugs (Grammen *et al.* 2014).

To further understand the role of drug transporters in the mucosal compartment, the relationship between the accumulation of topically applied PrEP drugs dapivirine, darunavir and TFV, and the expression of drug transporters was characterised in cervicovaginal cell lines (Hijazi *et al.* 2015). These included HeLa cell lines, VK2/E6E7, Ect1/E6E7 and End1/E6E7 derived from human cervical epithelial adenocarcinoma, primary vaginal, ectocervical and endocervical epithelial cells, respectively (Hijazi *et al.* 2015). Tenofovir significantly downregulated the mRNA expression of MPR5 in VK2/E6E7, while dapivirine significantly upregulated most MRP drug transporters in all cell lines. Darunavir stimulation also significantly upregulated the uptake drug transporter CNT3 in all cells, while MRP-3 was only significantly upregulated in VK2/E6E7 cell line (Hijazi *et al.* 2015). This characterisation by Hijazi *et al.* (2015) provided insight not only on the type of drug transporters present in the FGT but also how drug transporter disposition may be altered by the presence of certain drugs; which could assist in the assessment of ARV pharmacokinetics in the FGT. Furthermore, these findings could assist in the determination of suitable PrEP drug formulations that could provide sufficient drug concentrations to susceptible tissues and cells of the FGT (Hijazi *et al.* 2015).

1.6.2 Role of drug transporters in peripheral blood mononuclear cells (PBMCs):

A study by Turriziani *et al.* (2008) determined the mRNA expression levels of drug transporters in PBMCs (isolated from buffy coats) from HIV infected individuals failing ARV therapy and HIV negative individuals. The mRNA expression levels of P-gp, MRP-1, MRP-4 and MRP-5 was significantly higher in HIV infected individuals compared to HIV negative individuals (Turriziani *et al.* 2008). A higher inter-individual mRNA expression variability was also observed in HIV

infected individuals, indicating a correlation between the presence of ARVs and drug transporter expression levels (Turriziani *et al.* 2008). Similarly, Bousquet *et al.* (2009) investigated if the singular or combined (dual or triple) use of TFV, FTC and EFV on PBMCs isolated from healthy donors disrupts mRNA drug transporter expression levels (Bousquet *et al.* 2009). Following a 20-hour *in-vitro* incubation, a singular use of FTC induced MRP-5, while TFV reduced MRP-1, MRP-5, MRP-6 and P-gp mRNA expression in PBMCs (Bousquet *et al.* 2009). FTC was also shown to exhibit an inhibitory effect on the mRNA expression of efflux drug transporter MRP-1 in a dose-responsive manner. These findings suggest a correlation between the presence of FTC with MRP-1 expression (Bousquet *et al.* 2008). The use of ZDV was also previously shown to be associated with the upregulation of efflux drug transporters MRP-1 and MRP-5 expressed on PBMCs (Jorajuria *et al.* 2004). Findings from these studies showed that an interaction between ARVs and drug transporters may alter drug transporter disposition by affecting mRNA expression levels; subsequently affecting intracellular drug accumulation (Jorajuria *et al.* 2004, Bousquet *et al.* 2009).

Contrary to these studies, Falasca *et al.* (2011) and Giraud *et al.* (2010) showed no correlations between the mRNA expression levels of efflux drug transporters and the presence of ARVs ritonavir (RTV), ATV, and LPV in PBMCs isolated from HIV infected patients (Giraud *et al.* 2010, Falasca *et al.* 2011). The study found no variation in the mRNA expression levels of P-gp and MRP drug transporters before and after ARV intake (Falasca *et al.* 2011). However, in a more recent study, a significant association was observed between ARVs and drug transporters P-gp, BCRP, MRP-1, ENT-2 and OCT-1 expressed on monocytes and monocyte-derived macrophages isolated from HIV negative individuals and HIV infected individuals receiving ARV therapy containing either abacavir, ATV, EFV, rilpivirine, TFV, 3TC, FTC, elvitegravir, dolutegravir, and cobicistat (Hoque *et al.* 2021). These findings showed that these associations could lead to sub-optimal intracellular drug concentrations, subsequently allowing HIV infections in HIV negative individuals or further HIV replication in HIV infected individuals (Hoque *et al.* 2021).

1.6.3 Role of drug transporters in the renal system:

The entry of TFV into epithelial cells of the kidney tubule is mediated by the uptake drug transporters OAT-1 and OAT-3 expressed on its basolateral membrane (Cihlar *et al.* 2007), while the efflux of TFV into urine is mediated by efflux drug transporter MRP-4 expressed at

the apical side of renal proximal tubules (Ray *et al.* 2006). These data together provide evidence that TFV is a substrate of OAT-1, OAT-3 and MRP-4 drug transporters expressed in renal tubules (Ray *et al.* 2006, Cihlar *et al.* 2007). TFV is also a substrate of the efflux drug transporter MRP-8 expressed in renal proximal tubules since higher cytotoxic concentrations of the drug were observed in cells overexpressing MRP-8 (Tun-Yhong *et al.* 2017). The uptake of ARVs cidofovir, adefovir and TFV was evaluated in human embryonic kidney (HEK293) cells transfected with uptake drug transporters OCT-2, OAT-1 and OAT-3 (Uwai *et al.* 2007). Results showed higher uptake of all ARVs through OAT-1 compared to OAT-3, while OCT-2 exhibited no uptake, indicating that OAT-1 plays a significant role in renal transport of these ARVs (Uwai *et al.* 2007). Similarly, renal secretion of FTC was mediated by MATE-1 which functionally acts as an efflux drug transporter, expressed on the apical side of renal proximal tubules (Reznicek *et al.* 2017).

Varied expression of efflux and influx/uptake drug transporters may influence the TFV levels in cells such as PBMCs, colorectal, vaginal and kidney epithelial cells. Tenofovir has been previously shown to be a substrate of drug transporters such as MRP-4 and OAT-1 (Hu *et al.* 2015). Figure 1.3. is a hypothetical representation of how varying drug transporter expression levels may affect intracellular and plasma TFV drug levels. Figure 1.3.A suggests that low expression of MRP-4 may contribute to less effluxed TFV from cells, while high expression of MRP-4 may contribute to more effluxed TFV from cells. Figure 1.3B suggest that a low expression of OAT-1 may contribute to less influxed TFV into cells, while high expression of OAT-1 may contribute to more influxed TFV into cells. These vary levels of expression suggest that a homeostasis in the expression of ABC and SLC drug transporters is required to ensure optimal intracellular drug levels.

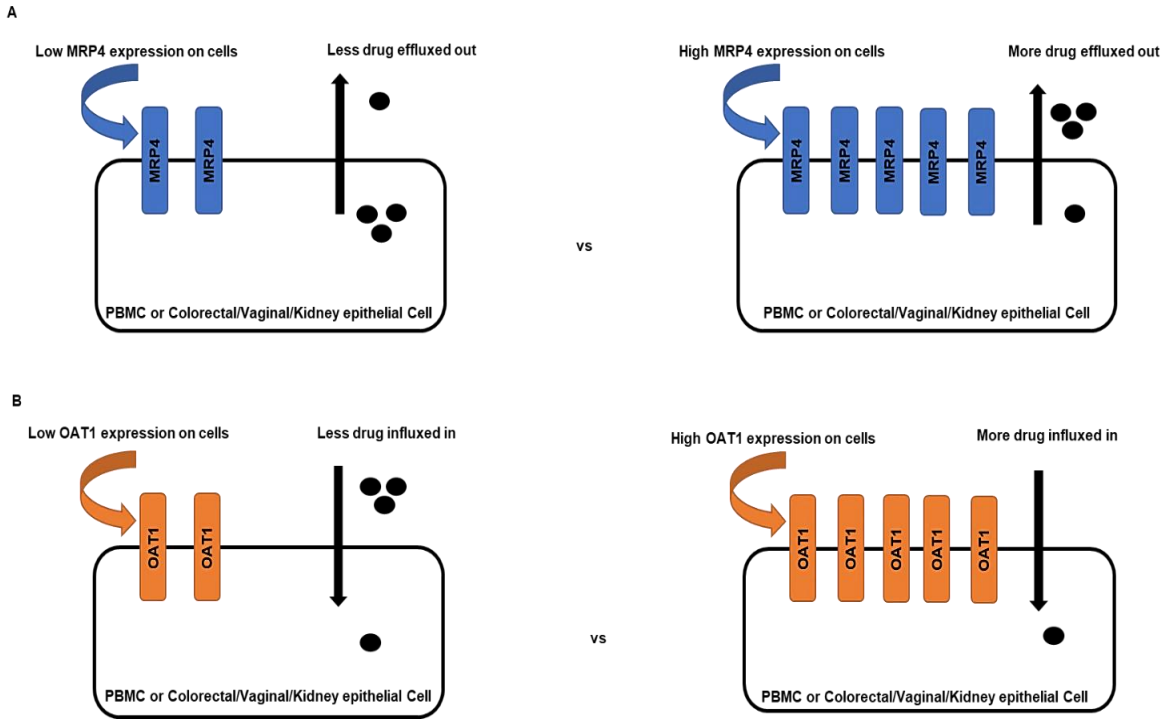

Figure 1.3: Hypothetical representation of how the expression of ABC and SLC drug transporters may influence TFV drug levels.
A) effects of low versus high expression of ABC drug transporter MRP-4 (blue- bars and arrows) on tenofovir levels**. B)** effects of low versus high expression of SLC drug transporter OAT-1 (orange -bars and arrows) on tenofovir level. Black arrows and dots represent tenofovir movement and accumulation, respectively.

These studies collectively provide insight that ARV drug levels are not only determined by drug adherence but also by other factors such as the presence of specific drug transporters and their expression levels. However, definitive conclusions on the full effects of drug transporters on ARV

pharmacokinetics in at-risk groups such as young women especially in Africa have not been drawn. The paucity of data on African women warrants the need for new studies to fully understand:

i) the effect of ARVs on drug transporter expression and,
ii) how varying drug transporter expression levels influence ARV penetration in vulnerable areas such as the FGT,

In addition, other factors that can also affect the mRNA expression levels and function of efflux and influx drug transporters include genetic polymorphisms, inflammation (the production of cytokines). We discuss these factors in detail below.

1.7 Biological factors modulating drug transporter expression and function

1.7.1 Genetic polymorphisms:

Pharmacogenetic research has been used as a tool to determine individuals' susceptibility to certain diseases and for the customisation of drug therapies according to patient's genetic blueprint (Sissung *et al.* 2012, Castellanos-Rubio and Ghosh 2019). As such, sequencing and genotyping technology have been widely used to identify and determine the effect of variants such as genetic polymorphisms in various genes. There are four types of genetic polymorphisms that have been shown to regulate genes (Ismail and Essawi 2012). These include:

I. small insertions and deletions (InDels) which is a deletion or insertion in the DNA sequence (Boschiero *et al.* 2015),
II. interspaced or tandem repeat polymorphisms which are tandemly repeated nucleotides of approximately ≥2 base pairs (bp) in DNA sequences (Ismail and Essawi 2012),
III. structure or copy-number variations (CNVs), polymorphisms which are various copies of differently sized segments of nucleotides in DNA sequences (Stankiewicz and Lupski 2010) and
IV. single nucleotide polymorphisms (SNPs), which are point mutations of nucleotide bases within DNA sequences (Sissung *et al.* 2012, Yee *et al.* 2018).

Types of SNP variations include missense mutations or nonsynonymous substitutions which is a single nucleotide change within a codon, subsequently resulting in the coding of a different

amino acid (Hunt *et al.* 2009). The presence of such mutations on protein binding sites may affect substrate binding, while those not found on protein binding sites may affect protein expression levels. For example, the missense mutation rs2273697 located on the efflux drug transporter gene *ABCC2* encoding MRP-2 results in a change from valine to isoleucine, on exon 417 (V417I), affecting its expression levels (Yee *et al.* 2018). Another type of SNP mutation is a silent mutation or synonymous substitutions, which are single nucleotide point mutations on a codon that do not result in an amino acid change (Hunt *et al.* 2009). However, these may still affect RNA transcription and stability that may affect mRNA expression levels and protein binding (Yee *et al.* 2018). For example, the SNP rs1045642 located on the efflux drug transporter *ABCB1* gene (3435C/T Ile1145Ile) encoding P-gp is a type of silent mutation that has been highly studied in drug pharmacokinetics (Sissung *et al.* 2012, Yee *et al.* 2018). This SNP has been also previously associated with low P-gp expression levels in the duodenum which correlated with an increase of digoxin plasma concentrations (Hoffmeyer *et al.* 2000). The presence of certain genetic variations in drug transporter genes has sparked a huge interest in further understanding their functional effect; especially since SNPs in certain drug transporter genes have been shown to modulate their function by affecting protein folding, expression levels, and their ability to bind substrates and regulate drug pharmacokinetics (Sissung *et al.* 2012, Yee *et al.* 2018).

SNPs involved in the pharmacokinetics of ARVs have led to adverse effects and varied ARV therapy outcomes amongst HIV infected patients (Shenfield 2004, Arruda *et al.* 2016). Arruda *et al.* (2016) showed the association between SNPs in drug transporter genes and intolerance to ARVs in a cohort of HIV infected Brazilian participants (Arruda *et al.* 2016). Results showed an association between variations in *ABCC2* genes (rs3740066 and rs4148396) encoding MRP-2 and intolerance in patients taking regimens containing either LPV, RTV, indinavir or ATV PIs; while variations in *SLCO2B1* genes (rs2712816, rs12422149, rs1676885 and rs949069) encoding OATP-2B1 caused intolerance in patients taking regimens containing stavudine or ZDV NRTIs (Arruda *et al.* 2016). The presence of the C allele on the *ABCC1* gene 198217C/T (rs212091) encoding MRP-1 and the TT genotype on the *ABCB1* gene 3435C/T (rs1045642) encoding P-gp; was also shown to be possibly associated with reduced gene expression in HIV infected Brazilian participants receiving highly active antiretroviral therapy (HAART) regimens; subsequently affecting the efflux of ARV regimens containing PIs, leading to an increased risk of virological failure (Table 1.3) (Coelho *et al.* 2013).

Fellay *et al.* (2002) showed that the TT genotype on the *ABCB1* gene 3435C/T in an HIV infected Caucasian population was associated with low P-gp expression in PBMCs, affecting ARV concentrations (Fellay *et al.* 2002). However, a subsequent study showed that virological failure was associated with the CC genotype of the *ABCB1* gene 3435C/T instead of the TT genotype in HIV infected patients from the province of British Columbia in Canada (Brumme *et al.* 2003). To elucidate variations of the *ABCB1* 3435C/T SNP observed in these studies; prior results by Ameyaw *et al.* (2001) that assessed the frequency of this SNP in ten ethnic groups can be used (Ameyaw *et al.* 2001). Results showed noticeable differences in the SNP frequencies between African, Asian and European populations. The C allele was highly present in the African populations compared to Asian and European populations which exhibited high frequencies for the CT and TT genotypes (Ameyaw *et al.* 2001). Schaeffeler *et al.* (2001) also supported these findings by reporting a high frequency of the CC genotype in the *ABCB1* gene 3435C/T of West African and African American populations compared to the T allele (Schaeffeler *et al.* 2001). Genetic variations in the *ABCC2* 1249G>A and *SLCO1B1* rs4149056 521G>C genes associated with altering plasma LPV concentrations in HIV infected Caucasian and Asian populations; were found to have no significant effect in black HIV infected Malawian and South African populations (Mpeta *et al.* 2016). These findings suggests that possible variations in drug transporter genes lead to varied ARV therapy outcomes in the African versus Caucasian and Asian populations (Ameyaw *et al.* 2001, Schaeffeler *et al.* 2001, Mpeta *et al.* 2016).

Pharmacogenetic studies conducted with African populations have also shown high genetic diversity (inter-ethnic and intra-ethnic diversity), which subsequently leads to varied drug transporter function and expression levels; impacting drug pharmacokinetics differently as reviewed by Rajman *et al.* (2020). The presence of the *SLCO1B1* SNP 463C/A rs11045819 encoding the OATP-1B1 protein was shown to impact rifampin pharmacokinetics differently in African populations (Weiner *et al.* 2010, Dompreh *et al.* 2018). A study by Weiner *et al.* (2010) showed that a high frequency of the CC genotype for the *SLCO1B1* 463C/A (rs11045819) gene was associated with low rifampin concentrations in African individuals during multidrug intensive therapy against TB (Weiner *et al.* 2010). However, in an African Ghanaian population also exhibiting a high frequency CC genotype for the same gene taking standard first-line TB therapy; no effect on rifampin was observed (Dompreh *et al.* 2018) Table 1.3. Similarly studies by Chigutsa *et al.* (2011) and Gengiah *et al.* (2014) on the *SLCO1B1* (rs4149032) SNP both reported an association between high SNP frequency and low rifampin plasma concentrations

in TB and HIV-TB co-infected South African individuals taking rifampin (Chigutsa *et al.* 2011, Gengiah *et al.* 2014). This association was however, not observed in a TB infected Ghanaian population taking standard first-line TB therapy containing rifampin which also exhibited a high frequency for this SNP (Dompreh *et al.* 2018). The presence of the *SLC22A6* SNP rs11568626 (728G>A) encoding the OAT-1 protein, which results in the amino acid change from arginine to histidine was associated with decreased transport affinity of adefovir, cidofovir, and TFV when compared to the wild type in HIV infected African individuals (Bleasby *et al.* 2005). This variation was however, not observed in Asian and Caucasian individuals (Bleasby *et al.* 2005). What these findings indicate is that certain SNPs may incur a functional deficit on drug transporters and lower effective drug levels in African populations taking certain ARVs (Bleasby *et al.* 2005).

The effect of the *ABCB1* SNP 4036G/G (rs3842) encoding P-gp on efavirenz was evaluated in different African populations. In an HIV infected South African population the AG and GG genotypes were significantly associated with decreased efavirenz plasma concentrations (Swart *et al.* 2012), however the GG genotypes in a healthy Ugandan population was associated with higher efavirenz plasma concentrations (Mukonzo *et al.* 2009). Similarly in Ethiopian and Tanzanian HIV infected populations the presence of the G allele was associated with higher efavirenz plasma concentrations, with higher frequency of the G allele observed in Tanzanians (Ngaimisi *et al.* 2013). These data indicated that the effects of SNPs may differ among African populations; therefore, in order to make definitive conclusions that a SNP affects the African population in a certain way, the functional or expressional effect of these SNPs should be tested among a wide range of different African populations (Rajman *et al.* 2020). Despite the small sample size and sparsity of these data in various studies with African populations as reviewed by Rajman *et al.* (2020) and Zondo *et al.* (2022); these data do add to the understanding of how SNPs can impact drug pharmacokinetics in the African population. Together these studies could be used to adjust the standard recommended dose of ARV and TB drug for the African population that accounts for the presence of SNPs (Dandara *et al.* 2011, Rajman *et al.* 2020).

The effects of SNPs on drug transporter genes have also been associated with increased plasma concentrations of TFV. Studies on an HIV-infected cohort in Thailand showed higher TFV plasma concentrations in patients with the CC genotype on the *ABCC2* 224C/T gene (rs717620) encoding MRP-2 compared to patients with the *ABCC2* TT or CT genotypes (Table

1.3) (Manosuthi *et al.* 2014). Similarly, another Thailand study by Rungtivasuwan *et al.*, (2015) reported higher TFV plasma concentrations in HIV infected patients with the *ABCC4* 4131 (rs3742106) TG or GG genotypes (encoding MRP-4) compared to patients with the *ABCC4* TT genotype (Table 1.3) (Rungtivasuwan *et al.* 2015). These studies proposed that polymorphisms in these drug transporter genes may alter their gene expression or function in renal tubules leading to more effluxed drug and reduced glomerular filtration which is involved in TFV renal clearance; resulting in higher plasma concentrations (Manosuthi *et al.* 2014, Rungtivasuwan *et al.* 2015). A more recent study also showed in an HIV infected Caucasian population a significant association of the CC genotype in the *ABCC2* 224C/T gene with high TFV plasma concentrations, resulting in an increased risk of TFV induced-kidney tubular dysfunction (KTD) (Table 1.3) (Danjuma *et al.* 2018). Nishijima *et al.* (2012) previously confirmed that the CC genotype in the *ABCC2* 224C/T gene leads to high TFV plasma concentrations resulting in the induction of KTD or renal toxicity in Japanese patients (Nishijima *et al.* 2012). While the presence of the TT genotype in the *ABCC4* 4131T/G gene was not associated with TFV induced-KTD, the study attributed these findings to inter-individual variability in genetic backgrounds, which may cause patients to respond differently to the same drug (Kerb 2006, Nishijima *et al.* 2012). Other SNPs on the *ABCC4* gene that have been associated with increased plasma TFV concentrations were evaluated in two studies; in an infected population from Thailand with the C allele on the *ABCC4* 4976C/T gene (Likanonsakul *et al.* 2016), and in a Caucasian population with GG genotype on the *ABCC4* 3436A/G gene (Salvaggio *et al.* 2017).

In some studies, the effects of haplotypes in drug transporter genes have been evaluated. Haplotypes are a group of SNPs that are near each other on the same chromosome (Liu *et al.* 2008). Haplotypes are inherited and may comprise of a large number of SNPs or only a select few (Liu *et al.* 2008). Studying haplotypes in more detail allows for the identification of variants in genes that could affect drug pharmacokinetics affecting disease treatment or prevention (Brunner *et al.* 2005, Jacobs *et al.* 2014). The impact of haplotypes has been shown in different studies to impact tenofovir (Izzedine *et al.* 2006, Nishijima *et al.* 2012). In HIV infected Thai individuals the CA haplotypes (-24C and 1249A SNPs) of the *ABCC2* gene was shown to be associated with tenofovir induced KTD (Nishijima *et al.* 2012). Similarly, in HIV infected Caucasian individuals, haplotypes in the *ABCC2* gene were associated with tenofovir induced renal proximal tubulopathy (rPT). The CATC (−24C, 1249A, 3563T, 3972C) haplotype was observed at a higher frequency in individuals with tenofovir rPT, suggesting that it could

predispose these individuals to this condition. In contrast, the CGAC haplotype (−24C, 1249G, 3563A, 3972C) for the *ABCC2* gene was not observed in rPT individuals but was present in the control group, suggesting that it could elicited a protective effect in these individuals (Izzedine *et al.* 2006). This data showed that allele changes on two of the SNPs could significantly alter the effect of the haplotype (Izzedine *et al.* 2006). The associations observed in these studies, highlight the importance of understanding the impacts of haplotypes in drug transporter genes. These types of studies could be further applied in studies evaluating the impact of SNPs on mRNA expression and function.

Reports obtained from these studies highlight the importance of understanding how the presence of SNPs or haplotypes may affect the efficacy of ARVs by affecting drug transporters' expression and function. Furthermore, these findings could be used to identify populations who are at a higher risk of developing adverse effects due to the presence of certain SNPs. However, most of these studies on SNPs in drug transporter genes affecting ARVs have been performed in non-African populations. Since SNP frequency differs significantly among different ethnic populations, more comprehensive investigations of SNPs in drug transporter genes are required, especially in the populations of African ethnicity. Data from populations of African descent will help us better understand how genetic diversity within these populations and SNPs influence drug transporter genes and subsequently lead to effective or ineffective therapy.

Pharmacogenetic research on polymorphisms present in drug transporter and drug-metabolizing genes is also vital in precision medicine, which enables the tailoring of effective therapies based on patients' genetic backgrounds (Hockings *et al.* 2020). The advantage of a precision medicine approach is the ability to predict putative ineffective therapies and possibly reduce adverse reactions (Hockings *et al.* 2020). Since there are reports of increased adverse reactions in patients in populations of African ethnicity taking ARVs, precision medicine is highly important in HIV prevention and treatment (Hockings *et al.* 2020). Patients taking ARV regimens containing EFV in SSA were predisposed to EFV-induced neuropsychiatric adverse reactions, due to specific genetic variants that reduced the functionality of cytochrome P450 2B6 (*CYP2B6)* the enzyme involved in EFV metabolism (Masimirembwa *et al.* 2016). One of the genetic variants of *CYP2B6* 516G>T (rs3745274) reported a frequency of 34% to 50% in African populations compared to 15 and 20% in Caucasian populations (Masimirembwa *et al.* 2016). However, when the EFV dosages in ARV regimens were further titrated and reduced,

there was improved EFV metabolism leading to significantly reduced neuropsychiatric adverse reactions (Gatanaga *et al.* 2007, Masimirembwa *et al.* 2016). These disparities in frequencies between the populations could lead to varied enzyme metabolism when similar drugs are used which may lead to ineffective drug metabolism and availability (Gatanaga *et al.* 2007, Masimirembwa *et al.* 2016) . This data highlights the importance of using pharmacogenetic research in guiding the development of precision medicine, especially in highly affected populations ensuring effective drug dosing, delivery, and metabolism.

There are also different scientific groups involved in precision medicine, these groups aim to identify chemical and biological targets to improve and/or accelerate the development of different drugs (Yang *et al.* 2021). These include FRAME which is an efficient screening tool and the Malaria Drug Accelerator (MalDA), which is a consortium of 15 leading academic and industrial scientific laboratories (Yang *et al.* 2021). One of the objectives of this consortium is to use different approaches such as reverse genetics to identify novel antimalarial therapies or develop target-based drugs to counteract drug resistant malaria strains such as *Plasmodium falciparum* (Yang *et al.* 2021). These systems could therefore be used to determine identify/screen drug that could be tailored to work efficiently in certain populations. However, the deficit is that these systems do not account for the presence of certain SNPs in drug transporters genes. This further highlights the importance of studies that identify clinically relevant SNPs and their functional effects on drug transporter genes and subsequently different drugs within different populations

Table 1.3: Effects of SNPs in drug transporter genes involved in the pharmacokinetics of ARVs in different ethnic groups.

SNPs	Ethnic group	ARVs	SNPs Effect	Genotype causing effect	Genotype frequency Number of patients n (%)	References
***ABCC2* 224C/T (rs717620)**	Thailand	TFV, Lamivudine Efavirenz	Increased TFV plasma concentration	CC	CC 67 (57); CT 45 (39); TT 5 (4) n=117	(Manosuthi *et al.* 2014)
	Japanese	TFV Emtricitabine Darunavir Ritonavir	TFV induced-KTD	CC	CC 18 (94.7); CT 1 (5.3); TT 0 (0) n=19	(Nishijima *et al.* 2012)
	Caucasian	TFV	TFV induced-KTD	CC	CC 9 (60.0); TT 1 (6.7); CT 5 (33.3) n=15	(Danjuma *et al.* 2018)
***ABCC4* 4131T/G (rs3742106)**	Thailand	TFV	Increased TFV plasma concentration	TG/GG	TT 34 (22.7); TG 80 (53.3); GG 36 (24.0) n=150	(Rungtivasuwan *et al.* 2015)
***ABCC1* 198217C/T (rs212091)**	Brazilian	Zidovudine Lamivudine Efavirenz/ Nevirapine; Lopinavir/ Ritonavir	Increased risk of virological failure	CC	CC 62 (84.9); TC 10 (13.7); CC 1 (1.4) n=73	(Coelho *et al.* 2013)
***ABCB1* 3435C/T (rs1045642)**				TT	CC 37 (50.7); CT 25 (34.2); TT 11 (15.1) n=73	
***ABCC4* 4976C/T (rs1059751)**	Thailand	TFV	TFV induced-KTD	CC	CC 20 (37.0); TT 9 (16.7); CT 25 (46.3) n=54	(Likanonsakul *et al.* 2016)
***ABCC4* 3436A/G (rs1751034)**	Caucasian	TFV	TFV induced-KTD	GG	AA 27 (64.3.); AG 9 (21.4); GG 6(14.3) n=42	(Salvaggio *et al.* 2017)
***SLCO1B1* 463C/A (rs11045819)**	African	Rifampin	Low plasma concentrations	CC	CC 30 (81); CA 7 (19) n=37	(Weiner *et al.* 2010)
	Ghanaian	Rifampin	High plasma concentrations	CC	CC 95 (84.1); CA 17 (15); AA 1 (0.09) n=113	(Dompreh *et al.* 2018)

1.7.2 Systemic inflammation and genital inflammation:

Systemic inflammation is defined as increased levels of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and IFN-γ. These cytokines are primarily expressed by activated immune cells such as macrophages, dendritic cells, mast cells, T-cells and natural killer cells in response to infections or injuries (Cressman *et al.* 2012, Saib and Delavenne 2021). These inflammatory cytokines have the ability to directly modulate the expression of drug transporters. This occurs through epigenetic modifications that regulate the expression and activity of genes that control drug metabolizing enzymes and transporter (DMET) (Lauschke *et al.* 2019). An additional consequence of this epigenetic effect includes the heterogeneity in responses to drugs among individuals (Ivanov *et al.* 2014). Inflammation in HIV infected individuals is a key factor that influences inter-individual pharmacokinetics of ARVs which may manifest clinically in terms of drug efficacy or toxicity. HIV infected individuals have a high inflammatory status. ARV treatment does not attenuate inflammation despite HIV viral load suppression (Neuhaus *et al.* 2010). Another layer of complexity when it comes to inflammation is that it is also affected by the level of adherence to drugs. Here increased inflammation is associated with sub-optimal ARV adherence. Whether systemic effects of inflammation induce a similar or different effect to ARV drug pharmacokinetics, disposition in an inflammatory mucosal environment like the genital tract remains largely unexplored.

Genital tract inflammation is defined as having an elevated profile of five of any of the nine pro-inflammatory cytokines (MIP-1α, MIP-1β, IP-10, IL-8, MCP-1, IL-1α, IL-1β, IL-6, and TNF-α) above the 75th percentile for each cytokine in the genital tract (Masson *et al.* 2015). The presence of either systemic or genital inflammation creates an environment that favours HIV infection and replication, thereby significantly increasing the risk of HIV acquisition in women (Cressman *et al.* 2012, McKinnon *et al.* 2018). Masson *et al.* (2015) showed that women with genital tract inflammation had more than a three-fold increased risk for HIV acquisition compared to women without genital inflammation (Masson *et al.*, 2015). The mechanism underpinning the increased risk is due to these cytokines having both pro-inflammatory and chemotactic properties. Both these properties attract HIV target T-cells, macrophages and dendritic cells, stimulating immune cell activation and differentiation which upregulates the expression of the CCR5 co-receptor which is necessary for HIV infection (Mueller and Strange 2004, Masson *et al.* 2015). MIP-1α and MIP-1β cytokines recruit HIV target cells through

binding the CCR5 receptor subsequently increasing HIV risk (Mueller and Strange 2004). In addition, McKinnon *et al.* (2018) showed, that the protective efficacy of the TFV gel was 57% in women with no genital inflammation compared to 3% in women with high genital inflammation (McKinnon *et al.* 2018). These findings indicated that women with no or low genital inflammation may in some way account for the success of the CAPRISA 004 trial (Abdool Karim *et al.* 2010, McKinnon *et al.* 2018). Although the mechanisms to explain why some people have comparatively high levels of genital inflammation while others do not are not fully understood, likely drivers of genital inflammation include bacterial vaginosis (BV), a microbial dysbiosis common in reproductively active women (Klatt *et al.* 2017), the presence of STI's (Kalichman *et al.* 2011, Passmore *et al.* 2016) and the use of hormonal contraceptives (Myer *et al.* 2007, Morrison *et al.* 2012).

1.7.2.1 Factors influencing genital inflammation

I. Bacterial vaginosis (BV)**:** is largely associated with genital inflammation (McKinnon *et al.* 2018). BV is characterised as an alteration in vaginal microbiota diversity. The displacement of *Lactobacilli* by anaerobic bacteria such as *Gardnerella vaginalis*, *Atopobium vaginae, Mycoplasma hominis* and those in the species *Peptostreptococcus*, *Prevotella,* and *Mobiluncus*, generally characterises the diagnosis of BV. In addition, it leads to genital inflammation which increases the risk of acquiring STI's such as HIV (Reis Machado *et al.* 2014, McKinnon *et al.* 2018). A study by Lennard *et al.* (2018) showed that South African women with BV (classified as a decreased *Lactobacillus crispatus* and *Lactobacillus iners* vaginal microbiome), had higher genital pro-inflammatory cytokines and therefore an increased risk of acquiring HIV (Lennard *et al.* 2018). Klatt *et al.* (2017) also showed that TFV gel was able to reduce HIV incidence, by 61% in women with a *Lactobacillus* dominant vaginal microbiome compared to an 18% reduction in women with a *non-Lactobacillus* dominant vaginal microbiome (Klatt *et al.* 2017). Furthermore, this *in-vitro* study suggested that *G. vaginalis, P. bivia, P. amnii, and M. mulieris*, metabolised and degraded TFV, undermining its efficacy, while no TFV metabolization was observed with *Lactobacillus iners* or *Lactobacillus crispatus* (Klatt *et al.* 2017). Contrary to this study, others showed that the efficacy of oral TFV/TDF-FTC PrEP drugs were not affected by vaginal microbiota, since no significant difference was observed in women with

predominantly *Lactobacillus* species compared to women with non-*Lactobacillus* species (*G. vaginalis* or *Bacteroides* species) (Heffron *et al.* 2017, Velloza and Heffron 2017). Similarly, a study in young, black South African women showed that shifts in the vaginal microbiota were not impacted by oral PrEP (Mazibuko-Motau *et al.* 2022). These findings suggested that the vaginal microbiota may be less likely to alter the efficacy of oral PrEP (Heffron *et al.* 2017) or that oral PrEP is also less likely to have an effect on the vaginal microbiome. However, the classes of drug administered or applied (oral versus topical vaginal formulations) may dictate the metabolism and effectiveness of drugs (Klatt *et al.* 2017). Together these studies affirm the need for further investigation to better inform on the optimal mode of delivery of PrEP that may confer the maximum benefit.

II. **Sexually transmitted infections (STIs):** are associated with increased acquisition of HIV by increasing the permeability of mucosal barriers leading to genital inflammation and the recruitment and activation of immune cells. These immune cells in turn are highly susceptible to HIV infection and replication, in the FGT (Kalichman *et al.* 2011, Passmore *et al.* 2016). HIV co-infection with other STIs pathogens (namely *Gonorrhoea, Chlamydia, Trichomoniasis* and *Syphilis*) also increases infectiousness and onward transmission thus making treatment for HIV challenging (Kalichman *et al.* 2011). Masese *et al.* (2015) showed that in high-risk Kenyan women, increased HIV acquisition was significantly associated with the pre-existing STI herpes simplex virus type 2 (HSV-2) (Masese *et al.* 2015). Increased genital tract pro-inflammatory cytokines IL-12, IL-10, TNF-α and IFN-γ were also observed with *Chlamydia, Gonorrhoea* and *Trichomoniasis* infections in women from India and South Africa (Reddy *et al.* 2004, Masson *et al.* 2014). Similar findings were shown when cervicovaginal epithelial cell lines from the ectocervix, vagina and endocervix were infected with the STI *Neisseria gonorrhoeae*, resulting in the stimulation of the genital tract pro-inflammatory cytokines IL-6 and IL-8 (Fichorova *et al.* 2001). Due to unprotected sexual activities, a high prevalence of STIs was also reported in high-risk African women participating in the Partners PrEP trial (Baeten *et al.* 2012) and in the previously terminated clinical trials VOICE (Marrazzo *et al.* 2015) and FEM-PrEP trial (Van Damme *et al.*

2012). This data emphasises consequences related to the presence of STIs which included the obstruction of HIV prevention interventions in the mucosal barriers of the FGT in HIV uninfected individuals and the high prevalence of STIs and HIV co-infection in HIV infected individuals.

III. **Exogenous hormonal contraceptives (HCs):** are the most commonly used methods for family planning globally, since young women of reproductive age are most prevalent to HIV infections and unintended pregnancies, the concurrent use of ARVs with HCs has become increasingly common in many SSA countries (Thurman *et al.* 2014, Scarsi *et al.* 2016). Studies examining the association between HCs use and the increased risk of HIV acquisition have varied (Myer *et al.* 2007, Morrison *et al.* 2012). Studies conducted on at-risk South African women using oral norethindrone enanthate (NET-EN) or DMPA showed no association with HIV acquisition (Myer *et al.* 2007, Morrison *et al.* 2012), while other studies demonstrated a higher two-fold increased risk of HIV acquisition (Heffron *et al.* 2012, Hapgood 2020). High-risk women from Kenya using DMPA were also shown to be at a significantly high risk of HIV acquisition (Baeten *et al.* 2007). The Evidence for Contraceptive Options and HIV Outcomes (ECHO) study also evaluated the risk of HIV infection and the use of hormonal contraceptive [intramuscular depot medroxyprogesterone acetate (DMPA-IM), copper intrauterine device (Cu-IUD) and levonorgestrel (LNG) implant] in HIV negative women from high HIV incidence areas in eSwatini, South Africa, Kenya and Zambia (Hofmeyr *et al.* 2017). However, results from this study showed no evidence of increased risk for HIV acquisition with all three methods (Hofmeyr *et al.* 2017). A sub-study of the ECHO further looked at the association between these contraceptives and the induction of cervical Th17-like cells, which are preferred targets for HIV due to their cell surface makers (Bunjun *et al.* 2022). Findings from this study showed that after one-month women assigned DMPA-IM had significantly higher Th17-like cells and a higher population of Th-17 cells co-expressing CCR5, CD38+, α4β7 and α4β7 receptors/proteins, when compared to women assigned CU-IUD and LNG. However, the accumulation of Th17-like cells in women assigned DMPA-IM was also associated with promoting mucosal barrier function. These data suggest that the accumulation of these Th17-like cells in the cervix did not

show a detrimental association simply due to these cells playing a role in maintaining mucosal barrier integrity. Furthermore there was no associations found between vaginal microbial dysbiosis and Th17-like cells mucosal barrier proteins (Bunjun *et al.* 2022). Deese *et al.* (2015) and Baeten *et al.* (2007) also showed high levels of genital tract pro-inflammatory cytokines MIP-1α, MIP-1β, IP-10, IL-6, IL-8 in women from the FEM-PrEP trial assigned TDF-FTC using long-acting injectable progestin-only DMPA, which could affect PrEP efficacy (Baeten *et al.* 2007, Deese *et al.* 2015).

The binding and activation of nuclear (steroid) receptors by certain hormonal contraceptives afford these receptors the potential to regulate drug transporter gene expression, which could result in low drug efficacy (Schindler *et al.* 2008, Hu *et al.* 2015). In mouse cervicovaginal tissues, Zhou *et al.* (2016) demonstrated that exogenous hormones- DMPA increased the mRNA expression levels of efflux drug transporters P-gp, BCRP and MRP-4. In the same study, endogenous hormones in the pregnant mare's serum gonadotropin (PMSG) decreased mRNA expression levels of these drug transporters (Zhou *et al.* 2016). The study attributed these findings to the specific interaction between certain HCs and nuclear receptors. However, these findings still need to be validated using appropriate experimental models (Zhou *et al.* 2016). Thurman *et al.* (2014) also previously noted that since both ARVs and HCs are transported to various compartments by similar or the same drug transporters, the role of HCs on the expression of drug transporters may lead to transporter-mediated drug-to-drug interactions, resulting in low drug efficacy. However, the exact mechanisms of how HCs may modify drug levels needs further elucidation (Thurman *et al.* 2014).

1.7.2.2 Role of inflammation-induced cytokines, in modulating drug transporter expression and function

The impact of inflammation on drug transporter expression and function has been examined in tissues of the intestines, kidneys, and blood-brain barrier (Petrovic *et al.* 2007, Saib and Delavenne 2021). Despite the lack of data regarding the direct mechanisms involved; inflammation-mediated changes in drug transporter expression

and function have been implicated in significantly impacting drug pharmacokinetics (Petrovic *et al.* 2007, Cressman *et al.* 2012). In an *in vitro* study, human brain cell lines (hCMEC/D3) treated with IL-6 and IL-1β, resulted in the downregulation of BCRP and P-gp expression levels (Poller *et al.* 2010). Additionally, the induction of IL-6 and IFN-γ on primary human hepatocytes was also shown to downregulate the mRNA expression levels of the efflux drug transporters BCRP, MRP-2, and MRP-3 and influx/uptake drug transporters OATP-2B1, OATP-1B1, OATP-1B3 (Le Vee *et al.* 2009, Le Vee *et al.* 2011). Previous studies corroborated similar findings of inflammation IL-6 induced downregulation of P-gp expression on rat hepatocytes and human hepatoma cell lines (Sukhai *et al.* 2000). Similarly human cell line Caco-2 pre-treated with TNF-α significantly decreased intestinal P-gp expression, while IFN-γ had no effect (Belliard *et al.* 2004). In rats with endotoxemia, high levels of IL-6 and IL-1β reduced the mRNA expression levels of P-gp and MRP-2 in intestinal tissues (Arana *et al.* 2017). This lipopolysaccharide-induced endotoxemia in rats model showed that there was IL-1β induced downregulation of MRP-2 in enterocytes (Arana *et al.* 2020). These various cellular and small animal models demonstrate how infection and inflammation-induced cytokines can modulate drug transporter disposition. The caveat to the methods used in these models is that mRNA expression levels may not directly reflect functional proteins expressed. Future investigations are therefore required and should include both mRNA expression to its corresponding protein. There are also other biological factors related to inflammation that could also affect drug transporter disposition.

1.7.3 Role of Toll-like receptors (TLRs) and pH in modulating drug transporter expression and function:

1.7.3.1 Toll-like receptors (TLRs)-induced inflammation

TLRs are pattern recognition receptors, these receptors recognise pathogen-associated molecular patterns located on various microbes for example Pam3CSK-4 and lipopolysaccharide (LPS) which are TLR-2 and TLR-4 agonists, respectively (Cario 2016, Suzuki *et al.* 2017). TLRs are activated by the binding to their respective agonists. This interaction causes the stimulation of appropriate signalling pathways in innate and adaptive immune cells which then regulate drug transporter expression levels (Cario 2016, Suzuki *et al.* 2017). There is limited data on how TLR mediated changes modify drug

transporter expression and HIV risk. However, progression of atherosclerosis has been evaluated in the context of TLR-mediated changes on drug transporter expression (Cario 2016, Suzuki *et al.* 2017). To determine which downstream transcriptional signalling pathways were involved in this interaction; *in-vitro* testing using mouse macrophage cell lines (Raw 264.7) stimulated with TLR-2 and TLR-4 agonists Pam3CSK-4 and Lipid-A, respectively; were performed (Suzuki *et al.* 2017). Expression of myeloid differentiation primary-response protein 88 (MyD88), Toll/interleukin-1-domain-containing adapter-inducing interferon β (TRIF), liver X receptors (LXR), interferon regulatory factor 3 (IRF3), and the phosphorylation of nuclear factor *kappa B* (NF-κB) were determined with TLR-2 and TLR-4 activation. These results showed a differential pattern of significantly increased MyD88, LXR and NF-κB expression and low TRIF and IRF3 expression (Suzuki *et al.* 2017). This coincided with the significant upregulation of *ABCA1* expression levels, while ABCG1 expression levels were downregulated. TLR-2 stimulated cells pre-treated with NF-κB and p38 inhibitors MG-132 and SB203580, respectively suppressed the expression of *ABCA1*. These data provided evidence of the sensitivity of drug transporter expression to signal transduction – the MyD88, LXR, NF-κB and p38 pathways. These data provide support to the hypothesis that inflammation modulates the expression of drug transporters which can then lead to disease pathogenesis (Suzuki *et al.* 2017). In addition, TLRs recognise a diverse range of microbial products and are important in immune activation and signalling pathways (Cho *et al.* 2009). The recruitment of the MYD88 protein during the MYD88 dependant pathways has been shown to lead to the production of the pro-inflammatory cytokine TNF-α, through the subsequent activation of TRAF6 and IL-1R-associated kinase (IRAK1) molecules (Cho *et al.* 2009). The TIR-domain-containing adapter-inducing interferon-β (TRIF) pathway also leads to the production of inflammatory cytokines. This occurs through interacting with tumor necrosis factor receptor (TNFR)-associated factor (TRAF)-3 and TRAF6, in which TRAF6 recruits the transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) complex and kinase and the receptor-interacting serine/threonine-protein kinase 1 subsequently inducing inflammatory cytokines and activating the mitogen-activated protein kinase (MAPKS) and NF-kb proteins (Kawasaki and Kawai 2014). Other pathways involved in TLR signalling such as IRF1 and IRF5 have also been shown to induce pro-inflammatory cytokines such as IFN-γ, through interacting with the MYD88 protein (Kawasaki and Kawai 2014).

1.7.3.2 Sensitivity of drug transporter function to pH

The level of acidity or alkalinity (pH) in extracellular fluids is an additional factor that has been shown to modulate drug transporter function (Breedveld *et al.* 2007). The function of the efflux drug transporter BCRP was determined in Madin-Darby canine kidney (MDCK II) cells grown in pH adjusted media and exposed to methotrexate (MTX). At acidic pH levels, the efflux transporter BCRP pumped out MTX more efficiently when compared to physiological and basic pH levels. This data highlighted the possible clinical implications that the function of BCRP is pH-sensitive in the extracellular environment, thereby affecting intracellular concentrations and the effectiveness of MTX (Breedveld *et al.* 2007). These data suggest that pH is an additional factor that can also modulate drug transporter function which can then affect the effectiveness of drugs (Breedveld *et al.* 2007).

Collectively these studies demonstrate how inflammation-induced cytokines and TLRs are involved in regulating the expression of drug transporters in various tissues; subsequently altering intracellular and plasma drug concentrations, thereby affecting drug pharmacokinetics and efficacy. Inflammation mediated changes in drug transporter expression are however mostly based on animal models and cell lines (Cressman *et al.* 2012, Saib and Delavenne 2021), thereby warranting the need for comparative *in-vivo* human studies. Future studies should also elucidate how BV, HC and STIs-induced genital inflammation contribute to drug transporter expression and function, subsequently, predisposing women to HIV infections, even during PrEP intake. Therefore, additional studies are needed to understand the interplay between inflammation and drug transporter expression, especially in sites highly susceptible to HIV such as the FGT and blood. Findings from such studies would provide a better understanding of how the presence of systemic and genital inflammation may alter drug transporters subsequently affecting ARV pharmacokinetics. Further elucidation of these factors either individually or collectively will aid in understanding disparities in PrEP efficacies observed in PrEP trials. This is especially important in highly susceptible groups such as African women from HIV endemic settings where PrEP is advocated as the standard of care for HIV prevention.

Together these studies show evidence that there may be an inextricable link between the expression and function of drug transporters with genetic polymorphisms, TLRs, pH and genital inflammation, which is further influenced by the presence of BV, STIs and HCs

(Figure 1.4). Subsequently these factors may significantly affect drug concentrations and potentially drug efficacies.

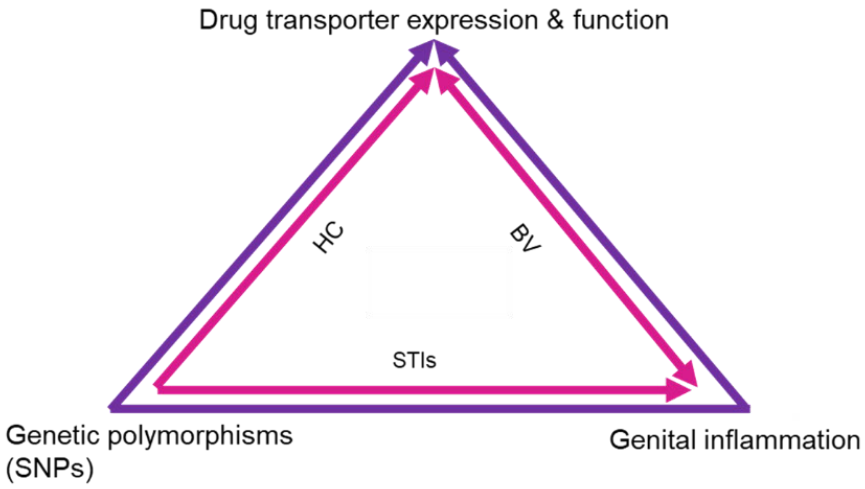

Figure 1.4: Proposed mechanism of effects on drug transporter expression and function.
The schematic shows the intersection of different biological factors and SNPs in drug transporter genes that affect drug transporter expression in the FGT, renal system and blood, subsequently affecting PrEP efficacy. Genital inflammation and SNPs are known to directly affect drug transporter expression and functionality, while the combined use of HCs and ARVs also affects drug transporter expression and function. Additionally, the presence of STIs and BV are shown to contribute to genital inflammation which in turn affects drug transporter expression and function. HC- Hormonal contraceptives, BV- Bacterial vaginosis, STIs- Sexually transmitted infections.

These studies provide evidence that the FGT, renal system and blood are subject to a variety of host biological factors that may undermine PrEP efficacy by affecting drug transporter expression levels and function. These afore-mentioned studies show how drug transporters are increasingly recognised as key determinants in drug pharmacokinetics and response. However, their contributions to the inconsistent efficacies seen in PrEP clinical studies in African women from regions with high HIV infection rates such as South Africa, have not been elucidated. Characterising the expression level of drug transporters in the blood and FGT from a vulnerable population will better define the biological factors underlying compartment variation in drug exposure during oral PrEP in at-risk African women. In turn, we may be able to better understand why African women remain susceptible to HIV despite PrEP interventions. Additionally, findings from such studies will shed an important light on how the genetics and the biology of the mucosal environment may play a pivotal role in modifying drug transporter expression, subsequently modulating HIV risk. Understanding these data may also aid in the development of more effective,

safe and optimal delivery systems that facilitate consistent effective dosage and usage of appropriate PrEP drugs.

In accordance with the University of KwaZulu-Natal guidelines for submitting a thesis via publication part of this literature review was published in *Frontiers in Genetics* on the 29th of September 2022 entitled: **"*Pharmacogenomics of drug transporters for antiretroviral long-acting pre-exposure prophylaxis for HIV*".** (2022). **Zondo NM**, Sobia P, Sivro A, Ngcapu S, Ramsuran V, Archary D. *Frontiers in Genetics*. 13:940661. *https://doi: 10.3389/fgene.2022.940661*. (See Appendix for full review paper- page 114).

1.8 Brief overview of study aim, design and methodologies

1.8.1 Hypothesis

We hypothesized that differential mRNA expression levels of efflux and influx drug transporter genes, SNPs in drug transporter genes and genital inflammation modify the mucosal environment of the FGT and the peripheral blood in African women from South Africa (KZN) taking oral PrEP (Truvada®) by directly or indirectly affecting drug delivery and absorption, leading to ineffective PrEP.

1.8.2 Aim

The overall aim of the study was to determine if biological factors (drug transporters, SNPs in drug transporter genes and genital inflammation) affect PrEP efficacy in African women from South Africa (KZN) taking oral PrEP (Truvada®).

Objectives

I. To determine if there is an association between the presence of SNPs in drug transporter genes and circulating plasma TDF-DP drug levels in African women taking PrEP.
II. To determine if there are differential drug transporter mRNA expression levels between the FGT (cytobrush) and blood (buffy coats) compartments in African women taking PrEP.

III. To determine if there is an association between drug transporter mRNA expression levels in the blood and circulating plasma TDF-DP drug levels in African women taking PrEP.

IV. To determine the association between:

   a. the presence of SNPs in drug transporter genes and drug transporter mRNA expression levels in the peripheral blood of African women taking PrEP.

   b. genital inflammation and drug transporter mRNA expression levels in the FGT of African women taking PrEP.

1.8.3 Methods and Materials

This retrospective study was granted ethics approval by the University of KwaZulu-Natal Biomedical Research Ethics Committee (UKZN BREC: (BREC/0002195/2020). The QuickExtract™ DNA Extraction Solution was used to extract genomic DNA and RNA from cytobrush (FGT) and buffycoat (blood) samples. SNP genotyping (Chapter 2) was conducted on 393 cytobrush samples using the TaqMan® SNP genotyping assays and TaqMan® genotyping master mix and run in the QuantStudio™ 5 Real-Time PCR System. The following assays were used to determine SNPs in ABC drug transporter genes: *ABCC4* (MRP-4) rs1059751 (assay ID: C___7461507_30); *ABCC4* (MRP-4) rs1751034 (assay ID: C___1901918_30); *ABCC4* (MRP-4) rs3742106 (assay ID: C___8059522_1_); *ABCB1* (P-gp) rs1045642 (assay ID: C___7586657_20); *ABCC1* (MRP1) rs212091 (assay ID: C___1003625_20) and *ABCC2* (MRP-2*)* rs2273697 (assay ID: C__22272980_20). To determine drug transporter mRNA expression levels in the FGT and blood (Chapter 3) quantitative Real-Time PCR in the QuantStudio™ 5 Real-Time PCR System was done on 134 matching FGT, and blood samples collected at baseline, 3 months and 6 months. The SYBR™ Green PCR Master Mix and gene specific primer pairs were used to determine the mRNA expression levels of the following genes: MATE-1, MRP-2, MRP-4, OAT-1 OAT-3 and P-gp. Expression values with only a C_T <35 were considered. Relative mRNA expression levels were normalised using the endogenous reference gene Beta-Actin and calculated using the comparative C_T ($2^{\Delta\Delta t\text{-}Ct}$) method. Cytokines concentrations were measured in 134 Soft Cup FGT samples using the Bio-Plex Pro Human Cytokine/Chemokine Magnetic Bead 27-Plex Panel. The Bio-Plex Manager software

version 6 was used to collect data, and the sample concentrations were calculated from standard curves by using a five- parameter (5PL) regression formula.

1.8.4 Statistical Analysis

Pearson's Chi-squared and Fisher's exact tests in the R statistical software (R Foundation for Statistical Computing, Vienna, Austria) were used to examine differences in demographic characteristics between participants. Linear mixed models were used to measure the associations between SNPs, drug transporter mRNA expression and logged plasma TDF-DP drug levels. The same models were used to determine the association between drug transporter mRNA expression and inflammation in the FGT. These analyses were performed in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Spearman's rank correlation coefficient was used to determine correlations between drug transporter mRNA expression levels in the FGT and blood. GraphPad Prism version 9.3.1 software for windows (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis and graphical representation. For all analyses p-value ≤0.05 was considered significant.

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2. CHAPTER TWO

2.1 Bridging Chapter

Besides PrEP drug adherence, single nucleotide polymorphisms (SNPs) also affect drug transporter expression levels and function. Therefore, SNPs have been implicated as key regulators of different antiretroviral drugs used as PrEP or treatment. Truvada® (a combination of tenofovir and emtricitabine) is the standard of care for HIV prevention in South Africa. Data from several studies underscore the importance of understanding how the presence of SNPs in drug transporter genes affect mRNA expression levels and the efficacy of ARVs containing tenofovir. In this study, we characterised SNPs in drug transporter genes in DNA samples from 393 black South African women taking oral PrEP (Truvada®) and determined their effects on circulating plasma tenofovir levels. In addition, the effect of these SNPs on mRNA expression levels was assessed. This study is important since SNP frequency and effects on drugs disposition and mRNA expression remain poorly characterised and understudied in African populations when compared to European and Asian populations. This paper, entitled: "***Single-nucleotide polymorphisms in ABC drug transporters alter expression and circulating tenofovir in healthy South African women exposed to pre-exposure prophylaxis***. (2023). **Zondo NM**, Sobia P, Sivro A, Ngcapu S, Mansoor LE, Mahomed S, Lewis L, Ramsuran V, Archary D. *Pharmacogenomics*. Jul;24(11):599-613. doi: 10.2217/pgs-2023-0058. Epub 2023 Jul 28. PMID: 37503696" has been published in the Future Medicine- *Pharmacogenomics Journal*. We identified SNPs that could potentially be the most clinically relevant in the pharmacokinetics of tenofovir in African women taking PrEP. Findings from this study support the concept of tailoring PrEP dosage to account for the presence of SNPs in drug transporter genes in order to increase PrEP effectiveness in at-risk African populations.

Research Article

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Pharmacogenomics

Single-nucleotide polymorphisms in ABC drug transporters alter expression and circulating tenofovir in healthy South African women exposed to pre-exposure prophylaxis

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Aim: We investigated if single-nucleotide polymorphisms (SNPs) in ATP-binding cassette (ABC) drug transporters alter gene expression and tenofovir disposition in South African women taking Truvada® for HIV prevention. **Materials & methods:** In 393 women, real-time PCR was used to determine the associations between six SNPs in ABC transporter genes, mRNA expression and circulating-tenofovir. **Results:** Univariable and multivariable analyses showed that CT and TT relative to CC genotypes for the *ABCC4*(3463C/T) SNP had significantly higher tenofovir levels. In contrast, the AA genotype for the *ABCC4*(4976A/G) SNP showed significantly less tenofovir, while mRNA expression was increased. **Conclusion:** SNPs in the *ABCC4* gene may differentially affect gene expression and circulating tenofovir. Their impact may inform on low pre-exposure prophylaxis efficacy and discern effective drugs in clinical trials of African women enriched for certain genotypes.

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Clinical trials testing oral Truvada® (tenofovir-disoproxil-fumarate [TDF]/and emtricitabine [FTC]) or topical (1% tenofovir [TFV] gel) formulations as pre-exposure prophylaxis (PrEP) in African women have produced inconsistent levels of protection against HIV, ranging from -49% to 14.5% and leading to many trial terminations [1–3]. The CAPRISA 004 1% tenofovir gel was the first trial to show 39% efficacy against HIV acquisition in African women [4]. These inconsistencies in results of PrEP clinical trials were primarily attributed to varying levels of adherence [3,5]. PrEP effectiveness is also dependent on adequate drug absorption and blood cellular delivery by drug transporter proteins in the female genital tract (FGT) and the peripheral blood [6]. Host biological factors that affect drug transporter expression levels and function such as single-nucleotide polymorphisms (SNPs) have been implicated as regulators of different antiretrovirals (ARVs) [7,8]. This association could, therefore, be applied to PrEP to understand how SNPs in drug transporter genes may contribute to the variability in PrEP effectiveness observed in previous clinical trials.

TDF, a nucleotide reverse-transcriptase inhibitor, is a prodrug of tenofovir [9,10]. HIV reverse transcriptase is inhibited by the active analogue tenofovir-diphosphate (TFV-DP), which is produced when intracellular phosphorylation of absorbed tenofovir occurs [9]. Tenofovir absorption into kidney tubular cells and secretion to the tubular lumen is facilitated by different influx solute carrier (SLC) and efflux ATP-binding cassette (ABC) drug transporter proteins [9]. MRP-2 and MRP-4 – encoded by the *ABCC2* and *ABCC4* genes, respectively – are key in tenofovir

Future Medicine

secretion across the luminal surface of renal proximal tubular cells [11]. Understanding factors that can affect drug transporters may help to define the pharmacokinetics of ARVs containing tenofovir relative to circulating tenofovir concentrations [12].

Genetic variations such as SNPs are known modulators of ABC (efflux) drug transporter gene expression and function [12]. SNPs have been previously shown to modulate drug transporter function by affecting protein folding, RNA transcription, mRNA expression levels and protein function, as well as their ability to bind substrates and regulate drug pharmacokinetics [12,13]. Together these factors converge, leading to varied drug levels and adverse reactions in individuals taking the same drugs [7,14]. Arruda *et al.* [7] showed a high intolerance to either lopinavir-, atazanavir-, ritonavir- or indinavir-containing regimens in HIV-infected Brazilian patients with variations in the *ABCC2* gene (rs3740066 and rs4148396), possibly leading to ineffective treatment outcomes [7].

The effects of SNPs in ABC drug transporter genes expressed in kidney renal tubules and peripheral blood mononuclear cells (PBMCs) relevant to tenofovir pharmacokinetics have been evaluated in several studies [9,15–17]. Certain SNPs in ABC genes have been shown to increase plasma tenofovir through altered drug transporter gene expression or function in renal tubules [9,16]. These studies showed that more drug was effluxed from cells and there was reduced glomerular filtration critical for tenofovir renal clearance, subsequently leading to higher plasma tenofovir [9,16]. In PBMCs, reduced P-gp expression was associated with the C allele on the *ABCB1* 3435C>T rs1045642 SNP in HIV-infected Brazilian and White populations [18]. In Asian [16,19] and White [20] populations, the CC genotype for the *ABCC2* (encoding MRP-2) 224C>T rs717620 SNP was associated with higher tenofovir plasma levels when compared with other genotypes, increasing the risk of developing tenofovir-induced kidney tubular dysfunction (KTD) [16,19,20]. Increased plasma tenofovir drug levels were also associated with the presence of other SNPs, such as the TG or GG genotypes for the *ABCC4* 4131T<G rs3742106 SNP in a Thai population [9]; the C allele for the *ABCC4* 4976T>C rs1059751 SNP [21]; and the GG genotype for the *ABCC4* 3436A>G rs1751034 SNP in White populations [17]. These studies proposed that certain polymorphisms in these drug transporter genes may alter gene expression or function, subsequently leading to more effluxed drug. These SNPs may result in higher plasma concentrations, toxicity, adverse side effects and reduced ARV efficacy, allowing the identification of populations who are at a higher risk of developing adverse effects owing to these SNPs. Importantly, African populations with the highest burden of HIV remain poorly characterized with regard to the effects of SNPs on ARVs compared with European and Asian populations [22].

Previous studies have highlighted inter-ethnic variability in the *ABCB1* 3435C>T SNP; these studies reported a significantly higher frequency of the C allele or CC genotype in African populations [23,24]. This contrasted with Asian and European populations, which exhibited high frequencies for the CT and TT genotypes for this SNP [25,26]. Among African populations, the frequency and functional effects of the *ABCB1* 4036G/G rs3842 SNP has also shown variability [27–29]. In HIV-infected Ugandan, Ethiopian and Tanzanian populations, high efavirenz plasma concentrations were associated with the GG genotypes [27,28], while in an HIV-infected South African population this genotype was associated with decreased efavirenz plasma concentrations [29]. Variability in SNP frequencies, functional effects between African and non-African populations, and genetic diversity among African populations warrants more comprehensive investigations. These may aid in understanding the role of SNPs on drug pharmacokinetics in African populations [24].

Collectively these data underscore the importance of understanding how the presence of SNPs may affect drug transporter expression and in turn the efficacy of ARVs containing tenofovir, especially in vulnerable African populations. In addition, noticeable inter-ethnic and intra-population genetic diversity reflects variability in ARV therapy outcomes, which may inform PrEP efficacy. This study aimed to determine the frequency of SNPs in ABC drug transporter genes relevant in tenofovir pharmacokinetics and investigate their role in potentially altering mRNA expression and plasma tenofovir drug levels in healthy South African women taking oral Truvada as PrEP.

Materials & methods

Study population, design & procedures

This retrospective study used samples collected from the CAPRISA 082 observational study [30]. Briefly, the study included healthy, sexually active black African women aged 18–30 years old from eThekwini (an urban area) and Vulindlela (a rural area) in South Africa. At enrolment and at each follow-up visit rapid HIV tests were done and women were offered oral PrEP. Women who elected to take PrEP were given Truvada, a combination of 300 mg TDF and 200 mg FTC. Blood (buffy coats) and genital specimens from the FGT (cytobrushes) were collected and stored every 3 months for all women who were enrolled. Plasma TFV-DP drug levels (fmol/punch) were

measured from dried blood spots in selected participants who had received oral PrEP. To measure drug levels, a modified liquid chromatography tandem mass spectrometry assay (Division of Clinical Pharmacology, University of Cape Town, Cape Town, South Africa) was used [31]. Adherence to oral PrEP was measured using pharmacy pill count, as described in Mansoor *et al.* [30]. The sub-study was reviewed and approved by the Biomedical Research Ethics Committee (BREC) at the University of KwaZulu-Natal (BREC/0002195/2020).

Selection of single-nucleotide polymorphisms

Previous literature [9,11,19,32] was used to select SNPs in ABC drug transporter genes that are relevant in the pharmacokinetics of tenofovir. Criteria for SNP selection was based on genes that encoded proteins involved in the pharmacokinetics (absorption, distribution, metabolism and excretion [ADME]) of ARVs that could affect drug disposition, bioavailability and treatment response. Furthermore, tenofovir had to be substrates of these drug transporters with selected SNPs. SNPs that previously showed the highest levels of significance in White and Asian populations when associated with altering drug transporter expression levels and function were included. In addition, SNPs that affected drug transporter mRNA expression levels, protein function and plasma tenofovir drug levels [9], and led to drug toxicity [32], tenofovir-induced KTD [11,33] and intolerance to therapy [7] in different ethnicities were included. Initially over 20 SNPs were selected; however, this number was narrowed down to six ABC SNPs that were the most significantly associated with affecting tenofovir drug levels when used as treatment or for prevention [9,19,21,32,34]. These six SNPs in ABC drug transporter genes included: *ABCB1* (encoding P-gp) 3435G>A rs1045642 SNP; *ABCC1* (encoding MRP1) 198217T>C rs212091 SNP; *ABCC2* (encoding MRP-2) 1249G>A rs2273697 SNP; *ABCC4* (encoding MRP-4) 3463T>C rs1751034 SNP; *ABCC4* (encoding MRP-4) 4131A>C rs3742106 SNP; and *ABCC4* (encoding MRP-4) 4976A>G rs1059751 SNP.

Genomic DNA & RNA extraction & cDNA synthesis

Genomic DNA was extracted from all cytobrush samples and RNA was extracted from matching cytobrush and buffy coat samples using the QuickExtract™ DNA Extraction Solution (lysogenic buffer; Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Briefly, either genomic DNA and RNA was extracted from the resuspended pellet post centrifugation using the QuickExtract DNA Extraction Solution and incubated in the Applied Biosystems SimpliAmp Thermal Cycler (Thermo Fisher Scientific, MA, USA). Genomic DNA and RNA concentrations were determined using the Nanodrop system (Thermo Fisher Scientific). DNA was treated with RNase, while RNA samples were treated with DNase (Agilent Technologies, CA, USA). Genomic DNA was standardized to 20 ng in nuclease-free water and stored at -20°C until further use in SNP genotyping, while RNA was standardized to 50 ng in nuclease-free water and cDNA synthesized using the SuperScript™ Vilo™ cDNA synthesis Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Briefly, standardized RNA was mixed with the Vilo reaction mix and SuperScript enzyme mix, incubated, and the reaction terminated accordingly. Resulting cDNA was diluted 1 in 5 and used to determine drug transporter mRNA expression levels.

Single-nucleotide polymorphism genotyping

SNP genotyping was conducted using the TaqMan® SNP genotyping assays (Thermo Fisher Scientific), which were run in the QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific). The following assays were used to determine SNPs in ABC drug transporter genes: *ABCC4* (MRP-4) rs1059751 (assay ID: C___7461507_30); *ABCC4* (MRP-4) rs1751034 (assay ID: C___1901918_30); *ABCC4* (MRP-4) rs3742106 (assay ID: C___8059522_1_); *ABCB1* (P-gp) rs1045642 (assay ID: C___7586657_20); *ABCC1* (MRP1) rs212091 (assay ID: C___1003625_20); and *ABCC2* (MRP-2) rs2273697 (assay ID: C__22272980_20). The final RT-PCR reaction volume for each reaction was 5.5 µl, consisting of: 20 ng of genomic DNA, TaqMan Genotyping Master Mix (Thermo Fisher Scientific) and a 20× drug-metabolizing genotype assay mix (this comprised a specific gene primer-probe; Thermo Fisher Scientific). The RT-PCR conditions were as follows: initial denaturation for 95°C for 10 min, followed by 40 cycles of denaturation at 92°C for 15 s and annealing for 60°C for 1 min using the RT-PCR system.

Quantitative RT-PCR mRNA expression

To conduct quantitative RT-PCR, gene-specific primers pairs and the SYBR Green PCR Master Mix were used (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, synthesized cDNA (diluted 1 in 5) was mixed with gene-specific primer pairs and the SYBR green master mix. Selected gene-specific primers (5′-3′ direction) included: P-gp (forward CCCATCATTGCAATAGCAGG, reverse TGTTCAAACTTCTGCTC-

CTGA); MRP-2 (forward TAATGGTCCTAGACAACGGG, reverse GGGCCTTCTGCTAGAATTT); MRP-4 (forward GGACAAAGACAACTGGTGTGCC, reverse AATGGTTAGCACGGTGCAGTGG); and the housekeeping gene β-actin (forward TCCTTCCTGGGCATGGAGT, reverse AGCACTGTGTTGGCGTACAG). Optimal conditions: denaturation at 95°C for 15 s, 40 cycles, annealing at 60°C for 1 min and extension at 72°C for 30 s were used. All reactions were conducted using the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific). Only expression with C_T <35 values were considered in the expression data. Relative mRNA expression levels of target genes were calculated using the comparative C_T ($2^{\Delta\Delta t\text{-}Ct}$) method, expression was normalized using the endogenous reference gene β-actin [35].

Minor allele frequencies

Criteria for studies included in minor allele frequency (MAF) analysis included studies of SNPs that have a functional effect on the drug transporter genes, which subsequently led to certain consequences, as stated in the 'Selection of SNPs' section above. In addition, these studies had to be evaluated in different ethnicities and most of these studies were evaluated in SNPs of drug transporter genes involved in ARVs pharmacokinetics (ARVs used for treatment or prevention, a few studies evaluated the impact of SNPs in other diseases such as cancer).

Statistical analyses

Pearson's Chi-squared and Fisher's exact tests using R statistical software (R Foundation for Statistical Computing, Vienna, Austria) were used to examine differences in demographic characteristics between participants that initiated and those that never initiated PrEP. Linear mixed models were used to measure the associations between SNPs and logged plasma tenofovir drug levels using a subsample of women who had sufficient pills to cover them during visits (i.e., medication possession ratio = 100%) and who had detectable drug levels. Pill count adherence was not well correlated with drug levels [30] and so could not be incorporated as a variable in regression analysis. We excluded participants with undetectable drug levels to remove those with poor adherence from analysis. The analysis adjusted for BMI and time since study enrolment, and was performed in SAS version 9.4 (SAS Institute Inc., NC, USA). For these analyses a p-value <0.05 was considered significant. The Kruskal-Wallis test with a Dunn's multiple comparison test was used to determine the associations between SNP genotypes and mean mRNA expression levels in cytobrush and buffy coats samples on GraphPad Prism version 9.3.1 software for Windows (GraphPad Software, CA, USA). Median mRNA expression was calculated using measurements from baseline, and 3 and 6 months. A two-tailed adjusted p-value <0.05 was considered significant. For all graphical representation, the GraphPad Prism was used.

Results

Sample description

Of the 429 women enrolled in the study, 413 had stored cytobrush (FGT) samples at baseline (Figure 1). A further 23 participants' cytobrush samples were excluded due to no informed storage and future use consent from participants (n = 7), poor DNA quality (negative DNA concentrations; n = 4) and undetermined SNP genotypes (n = 12). This provided a total of 393 participants with cytobrush samples from which SNP genotyping data were obtained. These 393 cytobrush samples were from 148 participants who did not initiate PrEP and 245 participants who volunteered to take oral PrEP. Of the 245 participants who initiated oral PrEP, 92 had plasma drug levels measured during follow-up. A further 25 participants were excluded from analysis because they had undetectable drug levels or did not have sufficient oral PrEP to last them between visits (i.e., medication possession ratio <100%), leaving 67 participants for analysis (this is the subset of participants with available drug levels at different time points). Of these 67 participants, 45 participants had matching cytobrush and buffycoat samples at baseline (45 samples each), 3 months (44 samples each) and 6 months (45 samples each). This provided a total of 134 cytobrush and buffy coat (blood) samples each from which mRNA was extracted and included in mRNA expression experiments and analysis.

Demographics table

Table 1 describes the demographic and clinical data of 148 (38%) participants who did not initiate PrEP and 245 (62%) participants who volunteered to take PrEP. There were no significant differences between those who initiated PrEP and those who did not initiate PrEP for age, BMI and prevalence of *Chlamydia trachomatis* and *Trichomonas vaginalis* at enrolment. At enrolment, the prevalence of *Neisseria gonorrhoea* was significantly higher in

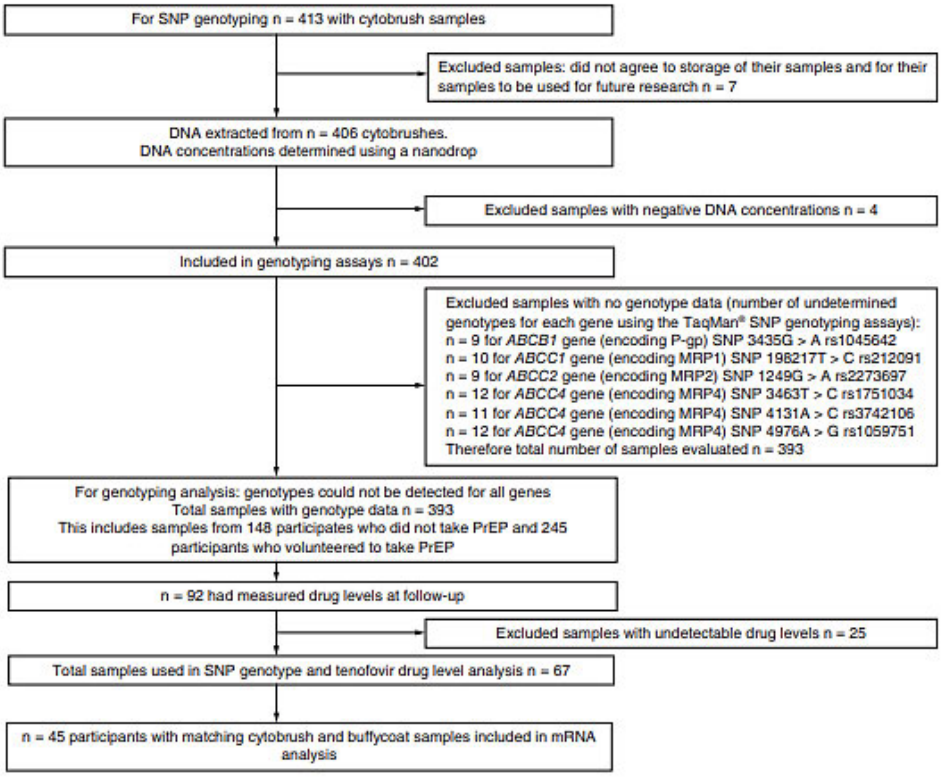

Figure 1. Sample description diagram. Description of the number of included and excluded cytobrush samples in this cohort study. The total number of cytobrush samples included in SNP genotyping (n = 393) and tenofovir drug analyses (n = 67) is shown. The total number of participants (n = 45) with matching cytobrush and buffycoat samples included in the mRNA expression experiments provided a total of 134 cytobrush and buffycoat samples each over several follow-up visits.
PrEP: Pre-exposure prophylaxis; SNP: Single-nucleotide polymorphism.

women who initiated PrEP (p = 0.016) compared with women who did not. Significantly more participants who initiated PrEP were from the Vulindlela site than those at the eThekwini site (p = 0.001). Among women who initiated PrEP, 67 (17%) had detectable plasma tenofovir levels. Of these 67 women, 45 (11.45%) were included for the mRNA expression analyses as they had samples for both cytobrushes and buffy coats at baseline, 3- and 6-months follow-up.

Minor allele frequencies & genotypes among different ethnic groups

Table 2 shows comparisons of MAF and genotype frequencies of six SNPs previously shown to have a functional effect on drug transporter genes among South African, other African/Afro-descendent, European/Euro-descendant and Asian populations. For the SNPs *ABCB1* 3435G>A (MAF-A allele), *ABCC1* 198217T>C (MAF-C allele) and *ABCC4* 4976A>G (MAF-G allele), variable differences in MAFs were observed when comparing the South African population with other populations. Low allele frequencies of 8%, 11% and 18% were observed for the *ABCB1* 3435G>A (MAF-A allele), *ABCC1* 198217T>C (MAF-C allele) and *ABCC4* 4976A>G (MAF-G allele) SNPs, respectively, in the South African population. For the *ABCC1* 198217T>C (MAF-C allele) similar MAFs were observed between the South African and African/Afro-descendent population. The MAF for the A allele for the *ABCC2* 1249G>A SNP was similar in African and European populations, while for the Asian population a slightly higher MAF of 28% was observed. For the *ABCC4* 3463T>C (MAF C allele) and 4131A>C (MAF

Table 1. Demographic and clinical characteristics of study participants.

Characteristic	Overall (n = 393), n (%)	Initiated PrEP (n = 245), n (%)	Never initiated PrEP (n = 148), n (%)	p-value†
Age category (years)				0.078
≤24	263 (67%)	156 (64%)	107 (72%)	
25–30	130 (33%)	89 (36%)	41 (28%)	
BMI category				0.900
Underweight (0–18.5)	12 (3.1%)	7 (2.9%)	5 (3.4%)	
Normal weight (18.5–25)	140 (36%)	84 (35%)	56 (38%)	
Overweight (25–30)	105 (27%)	67 (28%)	38 (26%)	
Obese (>30)	133 (34%)	85 (35%)	48 (33%)	
STI enrolment				
Chlamydia trachomatis	71 (18.1%)	48 (19.7%)	23 (15.5%)	0.303
Neisseria gonorrhoea	10 (2.5%)	10 (4.1%)	0 (0%)	0.016‡
Trichomonas vaginalis	16 (4.1%)	9 (3.7%)	7 (4.7%)	0.611
Site				0.001‡
eThekwini	156 (40%)	57 (23%)	99 (67%)	
Vulindlela	237 (60%)	188 (77%)	49 (33%)	
Detectable drug levels				
SNP genotyping	67 (17%)	67 (100%)	0 (0%)	
mRNA expression	45 (11.45%)	45 (100%)	0 (0%)	

mRNA expression includes matching cytobrush (female genital tract) and buffy coat samples (blood). From the 67 participants with detectable drug levels, four participants had HIV seroconverted.
†Pearson's Chi-squared and Fisher's exact tests.
‡p-value <0.05 (significant).
PrEP: Pre-exposure prophylaxis; SNP: Single-nucleotide polymorphism; STI: Sexually transmitted infection.

A allele) SNPs, a slightly higher MAF of 32% was observed in the South African population compared to other populations.

Associations between single-nucleotide polymorphisms in ABC drug transporter genes & plasma tenofovir drug levels

Table 3 & Figure 2 illustrate mean plasma tenofovir drug levels over time by genotype and SNP. To determine if there is an association between SNPs and plasma tenofovir levels, regression analyses were carried out within a subset of participants with available drug levels at different time points. In the first analysis, the model was adjusted for time in the study, BMI and each SNP separately, and for the second analysis, the model was adjusted for time in the study, BMI and all SNPs (Table 3). After adjusting additionally for all other polymorphisms, individuals presenting with the CT ($p = 0.002$) and TT ($p = 0.014$) genotypes for the *ABCC4* 3463T>C SNP had two- to three-fold significantly higher plasma tenofovir drug levels when compared with individuals with the homozygous CC reference genotype. For the *ABCC4* 4976A>G SNP, the analysis adjusting for all other SNPs and BMI and time in study showed that individuals exhibiting the AA genotype had a mean plasma tenofovir drug level of 46% less than those in the AG reference genotype ($p = 0.018$). Figure 2 illustrates how the mean plasma tenofovir drug levels varied over time, and we plotted these levels in relation to grouped genotype. No significant associations were observed for *ABCB1* (P-gp) 3435G>A, *ABCC1* (MRP1) 198217T>C, *ABCC2* (MRP-2) 1249G>A and *ABCC4* (MRP-4) 4131A>C rs3742106 SNPs.

Associations between single-nucleotide polymorphism genotypes & mean drug transporter mRNA expression levels in the female genital tract & blood

To determine if there was an association between mRNA expression levels and SNP genotypes, a subset of participants with matching FGT and blood samples at baseline, 3 and 6 months were used (Figure 3). After adjusting for multiple comparisons, differences in mRNA expression levels across SNP genotypes were determined. In the blood for the *ABCC4* 4976A>G SNP, individuals presenting with the AA genotype had significantly increased *ABCC4* mRNA expression levels when compared with individuals with the AG genotype ($p = 0.0132$). However, in cytobrush samples no significant associations between mRNA expression levels and genotypes for

Table 2. Minor allele frequencies and genotypes of single-nucleotide polymorphisms in ABC drug transporter genes among different ethnicities.

Genes		Minor alleles and genotypes	South African (%)	African/Afro-descendent (%)	European/Euro-descendent (%)	Asian (%)	Ref.
ABCB1 (P-glycoprotein) 3435G>A (rs1045642)	MAF	A	8	32.2	47	47	
	Genotypes	AA	1.53	15.1	21	21	
		GG	84.73	34.2	27	28	
		AG	13.74	50.7	52	51	
	Total samples		n = 393	n = 73	n = 263	n = 117	[15,16,36]
ABCC1 (MRP-1) 198217T>C (rs212091)	MAF	C	11	8.2	20	24	
	Genotypes	CC	0.77	1.4	26	6.6	
		TT	78.06	84.9	24	58	
		CT	21.17	13.7	50	35	
	Total samples		n = 392	n = 73	n = 160	n = 500	[15,37,38]
ABCC2 (MRP-2) 1249G>A (rs2273697)	MAF	A	13	15	19	28	
	Genotypes	AA	1.78	3	3	15.8	
		GG	75.32	74	64	57.9	
		AG	22.90	23	33	26.3	
	Total samples		n = 393	n = 209	n = 263	n = 19	[19,36]
ABCC4 (MRP-4) 4131A>C rs3742106	MAF	A	55	62	56	49	
	Genotypes	AA	28.65	37	33	22.7	
		CC	17.90	13	20	24	
		AC	53.45	50	47	53.3	
	Total samples		n = 391	n = 210	n = 265	n = 150	[9,36]
ABCC4 (MRP-4) 3463T>C rs1751034	MAF	C	32	22	25	19.3	
	Genotypes	CC	12.82	7	14.3	2.7	
		TT	48.21	63	64.3	64	
		CT	38.97	31	21.4	33.3	
	Total samples		n = 389	n = 211	n = 42	n = 150	[9,17,36]
ABCC4 (MRP-4) 4976A>G rs1059751	MAF	G	18	–	20	55	
	Genotype	AA	66.92		64	32	
		GG	2.31		4	42	
		AG	30.77		32	26	
	Total samples		n = 390		n = 286	n = 19	[8,37]

MAF: Minor allele frequency.

the *ABCC4* 4976A>G SNP were observed. In addition, no significant associations were observed for the *ABCB1* (P-gp) 3435G>A, *ABCC2* (MRP-2) 1249G>A, *ABCC4* (MRP-4) 3463T>C and *ABCC4* (MRP-4) 4131A>C SNPs.

Discussion

A large number of ABC drug transporters are known to be polymorphic, and these polymorphisms have been shown to affect the pharmacokinetics of tenofovir [20]. In this study, we investigated the frequencies and associations of SNPs in ABC drug transporter genes to understand their impact on plasma tenofovir drug levels and mRNA expression levels of drug transporters in healthy South African women taking Truvada as oral PrEP.

We observed marked variations in MAFs and genotype frequencies in our South African population relative to other populations. These data suggest that certain alleles for the *ABCB1* 3435G>A (MAF-A allele), *ABCC1* 198217T>C (MAF-C allele) and *ABCC4* 4976A>G (MAF-G allele) SNPs occurred at lower frequencies in our South African population when compared with other populations. This inferred that few individuals presented with these rare alleles. In addition, our findings also suggest that the GG/CC genotype for the *ABCB1* 3435G>A SNP occurred at a higher frequency for our population, while the AG genotype occurred at higher frequencies for European and Asian populations. Previous studies have also shown noticeable differences in genotype frequencies for the *ABCB1* 3435C>T SNP in African, Asian and European populations [25,36,39]. The T allele was found to be

Table 3. Associations between single-nucleotide polymorphisms and logged plasma tenofovir drug levels using linear mixed models.

SNP	Genotype	Model 1		Model 2	
		exp(Estimate)[1]	p-value[1]	exp(Estimate)[2]	p-value[2]
ABCB1 (P-glycoprotein)	AA	0.75	0.545	0.5	0.133
3435G>A rs1045642	AG	0.64	0.132	0.62	0.137
	GG	1		1	
ABCC1 (MRP-1)	CT	1.09	0.736	0.89	0.611
198217T>C rs212091	TT	1		1	
ABCC2 (MRP-2)	AG	0.88	0.647	0.92	0.784
1249G>A rs2273697	GG	1		1	
ABCC4 (MRP-4)	AA	1.11	0.761	0.69	0.329
4131A>C rs3742106	AC	1.25	0.469	0.98	0.952
	CC	1		1	
ABCC4 (MRP-4)	CT	2.21	0.020†	2.95	0.002†
3463T>C rs1751034	TT	2	0.024†	2.13	0.014†
	CC	1		1	
ABCC4 (MRP-4)	AA	0.68	0.121	0.54	0.018†
4976A>G rs1059751	GG	0.6	0.212	0.49	0.217
	AG	1		1	

Model 1 (exp 1): Univariable analyses; Model 2 (exp 2): Multivariable analyses.
†p-value <0.05 (significant).

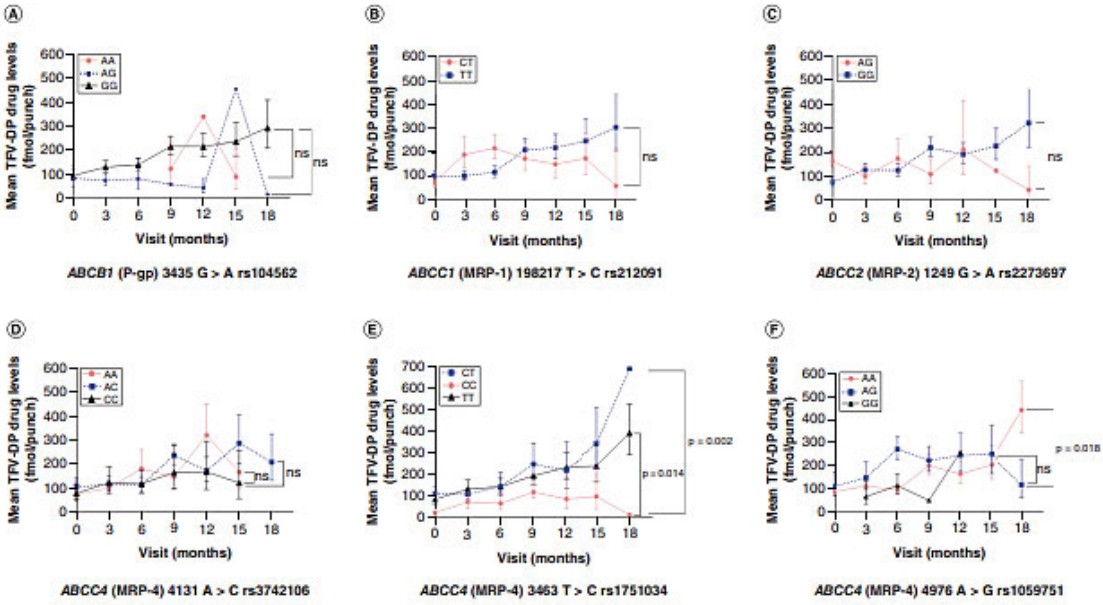

Figure 2. Graphical representation of correlations between mean plasma tenofovir-diphosphate drug levels (fmol/punch) over time, grouped by genotype. Relationship between TFV-DP drug levels over time by genotype of **(A)** *ABCB1* 3435G>A, **(B)** *ABCC1* 198217T>C, **(C)** *ABCC2* 1249G>A and **(D)** *ABCC4* 4131A>C SNPs were not significant. While **(E)** *ABCC4* 3463T>C (CT p = 0.002 and TT; p = 0.014) and **(F)** *ABCC4* 4976A>G (AA p = 0.018) were significant over time. Analysis includes findings after multivariant analysis (model 2). TFV-DP drug levels were measured from 3 months after PrEP initiation. Non-significant data is represented by ns, while significant data is represented by p-values. Mean standard error is represented by error bars.
PrEP: Pre-exposure prophylaxis; TFV-DP: Tenofovir-diphosphate.

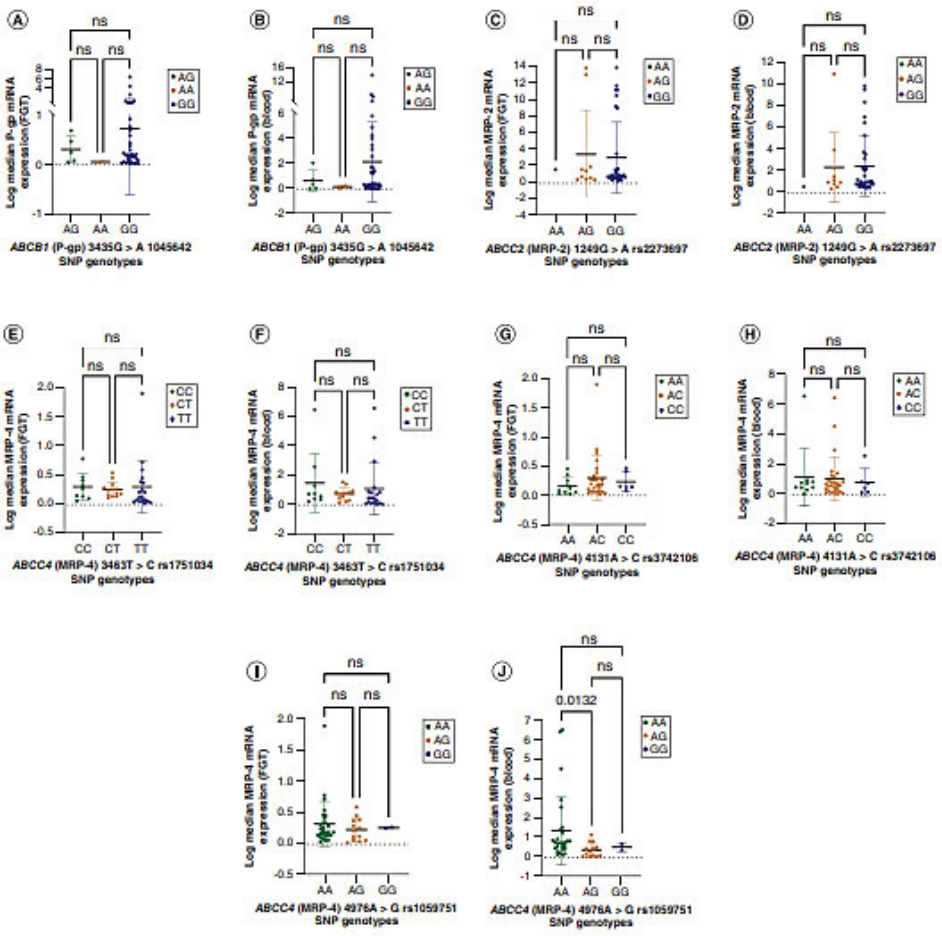

Figure 3. Associations between single-nucleotide polymorphism genotypes and median ABC drug transporter mRNA expression levels in the female genital tract and blood. The relationship between median mRNA expression and SNP genotypes were not significant for the **(A)** *ABCB1* (P-gp) 3435G>A SNP in the FGT; **(B)** *ABCB1* (P-gp) 3435G>A SNP in the blood; **(C)** *ABCC2* (MRP-2) 1249G>A SNP in the FGT; **(D)** *ABCC2* (MRP-2) 1249G>A SNP in the blood; **(E)** *ABCC4* 3463T>C SNP in the FGT; **(F)** *ABCC4* 3463T>C SNP in the blood; **(G)** *ABCC4* 4131A>C SNP in the FGT and **(H)** the *ABCC4* 4131A>C SNP in the blood. **(I)** The *ABCC4* 4976A>G SNP in the FGT was not significant, while in the blood the **(J)** *ABCC4* 4976A>G SNP showed significantly higher mRNA expression for the AA genotype ($p = 0.0132$) relative to the AG genotype. Analyses were done using the Kruskal-Wallis test and included FGT and blood samples collected at baseline, 3 and 6 months from women who initiated PrEP. Non-significant data are represented by ns, while significant data is represented by $p < 0.05$. Mean standard error is represented by error bars.
FGT: Female genital tract; PrEP: Pre-exposure prophylaxis; SNP: Single-nucleotide polymorphism.

rare, while the C allele was found at higher frequencies in populations of African/Afro-descendants compared to European and Asian populations [25]. Hoffmeyer *et al.* previously posited that the high frequency of the C allele or CC genotype for the *ABCB1* 3435C>T SNP in African populations could exert a functional effect on the *ABCB1* gene, resulting in the overexpression of the P-gp protein leading to more drug efflux [39]. Given the high frequency of the GG/CC genotype for the *ABCB1* gene in our cohort, these results further support the notion that this SNP could possibly alter tenofovir pharmacokinetics.

Using MAF analyses, our findings also suggested that the A allele in the *ABCC2* 1249G>A SNP was rare in African/Afro-descendants and European populations compared with Asian populations, while for the *ABCC4* 3463T>C (MAF C allele) and 4131A>C (MAF A allele) SNPs, similar MAFs were found across the populations. Contrary to these findings, a study by da Rocha *et al.* [36] reported that genotype frequencies and MAFs were only significantly different in African and European populations for the *ABCB1* 3435G>A SNP and not the *ABCC2* 1249G>A, *ABCC4* 4976A>G and *ABCC4* 4131A>C SNPs [36]. Collectively, these data highlight that there is variability in MAFs and genotype frequencies among populations, which may result in heterogeneous expression levels, which in turn may affect the function of drug transporter genes and drug levels.

Multivariable SNP analyses in our study suggested significant associations between increased plasma tenofovir drug levels and individuals exhibiting the CC and TT genotypes for the *ABCC4* 3463T>C SNP. Similarly, in an Italian HIV-infected population, the GG genotype for the *ABCC4* 3436A>G SNP was significantly associated with an increased risk of tenofovir-induced KTD in a bivariate analysis [17]. However, in a Thai HIV-infected population no significant association between the *ABCC4* 3436T>C SNP and plasma tenofovir drug levels was observed [9]. Furthermore, Kiser *et al.* showed that for the *ABCC4* 3463T>C SNP, HIV-infected White and African–American individuals with the G allele had higher intracellular drug levels when compared with those with the A allele [40].

For the *ABCC4* 4976A>G SNP, multivariable analyses in our study showed reduced plasma tenofovir in individuals exhibiting the AA genotype compared to those with the AG genotype. In addition, mRNA analyses for the *ABCC4* 4976A>G SNP showed significantly increased mRNA expression of the *ABCC4* gene in individuals with the AA genotype compared to individuals with the AG genotype. It follows that for AA individuals more *ABCC4* gene could be expressed, which may result in increased blood drug levels. However, our analyses for this SNP showed that individuals with the AA genotype presented with significantly less plasma tenofovir. This finding is counterintuitive to our mRNA expression analyses and suggests that other mechanisms or SNPs yet to be classified are at play. Previous studies in HIV-infected Thai [21] and Japanese [19] populations showed that the C allele for the *ABCC4* 4976A>G SNP was associated with increased risk for tenofovir-induced KTD [19,21]. However, similar effects were not observed in our African population. Notably, the A and not the C allele was more frequent in our population, indicating the heterogeneous impact of SNPs on mRNA expression and drug levels [19,21]. In addition, our study was designed to determine SNP frequencies and associations with plasma tenofovir and mRNA expression, and did not account for other biological factors that could affect drug transporter expression. The presence of haplotypes and/or high expression of other influx drug transporters may account for these contradictory findings [41]. We hypothesize that the haplotypes within the *ABCC4* gene and high expression of influx/uptake drug transporters that are tenofovir specific may have counteracted the effects of the *ABCC4* 4976A>G SNP on plasma tenofovir and *ABCC4* mRNA expression, leading to reduced plasma tenofovir [6,19,41–43]. In addition, we did not observe any significant associations for the other SNPs and mRNA expression levels. Other factors besides SNPs could affect drug transporter mRNA expression in these two sites, such as exposure to certain drug in the blood [44] and the presence of genital inflammation in the FGT [45–48].

Furthermore, these SNPs – *ABCB1* 3435G>A, *ABCC1* 198217T>C, *ABCC2* 1249G>A and *ABCC4* 4131A>C – showed no significant associations with increased or reduced plasma tenofovir in our study. Similarly, in Thai [16] and Japanese [19] HIV-infected populations, no associations between the *ABCB1* 3435G>A and *ABCC2* 1249G>A SNPs and high plasma tenofovir or tenofovir-induced KTD were shown [16,19]. However, in an HIV-infected White population, the TT genotype in the *ABCB1* 3435G>A SNP was associated with low mRNA transcription and protein expression, leading to reduced circulating tenofovir [18]. Another study showed significant associations between the TC and CC genotypes for the *ABCC1* 198217T>C SNP and the TT genotypes for the *ABCB1* 3435G>A with virological failure due to suboptimal drug levels in HIV-infected Brazilian participants [15]. However, the direct roles of the *ABCC1* 198217T>C and *ABCB1* 3435G>A SNPs in virological failure have not been clearly defined [15,49]. For the *ABCC4* 4131T>G SNP, Rungtivasuwan *et al.* showed that Thai HIV-infected individuals exhibiting the TG or GG genotypes 'had on average a 30% higher mean plasma tenofovir' compared to individuals with the TT genotype after multivariable adjustment [9]. More recently Cheli *et al.* reaffirmed in an HIV-infected European population the positive association of the G allele in the *ABCC4* 4131T>G SNP with increased plasma tenofovir [32]. These studies therefore suggested that there could be potentially high plasma drug levels in the presence of variants (the G allele) within the *ABCC4* gene, which may alter their gene expression or function [32,50]. However, we did not observe this phenomenon for all four SNPs in our study. Discrepancies in these studies highlight the need to further understand the role of SNPs in drug pharmacokinetics. Such data may aid in identifying populations who are at a high risk of developing adverse reactions and conditions such as

tenofovir-induced KTD while taking ARVs as PrEP or treatment. Our analysis indicates that further studies are warranted to determine the exact mechanisms of how these SNPs affect plasma tenofovir drug levels, especially in African women taking PrEP. More importantly, we need to further discern how these SNPs affect drug levels in the FGT.

Our study has several limitations. First, only ABC drug transporters were evaluated in this study; this limited our ability to compare the possible role of SNPs in SLC drug transporter genes in altering plasma tenofovir. This is particularly important, since tenofovir has been identified as a substrate of SLC drug transporters such as OAT-1 and OAT-3 [51]. Second, the analysis of the association between polymorphisms in drug transporters and plasma tenofovir was based on limited data from 67 women. Some of these women had no plasma tenofovir detectable at certain time points for one or more SNPs. Therefore, the two SNPs, *ABCC4* 3463T>C and *ABCC4* 4976A>G, that we found significant may be tenuously associated with plasma tenofovir. Furthermore, the significant finding of the TT genotype for the *ABCC4* 3463T>C SNP relative to plasma tenofovir at 18 months may be amplified by the lack of individuals with CT and CC genotypes. In our multivariable model, for the *ABCC4* 4976A>G SNP the heterozygous AG genotype was used as the reference genotype based on the frequency of this genotype in our study cohort. When we look at the SNP data relative to the plasma tenofovir, univariable vs multivariable analyses for the *ABCC4* 4976A>G SNP yield seemingly different results. Multivariable analyses would, however, be expected to be different when compared to univariable analyses, since a confounder variable such as adherence that could affect the variables being studies was not included. Last, since the analysis did not adjust for adherence to oral PrEP, which is known to be variable among women, to partially address this we removed participants with undetectable drug levels. We assumed that these participants had poor adherence to oral PrEP at these time points. In addition, although adherence is related to drug level, there is little reason to believe that adherence will be associated with SNP. This bidirectional relationship is required for adherence to be a confounder.

Adherence is significant since poor adherence in women poses a greater risk for HIV acquisition than men who have sex with men. These findings are supported by studies showing that following TDF administration, TFV-DP may reach concentrations that are 100-fold higher in rectal tissues than in vaginal tissues [52–54]. Furthermore, women require six out of seven doses of Truvada per week to be adequately protected [54,55]. These findings highlight that women require consistent use of oral PrEP to confer similar protection [52–56]. Collectively, these findings indicated that concentration thresholds for tenofovir that correlate with high levels of protection against HIV differ significantly for men compared to women.

Despite these limitations, to our knowledge this is the first study to identify SNP frequencies and elucidate their possible impact on both mRNA expression and plasma tenofovir in healthy South African women taking Truvada. This study also identified specific clinically relevant SNPs in tenofovir disposition, which could be used to tailor PrEP to limit high plasma tenofovir drug levels and ensure effective PrEP. In addition, the study cohort included only African women from the same country and region. This is especially important, since previous studies reviewed by Rajman *et al.* and Zondo *et al.* highlighted that there is high genetic diversity among African populations regarding the functional effects of SNPs on various genes including drug transporters [24,57]. Furthermore, these SNPs were evaluated in a high-risk homogenous population of women from an HIV hyperendemic region of South Africa, making our findings highly relevant. We also showed data for two compartments, the blood and the genital tract across time. This may help us further define if other epigenetic factors such as inflammation in the local milieu and drug levels can directly impact mRNA expression. Our significant findings could also be used to some extent to explain why certain women seroconverted while taking PrEP in our study [30], underscoring the importance of understanding how SNPs in drug transporter genes impact PrEP efficacy.

Conclusion

In conclusion, our study provided two important findings. First, we identified two SNPs in the *ABCC4* gene that may affect circulating tenofovir levels and one SNP that could alter mRNA gene expression in healthy South African women taking oral PrEP. These SNPs may play an integral role in low PrEP efficacies within this population. Second, four SNPs were not significantly associated with mRNA expression levels and circulating plasma tenofovir in our population. These findings further highlight the need to identify and investigate the role of other SNPs within the gene region of these drug transporters that may predispose African populations to insufficient drug levels. Understanding the role of SNPs in tenofovir disposition is important, especially since Truvada is the current standard of care for HIV prevention for at-risk African women. In addition, the possibility of conducting further research to investigate the role of other SNPs within the gene region of these drug transporters that

may predispose other populations to insufficient drug levels should be explored. These pharmacogenetic findings may therefore contribute to the identification of SNPs that are clinically relevant in tenofovir pharmacokinetics and can be used when tailoring PrEP drug dosages for African women. This precision medicine approach could potentially reduce the risk of HIV acquisition and improve health outcomes.

Summary points

- We investigated if single-nucleotide polymorphisms (SNPs) in ABC drug transporter genes alter their mRNA gene and circulating tenofovir in South African women taking the oral pre-exposure prophylaxis (PrEP) drug Truvada®.
- The association between six SNPs – *ABCB1* (*P*-gp) (3435A/G); *ABCC1* (MRP-1) (198217C/T); *ABCC2* (MRP-2) (1249A/G); *ABCC4* (MRP-4) (3463C/T); *ABCC4* (MRP-4) (4131A/C) and *ABCC4* (MRP-4) 4976A/G) – previously associated with tenofovir pharmacokinetics and HIV, mRNA expression P-gp, MRP-2 and MRP-4 and circulating tenofovir was determined using quantitative RT-PCR.
- For the six SNPs evaluated, we observed some degree of minor allele frequency variation between SNPs in our South African population compared with other ethnicities (African-/Afro-descendants, European populations and Asian populations).
- Among the six SNPs evaluated, both univariable and multivariable analyses showed that individuals with the CT ($p = 0.020$) and TT ($p = 0.024$) genotypes (univariable analyses) and the CT ($p = 0.002$) and TT ($p = 0.014$) genotypes (multivariable analyses) for the *ABCC4*(3463C/T) SNP had significantly higher plasma tenofovir (two- to threefold) relative to individuals with the CC genotype.
- In contrast, multivariable analyses for the *ABCC4* (4976A/G) SNP showed that individuals with the AA genotype had significantly less plasma tenofovir ($p = 0.018$) compared with individuals with the AG genotype.
- The *ABCC4*(4976A/G) SNP in the blood also showed significantly increased mRNA expression for individuals with the AA genotype compared with those with the AG genotype ($p = 0.0132$).
- No significant associations were observed for *ABCB1* (3435G>A), *ABCC1* (198217T>C), *ABCC2* 1(249G>A) and the *ABCC4* (4131A>C) SNPs with altering mRNA gene expression and plasma tenofovir.
- Our results showed that SNPs in the *ABCC4* gene may differentially affect circulating tenofovir levels and mRNA expression levels.
- Their combined impact may inform on low PrEP efficacy observed in clinical trials with African women and which drugs would be most effective in individuals presenting with certain genotypes with this gene.
- This study suggests that tailoring PrEP drug dosage for African women based on their ABC drug transporter polymorphisms could possibly improve the effectiveness of PrEP.

Author contributions

DA, NMZ, VR and PS were responsible for study design and conceptualization. NMZ was responsible for conducting research experiments, statistical data analysis, data interpretation, summarizing the main findings and writing the original draft for the research article. LL contributed in statistical data analysis, interpreting results, summarizing the main findings and final manuscript edits. DA, VR and PS contributed in data interpretation, summarizing the main findings and the final manuscript edits. AS, SN, SM and LEM all contributed in the final manuscript edits. All authors approved the final manuscript for publication.

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Disclaimer

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval for all human experimental investigations. The sub-study was reviewed and approved by the Biomedical Research Ethics Committee (BREC) at the University of KwaZulu-Natal (BREC/0002195/2020). In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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3. CHAPTER THREE

3.1 Bridging Chapter

Besides SNPs, varied drug transporter expression levels and the presence of pro-inflammatory cytokines in different compartments have been shown to modulate drug disposition. Data from several studies underscore the importance of understanding how certain ARVs can cause variations in drug transporter mRNA expression in different compartments targeted by HIV, which in turn affects ARV delivery and effectiveness. In addition, these studies highlighted consequences on ARVs arising from the interaction between pro-inflammatory cytokines and drug transporters. Cytokines have been shown to upregulate or downregulate drug transporter mRNA expression levels affecting ARV disposition. In this study, we determined if there is an association between drug transporter mRNA expression levels in the FGT and blood pre- and post- PrEP exposure in 45 black South African women taking oral PrEP (Truvada®) over time. In addition, we determined the association between pro-inflammatory cytokines and drug transporter mRNA expression levels. This study is relevant, since clinical trials testing PrEP in African women have shown low to no efficacy. Therefore, understanding the interaction between ARVs, drug transporter proteins and pro-inflammatory cytokines would provide a basis on how to improve PrEP efficacy for these women. We have earmarked to publish this manuscript entitled ***"Drug transporter expression levels in healthy South African women exposed to pre-exposure prophylaxis (PrEP)"*** in an international peer-reviewed journal - *Antimicrobial Agents and Chemotherapy*. Findings from this study showed that there are significant associations between drug transporter mRNA expression in the FGT and blood, both pre- and post- PrEP exposure for certain drug transporters. Furthermore, our findings suggested negatively trending associations between pro-inflammatory cytokines and drug transporter mRNA expression. These data further highlight the importance of studying drug transporters. Identifying those drug transporters in at-risk African populations that could be clinically relevant in PrEP pharmacokinetics is equally important to inform on drug effectiveness.

Drug transporter expression levels in healthy South African women exposed to pre-exposure prophylaxis (PrEP)

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3.2 Abstract

Pre-exposure prophylaxis (PrEP) formulations tested in African women, have produced varying results in preventing HIV infections. Drug transporter protein expression and function are proposed regulators of PrEP disposition. Additionally, ARV-specific drug transporters and inflammation are known modulators of drug transporter expression and function, affecting drug efficacy. We determined if there was concordance between drug transporter mRNA expression in the blood and female genital tract (FGT) of 45 women taking oral PrEP as Truvada® over 6 months. Additionally, we determined the associations between drug transporter mRNA expression, inflammation, and plasma tenofovir. mRNA expression of six drug transporters P-gp, MATE-1, MRP-2, MRP-4, OAT-1, and OAT-3 was conducted using quantitative RT-PCR. Cytokines were measured using multiplexed technology. Correlation analyses showed moderately significant associations between OAT-1 mRNA expression in the blood and FGT at baseline (r_s<1, p=0.0004), 3 months (r_s<1, p=0.0001) and 6 months (r_s<1, p=0.048). This was also observed for P-gp, MATE-1, MRP-2 and MRP-4 but only after PrEP initiation at 3 and 6 months (r_s<1, p<0.05). Linear mixed models showed trending associations between cytokines and drug transporters: IL-1β and MCP-1 and OAT-1 and OAT-3 (p<0.1); IL-1Rα and TNF-α and MRP-2 and MRP-4 (p<0.1); MIP-1β and MATE-1 (p<0.1). No significant associations were observed between drug transporter mRNA expression and plasma tenofovir at 3 or 6 months. Our results suggest that drug transporters may be similarly expressed in the blood and FGT. Furthermore, inflammation may alter drug transporter expression, which can modify PrEP disposition. Collectively, our data may be used to better understand factors that affect PrEP efficacy in African women.

3.3 Introduction

Antiretrovirals (ARVs) as oral pre-exposure prophylaxis (PrEP) Truvada® [a combination of tenofovir disoproxil fumarate (TDF)/tenofovir (TFV) and emtricitabine (FTC)] or topical tenofovir gels are one of the most effective strategies used to prevent HIV infections (Cottrell *et al.* 2015, Janes *et al.* 2018, Karim *et al.* 2022). Clinical trials testing these PrEP formulations in African women have shown discrepant efficacies to HIV ranging from -49% to 14.5%; which were attributed to varying levels of adherence and poor drug penetration at the sites of sexual transmission (Van Damme *et al.* 2012, Marrazzo *et al.* 2015, Corneli *et al.* 2016, Delany-Moretlwe *et al.* 2018). It is only the CAPRISA 004 1% tenofovir gel trial that conferred 39% protection against HIV in African women (Abdool Karim *et al.* 2010). To ensure optimal levels and penetration of PrEP drugs in HIV target cells across various compartments such as the female genital tract (FGT) and peripheral blood, drug transporter proteins have emerged as essential to drug disposition and pharmacokinetics (Cottrell *et al.* 2015).

Drug transporters are transmembrane proteins expressed in various cells of the body and comprise of two superfamilies: the ATP-binding cassette (ABC) efflux proteins and Solute Carrier (SLC) influx or uptake proteins (Hu *et al.* 2015, Zondo *et al.* 2022). PrEP candidates such as tenofovir and emtricitabine are substrates of various efflux and influx drug transporters: multidrug resistance-associated protein (MRP)-1, MRP-2 MRP-4, P-glycoprotein (P-gp), organic anion transporters (OAT)-1, OAT-3 and multidrug and toxin extrusion proteins (MATE)-1, respectively (Hu *et al.* 2015, Reznicek *et al.* 2017). This interaction between ARVs and drug transporters, has underscored drug transporter expression and function as critical in ARV delivery and availably to HIV vulnerable sites like the FGT and peripheral blood (Nicol *et al.* 2018).

The FGT offers a unique anatomy and physiology where locally expressed drug transporters are likely impacted by the milieu to support all necessary functions (Gunawardana *et al.* 2015). This is important since the sufficient delivery and absorption of tenofovir and emtricitabine is dependent on drug transporters. However, varying levels in the mRNA expression of drug transporters relevant in tenofovir pharmacokinetics, have shown to impact drug efficacy (Grammen *et al.* 2014, Nicol *et al.* 2014, Hijazi *et al.* 2015, Taneva *et al.* 2016). Studies comparing drug transporter mRNA expression levels between the liver, colorectal tissues and FGT showed moderate to low mRNA expression of influx drug transporter OAT-1 and OAT-3, and moderate to high expression of efflux drug transporters P-gp, BCRP, MRP-4 and MRP-2 in the FGT (Zhou *et al.* 2013,

Grammen *et al.* 2014, Nicol *et al.* 2014, Zhou *et al.* 2014, Taneva *et al.* 2016). In addition, laboratory manipulation of vaginal epithelial cells through *in-vitro* transfection with influx drug transporter OAT-1 increased intracellular drug accumulation through high tenofovir uptake (Taneva *et al.* 2016). These studies suggested that low expression of influx drug transporters could lead to insufficient drug uptake. In contrast, high expression of efflux drug transporters could lead to inadequate retention of intracellular drugs, impacting drug efficacy in the FGT (Grammen *et al.* 2014, Nicol *et al.* 2014, Hijazi *et al.* 2015, Taneva *et al.* 2016). The relationship between the accumulation of topical tenofovir and reduced MRP-1, MRP-5 and MRP-7 mRNA expression levels has also been shown in cervicovaginal cell lines derived from humans (Hijazi *et al.* 2015) and non-human primates (Hijazi *et al.* 2020).

Peripheral blood mononuclear cells (PBMCs) from HIV infected individuals failing ARV therapy or individuals taking tenofovir containing ARVs had significantly higher mRNA expression of efflux drug transporters P-gp, MRP-1, MRP-4, MRP-5, BCRP and influx drug transporters ENT-2 and OCT-1 (Turriziani *et al.* 2008, Hoque *et al.* 2021). These significant associations established that the correlation between ARVs and drug transporter expression levels could undermine therapy through sub-optimal intracellular drug concentrations. This would allow for further HIV replication in HIV infected individuals (Turriziani *et al.* 2008). Similarly, the mRNA expression levels of BCRP in CD8 T cells (Zhang *et al.* 2014a) and P-gp in PBMCs (Zhang *et al.* 2014b) were also significantly increased in HIV infected individuals on ARVs compared to healthy controls (Zhang *et al.* 2014a, Zhang *et al.* 2014b). In addition, previous in-*vitro* PBMC studies also showed that following incubation with tenofovir, significant reductions in MRP-1, MRP-5, MRP-6 and P-gp mRNA expression was found (Bousquet *et al.* 2009), while emtricitabine inhibited mRNA expression of MRP-1 in a dose-responsive manner (Bousquet *et al.* 2008). Collectively these FGT and PBMCs studies illustrated that an interaction between ARVs and drug transporters may alter drug transporter disposition by inducing or inhibiting mRNA expression levels; subsequently affecting intracellular drug accumulation. A large number of these studies have however, evaluated the effects of ARVs used as treatment and not PrEP and have not included at risk groups such as African women. In addition, the impact of other biological factors remains less well defined -for example how the local milieu in drug transporter genes affect expression and function of these proteins.

Besides ARVs, the effect of inflammatory cytokines on drug transporter expression levels and function have been evaluated and implicated in significantly impacting drug pharmacokinetics (Petrovic *et al.* 2007, Cressman *et al.* 2012). In studies associating cytokines with drug transporter

expression conflicting results have been observed, this is most likely due to the use of differences in cell types and experiment designs (Liptrott *et al.* 2009). In addition, cytokines affect cells and tissues differently, hence the effect of systemic inflammation cannot be linked with genital inflammation (Liptrott *et al.* 2009). Inflammation-derived cytokines can induce changes in gene mRNA expression and lead to increased or decreased expression of drug transporter proteins, such as P-gp, OAT-1 and a wide range of MRPs (Belliard *et al.* 2004, Le Vee *et al.* 2009, Poller *et al.* 2010, Le Vee *et al.* 2011). Genital inflammation has been previously defined as the elevation of any of these five of nine inflammatory cytokines and chemokines - MIP-1α, MIP-1β, IP-10, IL-8, MCP-1, IL-1α, IL-1β, IL-6, and TNF-α above the 75th percentile (Masson *et al.* 2015). Data from the CAP004 1% tenofovir gel trial underscores the key role of genital inflammation as a significant modifier for both HIV risk and for undermining PrEP efficacy (Klatt *et al.* 2017, McKinnon *et al.* 2018). The impact of local genital inflammation in the cervicovaginal compartment on drug transporter expression however remains less well defined. Different *in-vitro* studies have shown that the treatment of human cell lines derived from brain, colorectal adenocarcinoma and hepatocytes cells with inflammatory cytokines IL-6, IL-1β, TNF-α, or IFN-γ significantly downregulated the mRNA expression levels of efflux drug transporters P-gp, BCRP, MRP-2, MRP-3 and influx OATP-2B1, OATP-1B1, OATP-1B3 drug transporters (Belliard *et al.* 2004, Le Vee *et al.* 2009, Poller *et al.* 2010, Le Vee *et al.* 2011). These data suggest that inflammation affects the mRNA expression levels of various efflux and influx drug transporters in different tissues. Therefore, there is a need to further elucidate if there are inflammation-mediated effects on drug transporter expression levels and if they can have direct impact on circulating and local drug levels, toxicity, and possibly PrEP efficacy.

There is a paucity of studies evaluating the drug transporters mRNA expression profiles, and how these expression levels are impacted by inflammatory cytokines in African women taking PrEP. More so, there is scant data on the mRNA expression for drug transporters in the genital tract, the site for HIV sexual transmission relative to the blood especially given that PrEP is offered as an oral formulation. Further assessment of these factors could assist in the determination of suitable PrEP drug dosages and formulations that will provide sufficient drug concentrations to cells targeted by HIV in the FGT and blood. This study aimed, to therefore determine the mRNA expression profiles of efflux and influx drug transporter genes relevant in tenofovir pharmacokinetics disposition in the FGT and blood. Additionally, we wanted to determine if there are associations between these mRNA expression profiles, circulating tenofovir drug levels and

inflammatory cytokines in the genital tracts of healthy South African women offered oral PrEP Truvada®.

3.4 Methods

3.4.1 Study population, design, and procedures

This is a retrospective study. Previously collected blood and genital tract samples from the CAP082 observational study (Mansoor *et al.* 2022) were used. In the CAP082 observational study 18–30-year-old women who were, healthy and sexually active were included. All the women were black women from two high HIV incident sites- urban eThekwini and rural Vulindlela in KwaZulu-Natal, South Africa. Rapid HIV and STI tests were done at enrolment and at each follow-up visit, while STI tests were done only at enrolment. Women were offered oral PrEP as Truvada®, [300 mg TFV/TDF and 200 mg FTC]. Blood (buffy coat) and the female genital tract (cytobrushes) were collected and stored every three months for all women who enrolled. Participants who used Truvada® for more than three months had TFV-DP drug levels (fmol/punch) measured from dried blood spots using a Modified liquid chromatography tandem mass spectrometry assay (Division of Clinical Pharmacology, University of Cape Town, SA) (Mansoor *et al.* 2022). Adherence to oral PrEP was determined with a formula which included pharmacy pill count and TFV-DP drug level data as described in Mansoor *et al.* (2022). This sub-study was approved by the Biomedical Research Ethics Committee (BREC) at the University of KwaZulu-Natal (BREC/0002195/2020).

3.4.2 RNA extraction and cDNA synthesis

Previous literature was used to select ABC and SLC drug transporters relevant in the pharmacokinetics of tenofovir and emtricitabine (Hu *et al.* 2015, Taneva *et al.* 2016, Reznicek *et al.* 2017). These included efflux ABC drug transporters: P-gp, MRP-2, MRP-4 and MATE-1 and influx SLC drug transporters OAT-1 and OAT-3. RNA was extracted from the FGT and blood using the QuickExtract™ DNA Extraction Solution (lysogenic buffer) (Qiagen, Netherlands) according to the manufacturer's instructions. Briefly, after centrifugation at 4°C for 1 min at 10,000 rpm the pellet was resuspended in lysogen buffer and incubated for 5 min at 95°C and 4°C. RNA was then treated with DNase (Agilent Technologies, USA) for 10 min at 37°C to remove DNA; RNA concentrations were determined using the Nanodrop system

(Thermo Fisher Scientific, USA). RNA was standardised to 50 ng in nuclease-free water and cDNA synthesised using the SuperScript™ Vilo™ cDNA synthesis Kit (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. Briefly, standardized RNA was mixed with the Vilo™ reaction mix and SuperScript™ Enzyme Mix, incubated at 25°C for 10 min, followed by 42°C for 60 min and the reaction terminated at 85°C for 5 min. Resulting cDNA was diluted 1 in 5 and used to determine drug transporter mRNA expression levels.

3.4.3 Quantitative Real-Time PCR (RT-PCR)

Quantitative PCR was performed to compare the mRNA expression levels of specific drug transporter genes in FGT and blood. The QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific, USA) was used to conduct quantitative PCR, it included the SYBR Green PCR Master Mix (Thermo Fisher Scientific, USA), synthesised and diluted cDNA and gene specific primers (5'-3' direction): MATE-1 (forward ATGCTGTTTCCCACCTCTTTG; reverse TCCAACCTTCTGATTTCCACTC); MRP-2 (forward TAATGGTCCTAGACAACGGG, reverse GGGCCTTCTGCTAGAATTT); MRP-4 (forward GGACAAAGACAACTGGTGTGCC, reverse AATGGTTAGCACGGTGCAGTGG); OAT-1 (forward GGGCACCTTGATTGGCTATGTC, reverse GATGACAAGGAAGCCCACAAGC); OAT-3 (forward ACTCGGGTACTGCTACACCT, reverse CAGGTCACTTGCGGTGTACT); P-gp (forward CCCATCATTGCAATAGCAGG, reverse TGTTCAAACTTCTGCTCCTGA) and the housekeeping gene Beta-actin (forward TCCTTCCTGGGCATGGAGT, reverse AGCACTGTGTTGGCGTACAG). Optimal conditions included denaturation at 95°C for 15 sec, 40 cycles, annealing at 60°C for 1 min and extension at 72°C for 30 sec were used. Negative controls using nuclease fee water instead of the template and a melting curve (at 95°C) was performed after each run, to determine non-specific PCR products. Only expression with C_T<35 values were considered in the expression data. Relative mRNA expression levels of target genes were calculated using the comparative C_T ($2^{\Delta\Delta t\text{-}Ct}$) method, expression was normalised using the endogenous reference gene Beta-Actin as described previously by (Brugè *et al.* 2011).

3.4.4 Cytokine measurements

Cytokine concentrations were determined using soft-cup specimens derived from the FGT as previously described by (Archary *et al.* 2015) in 45 participants with matching samples at

baseline, 3 and 6 months. Cytokine concentrations were measured using the Bio-Plex Pro Human Cytokine/Chemokine Magnetic Bead 27-Plex Panel (California, USA). The Bio-Plex Manager software version 6 was used to collect data, and the sample concentrations were calculated from standard curves by using a five- parameter (5PL) regression formula.

3.4.5 Statistics analysis

To determine correlations between ABC and SLC drug transporter mRNA expression levels in the FGT and blood, the Spearman's rank correlation coefficient was performed using the GraphPad Prism version 9.3.1 software for windows (GraphPad Software, La Jolla, CA, USA). A two-tailed p-value of $p \leq 0.05$ was considered significant. Linear mixed models were used to test if there are linear associations between drug transporter mRNA expression and: cytokines in the FGT and plasma tenofovir in the blood. These analyses include participants with matching soft-cup specimens and participants with detectable drug levels at 3 and 6 months. These analyses were performed in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). For these analyses p-value ≤ 0.05 was considered significant. GraphPad Prism was used for all graphical representation.

3.5 Results

3.5.1 Sample description

Of the 429 women enrolled in the study, 200 women had matching stored cytobrush (FGT) and buffy coat (blood) samples at baseline, 3 and 6 months (Figure 3.1). Of these 200 participants 108 were excluded due to no measurable plasma tenofovir diphosphate (TFV-DP) drug levels. The remaining 92 participants had - plasma drug levels measured during follow-up, and available soft-cup, cytobrush and buffy coat samples at baseline, 3 and 6 months.

Of the 92 participants:

I. 45 participants had matching cytobrush and buffy coat samples at baseline (45 samples each), 3 months (44 samples each) and 6 months (45 samples each). This provided a total of 134 cytobrush and buffy coat samples each and were included in mRNA expression correlation analyses between the two compartments.

II. 45 participants had matching cytobrush and soft-cup samples which were included in mRNA expression relative to the presence of genital inflammation defined previously

using the proinflammatory cytokine levels (Masson *et al.* 2015). This provided a total of 134 soft-cup and cytobrush samples each.

III. 29 participants had detectable drug levels at 3 months and 6 months and were included in mRNA expression analyses relative to the plasma TFV-DP levels.

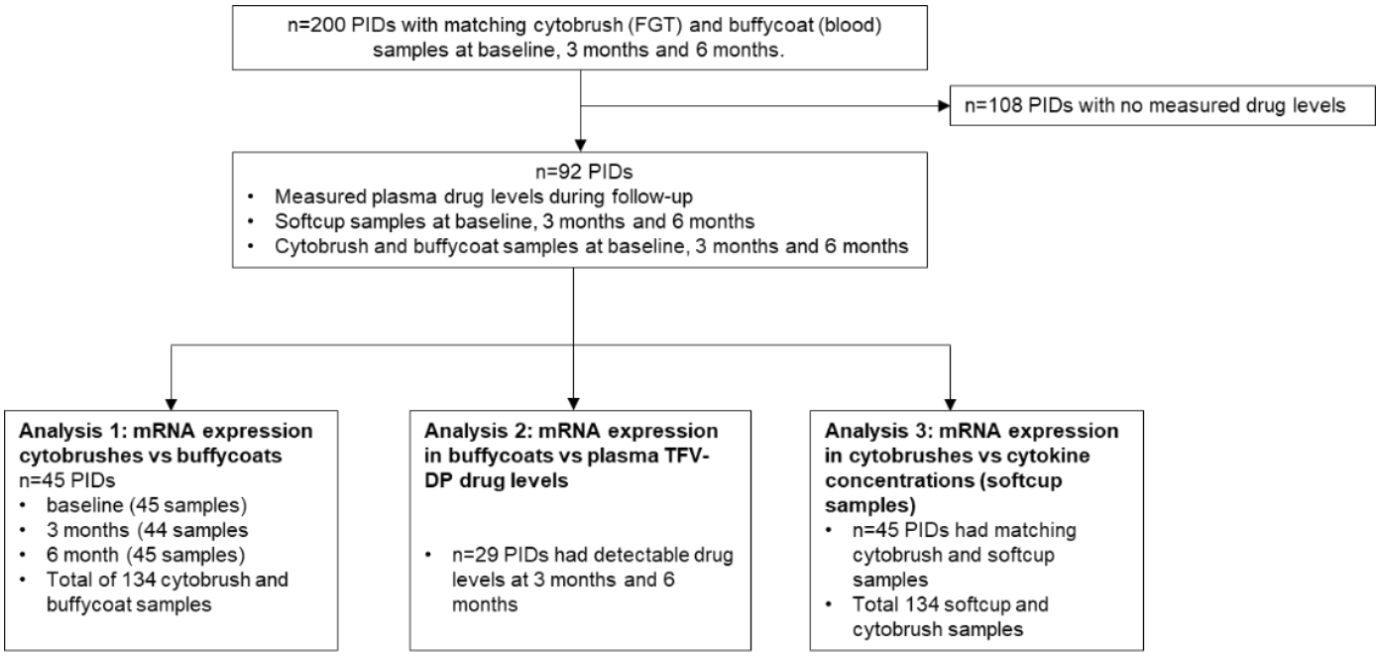

Figure 3.1: Description of the participant and sample allocation for three analysis plans in the study.

Description of the number of buffy coat and cytobrush samples included and excluded in this cohort study. A description of the analyses conducted, and the total number of samples included in each of the three analyses is shown.

3.5.2 Demographics table

Table 3.1 describes the demographic and clinical data of 45 participants who volunteered to take PrEP. For the following characteristics a large number of participants were aged <=24, had a matric education, were from the Vulindlela rural site and had an obese BMI of >30. At enrolment, prevalence of C. *trachomatis* was 20%, for *N. gonorrhoea* 7% and no participants presented with *T. vaginalis*. All of the 45 participants were included in mRNA expression and genital inflammation analyses, while 36 were included in TFV-DP drug levels analyses.

Table 3.1: Demographic and clinical characteristics of study participants

Characteristic	Number of participants n (%)
Age cat	
<=24	31 (69)
25-30	14 (31)
Education	
Matric	29 (64)
No Matric	10 (22)
Tertiary	6 (13)
Sites	
eThekwini (urban area))	19 (42)
Vulindlela (rural area)	26 (58)
BMI cat	
Underweight (0 - 18.5)	3 (7)
Normal weight (18.5 - 25)	15 (33)
Overweight (25 - 30)	8 (18)
Obese (>30)	19 (42)
STIs enrolment	
Chlamydia trachomatis	
Detected	9 (20)
Not Detected	36 (80)
Neisseria gonorrhoea	
Detected	3 (7)
Not Detected	42 (93)
Trichomonas vaginalis	
Negative	45 (100)
Positive	0 (0)
Analyses	
mRNA expression	45(100)
TFV-DP drug levels (detectable drug levels)	36 (80)
Genital inflammation	45 (100)

Abbreviations: Cat- category, BMI- Body Mass Index. mRNA expression includes matching cytobrush (FGT) and buffy coat samples (blood). TFV-DP drug levels analyses only included in buffy coat samples (blood). Genital inflammation analyses only included cytobrush (FGT). 36 participants were included in TFV-DP drug levels analyses this included 4 participants had HIV seroconverted.
.

3.5.3 mRNA expression levels of the OAT-1 drug transporter showed consistent and significant correlations from baseline to 6 months between the FGT and blood

Correlation analyses using the Spearman's rank correlation coefficient were done to determine if there are associations between drug transporter mRNA expression levels in the FGT and blood. Table 3.2 shows moderate and significant correlations for the OAT-1 gene at baseline Spearman's rank correlation coefficient (r_s) (r_s 0.53, p= 0.0004), 3 (r_s 0.54, p= 0.0001) and 6 (r_s 0.33, p= 0.048) months. A graphical representation of the correlations for OAT-1 mRNA expression between the FGT and blood are shown in Figures 3.2A, 3.2B and 3.2C. mRNA expression for P-gp however was moderately significant at 3 months only. Moderately significant correlations for mRNA expression were found between the FGT and blood for MATE-1, MRP-2 and MRP-4, post PrEP exposure i.e., at 3 and 6 months. No correlations were shown for the OAT-3 gene. Graphical representation for OAT-3, P-gp, MATE-1, MRP-2 and MRP-4 corelation analyses are shown in the appendices supplementary Figure 1S (page 130).

Table 3.2: Compartment correlations for mRNA expression of drug transporters between the FGT and blood

Timepoint	Baseline			3 Months			6 Months		
Drug transporters	**r_s**	**p**	**95%CI**	**r_s**	**p**	**95%CI**	**r_s**	**p**	**95%CI**
P-gp	0.33	ns	0.07 - 0.64	**0.56**	**0.002**	0.11 - 0.73	0.25	ns	-0.17 - 0.56
MATE-1	0.22	ns	-0.01 - 0.50	**0.46**	**0.002**	0.18 - 0.66	**0.42**	**0.004**	0.13 - 0.64
MRP-2	0.19	ns	-0.14 - 0.48	**0.35**	**0.023**	0.04 - 0.59	**0.38**	**0.022**	0.05 - 0.63
MRP-4	0.23	ns	-0.08 - 0.50	**0.37**	**0.016**	0.06 - 0.61	**0.38**	**0.015**	0.07 - 0.62
OAT-1	**0.53**	**0.0004**	0.26 - 0.73	**0.54**	**0.0001**	0.25 - 0.74	**0.33**	**0.048**	-0.01 - 0.60
OAT-3	0.28	ns	-0.04 - 0.54	0.11	ns	-0.22 - 0.41	0.18	ns	-0.15 - 0.48

r_s: Spearman's rank correlation coefficient, p<0.05 significant correlations, these values are highlighted in bold, 95%CI- confidence intervals (lower to upper limits)

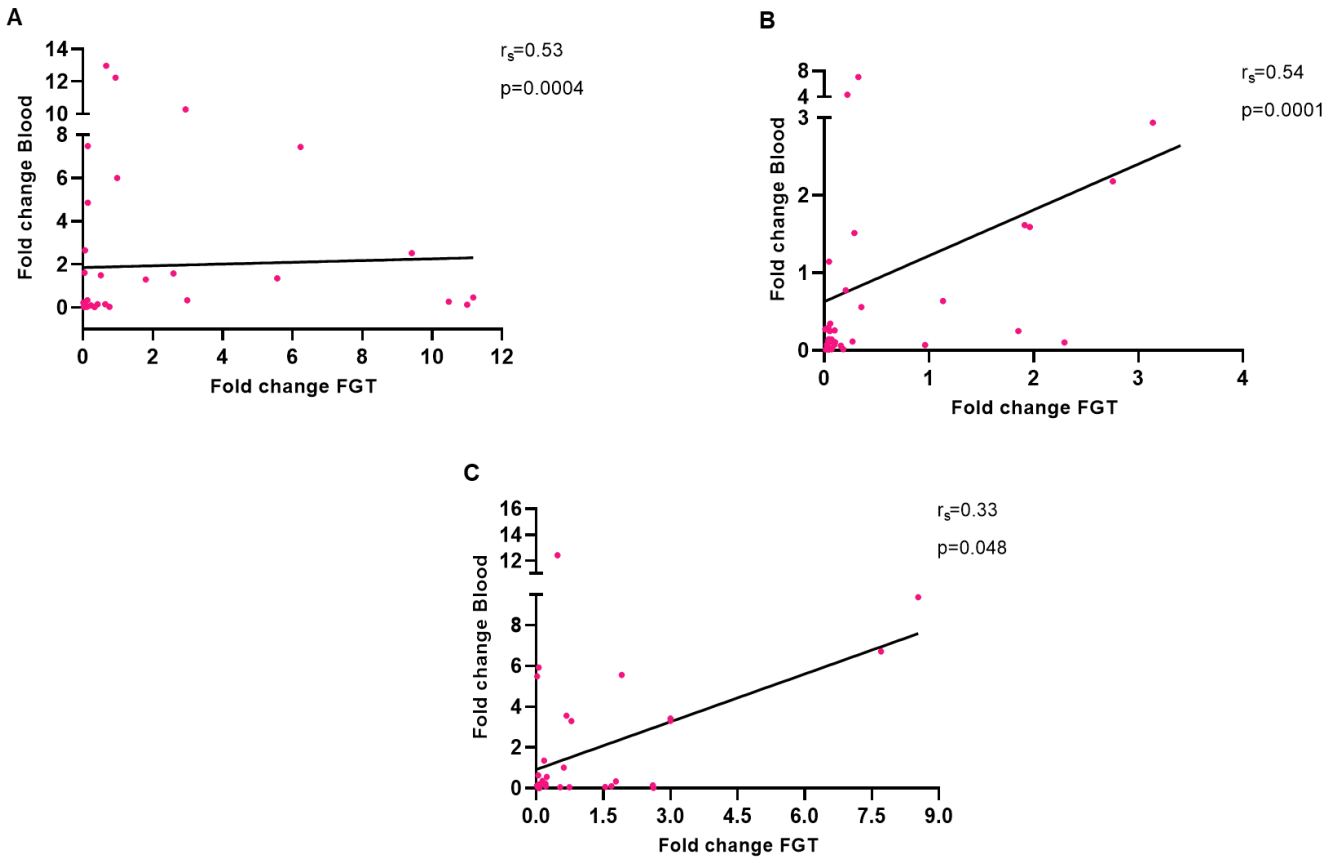

Figure 3.2: Graphical representation of correlations for OAT-1 mRNA expression between the FGT and blood.
Moderate and significant correlations are shown at three timepoints **A)** baseline, **B)** 3 months and **C)** 6 months for OAT-1 gene in cytobrushes (fold change FGT) and buffy coats (fold change Blood). Analyses were done using the Spearman's rank correlation coefficient r_s and a two-tailed p value of <0.05 was considered significant.

3.5.4 Association between pro-inflammatory cytokines and drug transporter mRNA expression

Linear mixed models were used to determine if there are associations between drug transporter mRNA expression and inflammation measured by cytokines in the FGT, in a subset of participants. Cytokines were grouped into quintiles according to concentrations, quintiles included below 20%; between 20-40%, 40-60%, 60-80% and 80-100%. Table 3.3 shows that there were no significant associations between any of the inflammatory cytokines and efflux drug transporters P-gp, MATE-1, MRP-2, MRP-4 and influx drug transporters OAT-1 and OAT-3 mRNA expression. Although these data were not significant, trends of negative associations were observed between IL-1β and influx drug transporters OAT-1 (p=0.06) and OAT-3 (p=0.04). A similar trend was also observed

between IL-1Rα and efflux drug transporters MRP-2 (p=0.05) and MRP-4 (p=0.03). Similarly, MCP-1, and TNF-α showed trends of negative associations with OAT-3 (p= 0.06), and MRP-2 (p=0.07), respectively. MIP-1β was the only chemokine showing a positive trend with efflux drug transporter, MATE-1 (p=0.07). For the other cytokines there were no trends observed for IL -6, IL-8, IP-10, MIP-1α with all drug transporters.

Table 3.3:Associations between drug transporter mRNA expression and cytokines (quintiles)

	Log P-gp			Log MATE-1			Log OAT-1			Log OAT-3			Log MRP-2			Log MRP-4		
Cytokine (quintile)	**Est**	**95% CI**	**p**	**Est**	**95% CI**	**p**	**Est**	**95% CI**	**p**	**Est**	**95% CI**	**p**	**Est**	**95% CI**	**p**	**Est**	**95% CI**	**p**
IL-1β	-0.14	0.35 - 0.09	0.24	0.08	-0.02 - 0.18	0.13	-0.22	-0.45 - 0.01	**0.06**	-0.17	-0.34 -0.01	**0.04**	-0.03	-0.13 - 0.06	0.48	-0.02	-0.16 - 0.12	0.79
IL-1Rα	-0.20	-0.48 - 0.08	0.16	0.15	-0.08 - 0.37	0.20	-0.16	-0.51 - 0.20	0.38	0.04	-0.26 - 0.35	0.79	-0.20	-0.41 - 0.00	**0.05**	-0.15	-0.29 - 0.01	**0.03**
IL-6	0.13	-0.11- 0.37	0.30	0.00	-0.13 - 0.13	0.95	0.03	-0.20 - 0.26	0.77	-0.04	-0.23 - 0.16	0.71	-0.05	-0.17 - 0.07	0.42	-0.02	-0.15 - 0.10	0.71
IL-8	0.12	-0.15 - 0.39	0.39	0.08	-0.06 - 0.22	0.25	-0.06	-0.29 - 0.17	0.58	-0.15	-0.32 - 0.03	0.10	-0.07	-0.17 - 0.03	0.18	0.04	-0.09 - 0.18	0.52
MCP-1	0.11	-0.14 - 0.35	0.38	0.09	-0.04 - 0.21	0.17	0.02	-0.23 - 0.27	0.89	-0.15	-0.31 - 0.01	**0.06**	-0.05	-0.17 - 0.08	0.44	-0.01	-0.17 - 0.14	0.86
IP-10	0.13	-0.09 - 0.35	0.26	0.05	-0.07 - 0.17	0.43	-0.06	-0.29 - 0.16	0.58	-0.04	-0.20 - 0.11	0.60	-0.05	-0.17 - 0.08	0.47	0.09	-0.05 - 0.22	0.20
MIP-1α	0.15	-0.11 - 0.41	0.26	-0.02	-0.15 - 0.11	0.74	-0.13	-0.37 - 0.11	0.29	-0.05	-0.22 - 0.13	0.62	-0.08	-0.20 - 0.03	0.14	0.02	-0.12 - 0.16	0.76
MIP-1β	0.09	-0.18 - 0.36	0.52	0.12	-0.01 - 0.25	**0.07**	-0.12	-0.47 - 0.22	0.48	-0.03	-0.20 - 0.15	0.75	-0.08	-0.22 - 0.07	0.31	0.08	-0.06 - 0.23	0.27
TNF-α	-0.09	-0.34 - 0.15	0.46	0.00	-0.09 - 0.10	0.92	-0.20	-0.46 - 0.05	0.12	-0.11	-0.27 - 0.04	0.15	-0.10	-0.20 - 0.01	**0.07**	0.03	-0.12 - 0.17	0.74

Est: estimated value, p <0.1 trending towards significance, these values are underlined and highlighted in bold, 95%CI: confidence intervals (lower to upper limits)

3.5.5 Associations between plasma tenofovir and drug transporter mRNA expression

Linear mixed models were also used to determine if there are associations between drug transporter mRNA expression and plasma tenofovir. These analyses were conducted in a sub-set of participants that had detectable drug levels at 3 and 6 months, after electing to take PrEP. At 3 and 6 months no significant associations were found between all mRNA efflux and influx drug transporter expression and plasma tenofovir measured at 3 and 6 months (Table 3.4).

Table 3.4: Associations between drug transporter mRNA expression and plasma TFV-DP at 3 and 6 months

Drug transporter	Estimate	95% CI	p value
Log P-gp	0.12	-0.19 - 0.42	0.31
Log MATE-1	-0.12	-0.63 - 0.39	0.51
Log OAT-1	-0.03	-0.28 - 0.21	0.71
Log OAT-3	-0.07	-0.30 - 0.17	0.43
Log MRP-2	0.01	-0.28 - 0.48	0.47
Log MRP-4	-0.15	-0.67 - 0.38	0.44

Est: estimate, p significance <0.05, 95%CI: confidence intervals (lower to upper limits)

3.6 Discussion

In our study, we showed consistent and significant correlations between mRNA expression of ABC and SLC drug transporters in the FGT and blood. Additionally, we determined if there were relationships between drug transporter mRNA expression levels, circulating tenofovir drug levels and genital inflammation in healthy South African women offered oral PrEP (Truvada®).

The crosstalk or concordance between the influx and efflux drug transporters in the blood to that in the genital tract remains relatively poorly characterised. In our study, we established that there is some degree of positive associations for four efflux drug transporters namely P-gp, MATE-1, MRP-2 an MRP-4 and one influx drug transporter OAT-1. What we show convincingly is that these correlations between the blood and FGT are maintained moderately over time with exposure to PrEP. This pattern of correlations between the two compartments after drug exposure may suggest that the cells in the blood may in part traffic to the genital tract. Although cell line and *in vitro* studies show high expression of P-gp, MRP-4 and OAT-1 drug transporters in human cervicovaginal tissues relative to mice cervicovaginal tissues (Zhou *et al.* 2013), no direct comparisons have been shown between the blood and the genital compartments in humans. Furthermore, the study by Zhou *et al.* (2013) showed high baseline expression of these efflux drug transporters P-gp and MRP-4 in human and mouse vaginal tissue models (Zhou *et al.* 2013).

Drug transporters have also shown heterogeneous expression profiles for vaginal tissues compared to colorectal tissues (Nicol *et al.* 2014). Whereas high mRNA expression of P-gp and MRP-2 was found in human vaginal compared to colorectal tissues, the opposite was found for MRP-4. Taneva *et al.* 2016 also demonstrated low expression of OAT-1 and OAT-3 influx drug transporters in human vaginal epithelial and T cells, which accounted for the poor permeability of TFV across the cell membranes and into the cells (Taneva *et al.* 2016). These patterns of expression may be indicative of why certain ARVs such as tenofovir are maintained at higher intracellular levels in colorectal tissues compared to vaginal tissues (Nicol *et al.* 2014, Taneva *et al.* 2016). In contrast, our findings differ by the high baseline OAT-1 expression (influx drug transporter) before PrEP exposure in the FGT. These data indicated that more in-depth analyses are still required to fully elucidate the correlations between drug transporters expressed in the blood and FGT, if they differ significantly or affected by biological factors in the same manner.

In our study, we found associations that trended towards significance between drug transporter mRNA expression and pro-inflammatory cytokines. These negative correlations were observed

between: IL-1β and influx drug transporters OAT-1 and OAT-3, IL-1Rα and efflux drug transporters MRP-2 and MRP-4, MCP-1 and OAT-3 and TNF-α and MRP-2. While no strong associations were observed, we can make certain inferences from these findings. These trends suggest that inflammatory cytokines in the FGT can shape drug transporter mRNA expression patterns. In addition, our findings support our hypothesis that certain cytokines can affect the mRNA expression of drug transporter families differently. This is corroborated by studies in animal and human *in vitro* and *in vivo* models showing that efflux and influx drug transporters are affected by pro-inflammatory cytokines, which in turn affect drug levels (Saib and Delavenne 2021). These studies also showed that modulation of drug transporters by pro-inflammatory cytokines varies according to tissue type (Saib and Delavenne 2021). Studies done on PBMCs stimulated with various cytokines IL-2, IL-4, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, IFN-γ, TGF-β and TNF-α showed significantly upregulated expression of efflux drug transporters P-gp, MRP-1 and MRP-4 (Liptrott *et al.* 2009). In contrast, in the liver (*in vitro* and *in vivo* hepatic animal and human models) showed that pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, significantly upregulated the expression of influx drug transporters OATP-B, OATP-C and OATP-8, while the mRNA expression of the efflux drug transporter MRP-2 was significantly downregulated (Hinoshita *et al.* 2001, Le Vee *et al.* 2009, Le Vee *et al.* 2011). These data highlight that further elucidation of pro-inflammatory cytokines relative to drug transporter expression in different tissues is needed. This is especially important since genital inflammation can directly impact drug transporter expression, thereby affecting drug disposition (Carserides *et al.* 2022).

In addition to inflammation, we also evaluated the associations between drug transporter mRNA expression and plasma tenofovir. We did not observe any significant associations. Most previous studies evaluating such associations for ARVs used as treatment rather than for prevention. High mRNA and protein expression of efflux drug transporters P-gp and MRP-4 were found in HIV infected individuals taking therapy compared to healthy donors (Turriziani *et al.* 2008, Kis *et al.* 2010). These results suggest that taking ARVs as treatment can differentially impact mRNA expression of various efflux drug transporters but particularly in the background of HIV infections. HIV infected individuals, ARV experienced or naïve have higher inflammatory status compared to healthy individuals, which likely also impacts drug transporter expression (Turriziani *et al.* 2008, Kis *et al.* 2010). ARVs have been directly implicated as inducers and/or inhibitors of drug transporters affecting corresponding protein expression and function (Kis *et al.* 2010, Zhou *et al.* 2013). Furthermore, i*n vitro* studies using cervicovaginal tissues from drug naïve macaques exposed to tenofovir and darunavir, showed significantly increased efflux drug transporter MRP-

2 expression (Hijazi *et al.* 2020). However, human cervicovaginal cell lines VK2/E6E7 stimulated with tenofovir, showed significantly reduced MRP-5 and not MRP-2 mRNA expression (Hijazi *et al.* 2015). Such discrepancies suggest that the relationship between drug transporter expression and drugs is complex. Therefore, it remains imperative to identify the combinations and dosages of candidate drugs for PrEP conferring good drug penetration in the FGT for preventing sexual transmission of HIV.

Collectively, what these data suggest is that we need comprehensive and detailed understanding of interactions between ARVs and various drug transporter genes in the blood and more especially in the FGT, to exploit the limited drugs we have available to prevent HIV infections. Understanding the impact that ARVs have on drug transporter mRNA expression levels is particularly important in women. This is because tenofovir levels even at the same dosage were significantly higher in colorectal compared to vaginal tissue, which directly impacted PrEP efficacy (Cottrell *et al.* 2016). These data support why more research is needed in women to optimise PrEP and dosage to confer sufficient protection against HIV in the FGT (Patterson *et al.* 2011, Louissaint *et al.* 2013, Cottrell *et al.* 2016, Sheth *et al.* 2016, Bailey *et al.* 2017).

The limitation of our study is that we did not determine drug transporter mRNA expression relative to the expressed protein. This precludes the understanding of a direct relationship between protein translation, expression, or function. However, mRNA expression analyses alone still provide a basis for the selection of genes (Zhou *et al.* 2013, Hijazi *et al.* 2015, Seifert *et al.* 2016). We included only participants with detectable drug levels which further impacted our modest sample size. Despite the limited sample size, specimens were from two different compartments, the genital tract and blood. Additionally, these samples were collected longitudinally and at the same time points. Also, because we collected specimens pre- and post-PrEP exposure, we have the advantage of comparing drug transporter mRNA expression accordingly. An added strength is the matching soft-cup specimens that we used to measure genital inflammation and investigate potential interactions on drug transporter mRNA expression. mRNA expression, pro-inflammatory cytokines and plasma tenofovir associations were done in a limited sample size making it challenging draw definitive conclusions and using more stringent and robust analyses. Finally, our study was conducted in high-risk young African women from a hyperendemic HIV region, making it highly relevant to understanding how biology can potentially modify the efficacy of ARVs as PrEP.

In conclusion, our study provided two important findings, firstly only the influx drug transporter OAT-1 correlated significantly between the FGT and blood irrespective of PrEP exposure. In contrast, efflux drug transporters P-gp, MATE-1, MRP-2 and MRP-4 only showed correlations after PrEP exposure. Secondly a modest relationship was observed between the presence of certain cytokines and drug transporter mRNA expression in the FGT. This may be a signal that alterations in drug transporter mRNA expression in the blood and FGT may be sensitive to PrEP and specific cytokines, respectively. Our study provides a basis for understanding how and which drug transporters are modulated in African women using PrEP and provides a pathway to evaluating other drug candidates for PrEP.

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3.8 Author contributions

DA, NMZ, VR and PS were responsible for study design and conceptualization. NMZ was responsible for conducting research experiments, statistical data analysis, data interpretation, summarizing the main findings and writing the original draft for the research article. LL contributed in statistical data analysis, interpreting results, summarizing the main findings and final manuscript edits. DA, VR and PS contributed in data interpretation, summarizing the main findings and the final manuscript edits. AS, SN, SM, LEM all contributed in the final manuscript edits.

3.9 Conflict of interest

The authors declare no competing interests.

3.10 Data Availability Statement

Materials described in this manuscript, including all relevant raw data, will be freely available to any researcher wishing to use them for non-commercial purposes, without breaching participant confidentiality.

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4. CHAPTER FOUR: FINAL SUMMARY

Our study explored the effects of three biological factors that may affect tenofovir disposition in black South African women taking oral PrEP - Truvada®. These include SNPs in drug transporter genes, drug transporter mRNA expression and genital inflammation.

In the first part of our study, we discuss the impact of SNPs in drug transporter genes on circulating tenofovir and mRNA expression. SNPs are genetic mutations, leading to allelic variations, resulting in altered mRNA transcription, protein translation, expression and function which differ among populations (according to ethnicities) (Ismail and Essawi 2012, Yee *et al.* 2018). The presence of certain SNPs in drug transporter genes have been associated with affecting drug transporter expression and function, which in turn affects ARV pharmacokinetics and efficacy (Danjuma *et al.* 2018). In our study we hypothesized that SNPs in ABC drug transporter genes affect drug transporter mRNA expression and circulating tenofovir in South African women taking PrEP. Our findings suggested that SNPs in the *ABCC4* gene affect drug transporter expression and circulating tenofovir differently (chapter two). The CC genotype for the *ABCC4* 3436T>C SNP was associated with increased plasma tenofovir, suggesting that it could subsequently reduce intracellular tenofovir and potentially its efficacy. Previous studies have also found an association between high plasma tenofovir and tenofovir-induced KTD, resulting in low drug efficacy for this SNP (Kiser *et al.* 2008, Nishijima *et al.* 2012, Manosuthi *et al.* 2014, Rungtivasuwan *et al.* 2015, Likanonsakul *et al.* 2016). The AA genotype for the *ABCC4* 4976A>G SNP was associated with increased *ABCC4* gene mRNA expression with an inverse correlation to plasma tenofovir. We therefore hypothesized that the presence of either tenofovir-specific influx/uptake drug transporter genes or haplotypes in the *ABCC4* gene could counteract the effect that this SNP has on drug levels by decreasing circulating drug levels. Additionally, many studies have shown the effects of the CC/GG genotype for this SNP and its association with increased plasma tenofovir (Nishijima *et al.* 2012, Likanonsakul *et al.* 2016). This data further supported our findings that the C/G allele is associated with increased plasma tenofovir and not the A allele.

Other SNPs showed no associations with plasma tenofovir and drug transporter mRNA expression. This data suggested that while these SNPs impact ARVs and drug transporter mRNA expression in other ethnicities, this may not be the case for our population. Furthermore, this data suggested that other yet to be discovered SNPs in our population may play an integral role in low PrEP efficacy or may even exert a protective effect by increasing PrEP efficacy.

A review by Bruckmueller and Cascorbi (2021), highlighted that two important factors must be considered when evaluating the effects of SNPs on drug transporter mRNA expression: function and drug levels. The drug being studied should be substrate/drug transporter specific to the functionality related to the SNP (Bruckmueller and Cascorbi 2021). For our study, we included efflux drug transporters P-gp, MRP1, MRP-2 and MRP-4 that were specific for tenofovir metabolism (Hu *et al.* 2015, Zondo *et al.* 2022). For the second factor evaluating the effects of SNPs on drug transporter mRNA expression provided a basis that could be further explored in functional and pharmacokinetics studies, while the direct functional effects of the SNPs were not determined.

Data evaluating the impact of SNPs in African populations taking ARV as treatment or PrEP is very limited, many studies have drawn major conclusions on SNPs based on European and Asian populations. In addition, studies that have evaluated the impact of SNPs on ARV have shown that among African populations there is high genetic diversity (Ikediobi *et al.* 2011, Rajman *et al.* 2020).This highlights that the data currently available does not provide sufficient evidence, on the impact of SNPs in African women and how they can used as predictive biomarker for PrEP bioavailability or response. We acknowledge our study limitations; we only studied SNPs in efflux ABC drug transporter genes and did not include SNPs in SLC drug transporter genes. Our sample size for plasma tenofovir and mRNA expression experiments were reduced due to the exclusion of participants with undetectable drug levels over time. Despite these limitations our study was still able to show a significant, direct association between *ABCC4* SNPs and plasma tenofovir and mRNA expression. Conversely, *ABCB1*, *ABCC1*, *ABCC2* SNPs showed no such correlations. The frequency of these SNPs in vulnerable populations should be considered when tailoring PrEP dosages especially for African populations.

In the second part of our study, we evaluated drug transporter mRNA expression levels in two compartments, the FGT and blood. Furthermore, we determined if there are associations between drug transporter mRNA expression levels with circulating tenofovir in the blood and genital inflammation in the FGT.

Drug transporters are transmembrane proteins expressed in most cells and tissues targeted by HIV. Most ARVs are substrates of these proteins and affect drug transporter expression and function, subsequently impacting on ARV efficacy (Kis *et al.* 2010, Zhou *et al.* 2013). However, there is a paucity of data regarding the effects and implications of ARVs on drug transporters expressed in the FGT (Nicol *et al.* 2014). In our study we aimed to characterise the mRNA

expression levels of efflux and influx drug transporters in the FGT and blood from African women exposed to oral PrEP. We aimed to determine if similar mRNA expression levels are observed in the FGT and blood pre- and post- PrEP exposure. We showed moderately significant associations between the FGT and blood for the influx drug transporter OAT-1 at baseline (pre-PrEP) and at 3 and 6 months (post and during-PrEP). For efflux drug transporters MATE-1, MRP-2 and MRP-4 moderately significant associations between the FGT and blood were observed only post PrEP exposure at 3 and 6 months, while for the P-gp this association was only observed at 3 months. These data indicated that the presence of PrEP may modulate drug transporter mRNA expression. Identifying drug transporter mRNA expression levels in the FGT and blood pre- and post- PrEP exposure, will aid in the tailoring of PrEP to ensure maximum mucosal exposure and efficacy. Additionally, findings from this study could be used to determine if PrEP modulates drug transporter mRNA expression similarly in the FGT and blood.

In studies of the cervicovaginal compartment, ARVs were shown to modulate drug transporter mRNA expression. These findings suggest that efficient drug transfer across cervicovaginal epithelial barrier may be highly dependent on drug transporter expression (Hijazi *et al.* 2015, Hijazi *et al.* 2020). Findings from our study differed from previous studies that showed low expression of influx drug transporters OAT-1, we showed high expression of OAT-1 in the FGT. We corroborate our findings of high expression of efflux drug transporters MRP-4, MRP-2 and P-gp, in vaginal tissues (Zhou *et al.* 2013, Nicol *et al.* 2014, Taneva *et al.* 2016). Collectively our data suggest that convergence of low influx drug transporters and high efflux drug transporters in vaginal tissues could limit both drug uptake or retention, resulting in increased plasma drug levels. This leads to greater understanding of how variability in drug transporter expression can affect drug disposition and efficacy (Zhou *et al.* 2013, Nicol *et al.* 2014, Taneva *et al.* 2016). The involvement of drug transporter in drug pharmacokinetics indicates their ability to restrict drug delivery and absorption in different sites targeted by HIV, thereby affecting drug efficacy. This interaction can therefore be exploited to tailor ARVs dosage to ensure sufficient intracellular retention (Carserides *et al.* 2022).

Besides ARVs impact on drug transporter expression, inflammatory cytokines can directly modulate drug transporter expression. We support this hypothesis because we observed negatively correlated trends between: IL-1β and OAT-1 and OAT-3, IL-1Rα and MRP-2 and MRP-4; MCP-1 and OAT-3, TNF-α and MRP-2. However, between MIP-1β and MATE-1 a positive trend was found. Findings from these data indicated that pro-inflammatory cytokines could have

differential effects on efflux and influx drug transporters in the FGT. This has also been observed in other studies, stimulation of human hepatocytes with pro-inflammatory cytokines IL-1β, IL-6 and TNF-α significantly upregulated the expression of influx drug transporters OATP-8, OATP-B and OATP-C. However, in PBMCs stimulated with pro-inflammatory cytokines:IL-2, IL-4, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, IFN-γ, TGF-β and TNF-α, efflux drug transporters P- gp, MRP-1 and MRP-4 were significantly upregulated (Le Vee *et al.* 2009, Liptrott *et al.* 2009, Le Vee *et al.* 2011). Modulation of drug transporters by cytokines has been shown in various cells, however this interaction has not been demonstrated in cells of the FGT (Carserides *et al.* 2022).

Findings from the second part of our study highlight that drug transporter mRNA expression can be affected by oral PrEP and by specific cytokines. Our mRNA expression analyses to plasma tenofovir levels were not significantly associated, and this may be mainly attributed to our limited sample size. However, the significant association of drug transporter mRNA expression in the FGT relative to the blood provides an understanding that the expression between these compartments are similar for influx drug transporter OAT-1 in a PrEP naïve scenario. For the efflux drug transporters P-gp, MATE-1, MRP-2 and MRP-4, these significant associations are apparent after PrEP exposure. These data highlight the heterogenous effect that PrEP can have on various drug transporters which may impact drug disposition. Cytokines also differentially associate with influx and efflux drug transporters and our data reflects this. OAT-1 and OAT-3 showed inverse relationships to IL-1β and MCP-1, while MRP-2 and MRP-4 showed this pattern with IL-1Rα and TNF-α. Our findings support our hypothesis that differential mRNA expression levels of efflux and influx drug transporter genes, SNPs in drug transporter genes and genital inflammation modify the peripheral blood and the mucosal environment of the FGT.

Conclusions and recommendations

Our study comprised of three major findings. Firstly, there is a correlation between efflux and influx drug transporter mRNA expression in the FGT and blood pre- and post- PrEP exposure. Secondly, pro-inflammatory cytokines produced in the FGT could affect drug transporter mRNA expression. Thirdly, SNPs in drug transporter genes affect plasma tenofovir and drug transporter mRNA expression. Collectively these data suggest that the presence of PrEP, pro-inflammatory cytokines and SNPs in African women could converge to modify drug transporter mRNA expression, potentially limiting PrEP delivery and efficacy. These findings add to the limited data among African populations on factors that affect PrEP efficacy. Limitations of our study included

the small sample size, and further attrition due to exclusion of participants because of undetectable drug levels and the lack of functional and mechanistic studies. Despite these limitations our findings informed on the types of drug transporters, SNPs and pro-inflammatory cytokines that can be further evaluated in African women. For the future, firstly we recommend that SNPs in both ABC and SLC drug transporter genes be evaluated. Secondly, we recommend that haplotypes within drug transporter genes be included in these studies to understand how they may impact drug disposition. This would allow for dissection of each region of the gene individually to discover more SNPs that may affect drug levels and possibly PrEP efficacy. To discern in greater detail the relationships between pro-inflammatory cytokines and drug transporter expression we recommend more stringent, principal component analyses to account for the confounding of highly correlated cytokines. Ultimately the goal of these studies is to exploit existing ARVs for PrEP to augment efficacy in these vulnerable and at-risk African women.

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APPENDICES

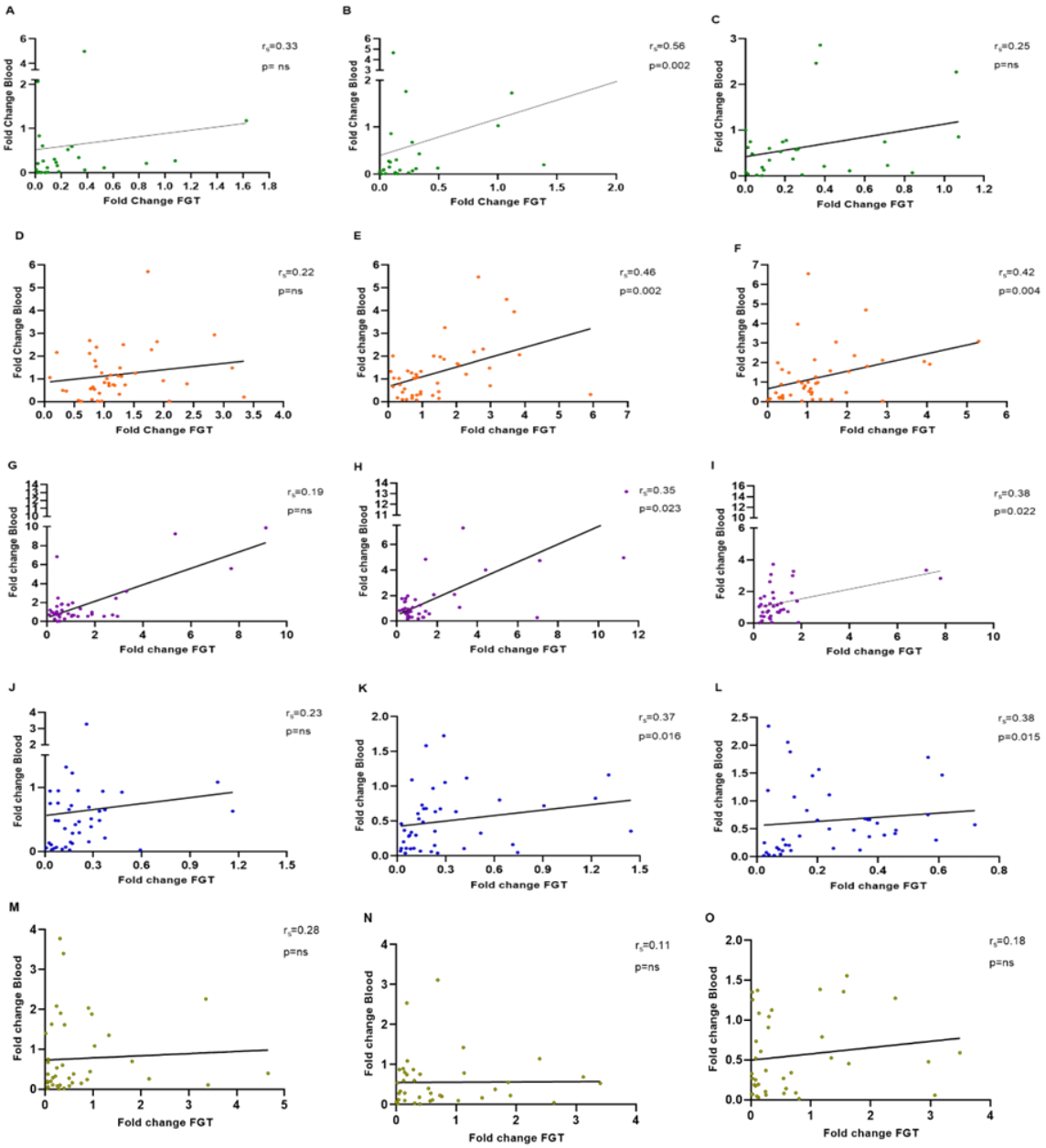

Figure 1S: Graphical representation of correlations between all ABC and SCL mRNA expression between the FGT and Blood

Correlations between the FGT and blood at **A)** baseline (ns) **B)** 3 months (moderately significant) and **C)** 6 months (ns) for P-gp. At **D)** baseline (ns) **E)** 3 months (moderately significant) and **F)** 6 (moderately significant) for MATE-1. At **G)** baseline (ns) **H)** 3 months (moderately significant) and **I)** 6 (moderately significant) for MRP-2. At **J)** baseline (ns) **K)** 3 months (moderately significant) and **L)** 6 (moderately significant) for MRP-4 and at **M)** baseline (ns) **N)** 3 months (ns) and **O)** 6 months (ns) for OAT-3. Analyses were done using the Spearman's rank correlation coefficient r_s, ns represents not significant and a two-tailed p value of <0.05 was considered significant.

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Pharmacogenomics of drug transporters for antiretroviral long-acting pre-exposure prophylaxis for HIV

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The use of antiretrovirals (ARVs) as oral, topical, or long-acting pre-exposure prophylaxis (PrEP) has emerged as a promising strategy for HIV prevention. Clinical trials testing Truvada® [tenofovir disoproxil fumarate (TDF)/tenofovir (TFV) and emtricitabine (FTC)] as oral or topical PrEP in African women showed mixed results in preventing HIV infections. Since oral and topical PrEP effectiveness is dependent on adequate drug delivery and availability to sites of HIV infection such as the blood and female genital tract (FGT); host biological factors such as drug transporters have been implicated as key regulators of PrEP. Drug transporter expression levels and function have been identified as critical determinants of PrEP efficacy by regulating PrEP pharmacokinetics across various cells and tissues of the blood, renal tissues, FGT mucosal tissues and other immune cells targeted by HIV. In addition, biological factors such as genetic polymorphisms and genital inflammation also influence drug transporter expression levels and functionality. In this review, drug transporters and biological factors modulating drug transporter disposition are used to explain discrepancies observed in PrEP clinical trials. This review also provides insight at a pharmacological level of how these factors further increase the susceptibility of the FGT to HIV infections, subsequently contributing to ineffective PrEP interventions in African women.

KEYWORDS

African women, PrEP (pre-exposure prophylaxis), female genital tract (FGT), drug transporters, single nucleotid polymorphism (SNP), inflammation

1 Introduction

HIV remains a formidable public health challenge, with currently over 37.7 million people living with HIV globally (UNAIDS, 2021). At the end of 2020 globally, an estimated 1.5 million new HIV infections were reported, of which 60% occurred in sub-Saharan African (SSA) countries (UNAIDS, 2021). The use and coverage of (antiretrovirals) ARVs has, however, had a positive impact in many SSA countries

including South Africa. Despite the advent and use of ARVs, new HIV infections (~4,000 daily) continue and remain a major concern (UNAIDS, 2021).

The increasing challenge of providing ARVs to a rapidly growing HIV population prompts the need for new interventions to decrease HIV incidence rates (Nicol et al., 2018). Previously tested HIV prevention methods have included the use of ARVs as oral, topical gels or long-acting pre-exposure prophylaxis (PrEP) formulations in uninfected individuals (Abdool Karim et al., 2010; Janes et al., 2018; Nicol et al., 2018). Clinical trials using oral and topical PrEP regimens in high risk heterosexual HIV-serodiscordant couples (Baeten et al., 2012; Thigpen et al., 2012) and men who have sex with men (MSM) (Grant et al., 2010; Molina et al., 2015; Mccormack et al., 2016) reported high levels of protection against HIV acquisition ranging from 44 to 86% (Grant et al., 2010; Thigpen et al., 2012; Mccormack and Dunn, 2015; Molina et al., 2015). However, clinical trials using the same PrEP regimens that focused primarily on at-risk African women, produced inconsistent levels of protection against HIV, ranging from −49 to 39%, with a majority leading to trial termination (Abdool Karim et al., 2010; Van Damme et al., 2012; Marrazzo et al., 2015; Delany-Moretlwe et al., 2018).

The main contributory factor for these low efficacies was identified as low to no adherence to PrEP. However, underlying biological factors beyond adherence have been proposed to play an integral role in low PrEP efficacies (Hu et al., 2015; Nicol et al., 2018). These include drug transporters, which are transmembrane proteins that are expressed ubiquitously in various cells and tissues of the body. Various ARVs used as PrEP have been identified as substrates of different drug transporters (Hu et al., 2015; Taneva et al., 2016; Reznicek et al., 2017). Therefore, drug transporter expression levels and functionality are considered essential for optimal PrEP delivery and for maintaining optimal drug concentrations in cells and tissues targeted by HIV (Hu et al., 2015; Nicol et al., 2018). In addition, there are also host biological factors such as inflammation (Saib and Delavenne, 2021) and genetic polymorphisms (Shenfield, 2004; Arruda et al., 2016) affecting drug transporter disposition, which subsequently affects drug efficacy (Shenfield, 2004; Arruda et al., 2016; Saib and Delavenne, 2021). These findings underscore drug transporters as critical determinants of drug pharmacokinetics. However, there is limited data on drug transporter expression profiles and host factors affecting drug transporter expression and function in anatomical compartments such as the FGT, the predominant site for HIV infection in women during heterosexual intercourse (Hu et al., 2015; Nicol et al., 2018). This warrants the need for further studies that will evaluate these factors, especially in high-risk groups such as African women. The current review, therefore, evaluates various biological factors affecting PrEP pharmacokinetics [absorption, distribution, metabolism and excretion (ADME)] to better understand inconsistencies in PrEP effectiveness observed in clinical trials with at-risk African women.

2 Biological, behavioural, and socio-economic factors that increase women's susceptibility to HIV

Despite noticeable reductions in HIV infections and increases in ARVs accessibility, there are several biological, behavioural and social factors that contribute to higher HIV prevalence rates in women (Ramjee and Daniels, 2013; Abdool Karim et al., 2020). Socio-economic factors that drive high HIV incidence rates in women include sexual abuse, lack of education, lack of food security and the lack of proper social services such as education on HIV and insufficient provision of health services; especially in highly affected regions (Abdool Karim et al., 2012; Ramjee and Daniels, 2013; Nicol et al., 2018; Durevall et al., 2019).

Behavioural factors also play an integral role in high rates of HIV acquisition in young women. These include early age of sexual debut (Mabaso et al., 2018), multiple concurrent sex partners, intergenerational sexual partnering with older men and transactional sexual encounters (Maartens et al., 2014; De Oliveira et al., 2017; Mabaso et al., 2018). Other factors include low marriage rates (Alcaide et al., 2014), intravaginal practices, and low to no condom use due to the inability to negotiate safe sexual practices with their male partners (Ramjee and Daniels, 2013; De Oliveira et al., 2017; Mabaso et al., 2018). Additionally, the use of injectable drugs and alcohol have also been associated with increased HIV transmission through shared needles and high-risk sexual behaviour, respectively (Maartens et al., 2014). Together, these factors suggest that the economic and social disempowerment of young women especially in a developing country such as South Africa contributes largely to high HIV prevalence rates within this population.

Apart from behavioural and socio-economic factors that fuel HIV infections, biological factors also drive higher rates of HIV infections in women. The greater mucosal surface area of the female genital tract (FGT) makes this surface highly susceptible through increased opportunities for target $CD4^+$ T cells to become infected with HIV and other sexually transmitted infections (STIs) during sexual intercourse (Ramjee and Daniels, 2013). Other biological factors that increase women's susceptibility to HIV include bacterial vaginosis (BV) (Heffron et al., 2017; Klatt et al., 2017), vaginal micro-abrasions (Stanley, 2009), cervical ectopy (Critchlow et al., 1995) and genital inflammation (Masson et al., 2015; Mckinnon et al., 2018). Additionally, the use of long-acting injectable progestin hormonal contraceptives (particularly DMPA) has also been associated with increased women's susceptibility to HIV, however, this remains a topic of ongoing debate, with some studies showing an increased HIV risk (Heffron et al., 2012; Hapgood, 2020) while, others showed no differences (Myer et al., 2007; Shen et al., 2017).

TABLE 1 PrEP clinical trials demonstrating various efficacies in high-risk populations from different regions.

Clinical trials	Study population (regions)	PrEP drugs	PrEP efficacy -reduction in HIV incidence (%)	References
CAPRISA 004	African women (South Africa)	1% TFV gel	39	Abdool Karim et al. (2010)
Partners PrEP	Heterosexual couples (Kenya and Uganda)	Oral TDF-FTC	75	Baeten et al. (2012)
		Oral TDF alone	67	
TDF2	Heterosexual couples (Botswana)	Oral TDF-FTC	62	Thigpen et al. (2012)
iPrEx	MSM (South America, the United States, South Africa, and Thailand)	Oral TDF-FTC	44	Grant et al. (2010)
PROUD	MSM (England)	Oral TDF-FTC	86	McCormack and Dunn, (2015)
IPERGAY	MSM (France and Canada)	Oral TDF-FTC	86	Molina et al. (2015)

3 PrEP in HIV prevention

Since the main route of HIV infection in women is through sexual intercourse, many prevention strategies are aimed at protecting the FGT (Celum, 2011; Nicol et al., 2018). As a result, many different modalities have been tested, which include a vaginal ring containing dapivirine (Baeten et al., 2016), various microbicide gel formulations such as Carraguard (Skoler-Karpoff et al., 2008) and PRO2000 vaginal gels (McCormack et al., 2010) and spermicide gel formulations such as nonoxynol-9 (N-9) (Wilkinson et al., 2002). Besides topical gels and rings, implants containing tenofovir alafenamide (TAF) (Gunawardana et al., 2015) and long-acting injectable formulations containing for example cabotegravir (CAB) (Landovitz et al., 2018) are currently being tested for HIV prevention in PrEP clinical trials. Truvada® which is the co-formulated tenofovir disoproxil fumarate (TDF)/TFV and emtricitabine (FTC)) is a licensed oral PrEP drug that is also used for HIV prevention (Alvarez et al., 2011).

The concept of using TFV as PrEP in preventing HIV infections was initially investigated in macaques with simian immunodeficiency virus (SIV) (Person et al., 2012). During the 1990s, studies on TFV (a nucleotide reverse transcriptase inhibitor of HIV) previously known as (R)-9-(2-phosphonylmethoxypropyl)adenine (PMPA) demonstrated evidence of complete protection against SIV infections (Tsai et al., 1995; Person et al., 2012). The success observed in animal models was, however not fully translated in human HIV prevention clinical trials (Person et al., 2012). One of the major factors that attributed to these inconsistencies was low to no adherence, which limited PrEP exposure to tissues and cells targeted by HIV in areas such as FGT (Rohan and Sassi, 2009; Hu et al., 2015).

4 PrEP clinical trials: Efficacy in African women

Significant breakthroughs in using PrEP to prevent HIV infections have been observed in PrEP trials focused-on high-risk HIV-serodiscordant heterosexual couples and MSM (Baeten et al., 2012; Thigpen et al., 2012) (Table 1). The Partners PrEP trial, performed in HIV-serodiscordant heterosexual couples in Kenya and Uganda showed significant HIV reductions of 75 and 67% respectively, with oral TDF-FTC and with TDF alone (Baeten et al., 2012). The TDF2 trial which evaluated the effectiveness of TDF-FTC drugs in sexually active HIV negative heterosexual adults from Botswana; showed that TDF-FTC prevented new HIV infections, by demonstrating a 62% reduction in HIV incidence (Thigpen et al., 2012). In the Pre-exposure Prophylaxis Initiative trial (iPrEX) in MSM from South America, the United States, South Africa and Thailand, a daily single oral dose of Truvada® demonstrated a 44% reduction in HIV incidence (Grant et al., 2010). Similarly, in other MSM European studies testing Truvada® the Pragmatic open-label randomised trial of pre-exposure prophylaxis (PROUD) (Mccormack and Dunn, 2015) and the On-Demand Antiretroviral Pre-exposure Prophylaxis for HIV Infection (IPERGAY) in men who have sex with men (MSM) (Molina et al., 2015), both showed an 86% reduction in HIV incidence (Mccormack and Dunn, 2015; Molina et al., 2015). Results from these studies further supported the effectiveness of PrEP among MSM who are at risk of acquiring HIV (Mccormack and Dunn, 2015; Molina et al., 2015). Currently, in African women, the CAPRISA 004 trial remains the only trial that showed an overall 39% efficacy with a topical gel containing ARV (Table 1) (Abdool Karim et al., 2010). Furthermore, when the data was stratified according to

TABLE 2 PrEP clinical trials demonstrating low PrEP efficacies in high-risk populations from different regions.

Clinical trials	Study population (regions)	PrEP drugs	PrEP efficacy -reduction in HIV incidence %	References
FEM-PrEP	African women (South Africa, Kenya and Tanzania)	Oral TDF-FTC	4.7%	Van Damme et al. (2012)
VOICE	African women (South Africa, Uganda and Zimbabwe)	Oral TDF-FTC	−4.4%	Marrazzo et al. (2015)
		Oral TDF alone	−49%	
		1% TFV gel	14.5%	
FACTS-001	African women (South Africa)	1% TFV gel	6.52%	Delany-Moretlwe et al. (2018)

degree of adherence, women with high adherence had a corresponding reduction in HIV incidence by 54%, while women with low adherence had only a 28% reduction in HIV incidence (Abdool Karim et al., 2010).

Other PrEP clinical trial studies that focused primarily on at-risk African women demonstrated inconsistent levels of protection showing HIV incidence of −49% to 14.5% (Table 2). These include the FEM-PrEP trial (Van Damme et al., 2012) which evaluated, daily Truvada® in women from high-risk areas in South Africa, Kenya and Tanzania. This trial was, however, terminated following low efficacy largely attributed to lack of adherence (Corneli et al., 2016) and low drug concentrations in the FGT (Abdool Karim et al., 2011). The Vaginal and Oral Interventions to Control the Epidemic (VOICE) Microbicide Trial Network (MTN 003) trial was also conducted in women from high HIV prevalence areas in South Africa, Uganda and Zimbabwe (Marrazzo et al., 2015). Women were randomised to either oral TDF, oral TDF-FTC, TFV gel, or respective oral or vaginal placebos. Similar to the FEM-PrEP study, results from this trial showed no efficacy (Marrazzo et al., 2015). The moderate success of the topical CAPRISA 004 1% TFV gel trial led to the Follow on African Consortium for Tenofovir Studies (FACTS-001) (Delany-Moretlwe et al., 2018). The study was conducted at nine community-based clinical trial sites where it assessed the safety and efficacy of the precoitally applied 1% TFV gel in high-risk South African women. Here too, the FACTS-001 trial showed no significant reduction in HIV incidence between the active arm and the control (Delany-Moretlwe et al., 2018).

A potential explanation for the disparities in efficacy observed in these PrEP clinical trials could be due to differential drug penetration levels in the rectal compared to the vaginal mucosal tissues (Patterson et al., 2011; Janes et al., 2018). Data from Cottrell et al. (2015), observed that similar levels of adherence of two doses per week of Truvada® reduced HIV incidence by 90% in the MSM population of the iPrEX study, whereas in heterosexual women populations of the FEM-PrEP and VOICE studies low to no protection was observed (Cottrell et al., 2015). Additionally, the complex composition of the vagina's microbiome and inflammation may affect PrEP disposition in women (Patterson et al., 2011; Klatt et al., 2017; Janes et al., 2018; Mckinnon et al., 2018). These findings urge the need to better understand the mechanisms of drug availability and metabolism within the area of vulnerability, the FGT (Rohan and Sassi, 2009; Hu et al., 2015).

5 Compartmental heterogeneity in PrEP drug disposition

The FGT is a highly active and diverse immune environment, with a wide range of heterogeneous immune cells such as macrophages, dendritic cells, Langerhans cells, natural-killer cells, B and T-cells, making it highly susceptible to HIV infections (Hu et al., 2015; Nicol et al., 2018). The increased vulnerability of the FGT to HIV infections is also largely ascribed to the presence of a single columnar epithelium cell layer in the endocervix as opposed to the multi-layered squamous epithelium of the ectocervix (Nicol et al., 2018). These cell layers are vulnerable to micro-abrasions caused by friction during heterosexual intercourse allowing for easy access of HIV (Nicol et al., 2018). A previous study by Shen et al. (2014) also observed that even with intact epithelium, vaginal myeloid dendritic cells expressing HIV receptors can facilitate the capture and dissemination of HIV into the deeper mucosal tissue layers (Shen et al., 2014). The vulnerability of the FGT prompts the need for HIV prevention interventions that provide sufficient protection within all compartments exposed to the virus (Nicol et al., 2018).

Interventions such as PrEP should therefore provide optimal ARV drug concentrations that are sufficient in preventing HIV viral entry, transcription, and replication in HIV targeted cells (Cottrell et al., 2015). Drug exposure of PrEP in the FGT has previously shown variability in drug delivery and availability (Cottrell et al., 2015). To understand this variability; a 14-days open-labelled study by Patterson et al. (2011), demonstrated varying concentration levels of TFV, FTC and their respective active metabolites TFV-DP and FTC triphosphate (FTC-TP) in rectal, vaginal and cervical tissues, following a single dose of Truvada®. In rectal tissues, TFV and TFV-DP were detected throughout the 14-day period and concentrations were 100-fold higher when compared to cervical and vaginal tissues; while FTC

concentrations were 10 to 15- fold higher in vaginal and cervical tissue when compared to rectal tissue. The active metabolite FTC-TP was, however, only detected for 2 days in all tissues (Patterson et al., 2011). A prior study also compared ARVs drug exposure in cervicovaginal fluid and blood. In the FGT, NRTIs lamivudine (3TC), zidovudine (ZDV), FTC and TFV exhibited high drug concentrations relative to the blood. However, non-nucleoside reverse transcriptase inhibitor (NNRTI) efavirenz (EFV) and protease inhibitors (PIs) lopinavir (LPV) and atazanavir (ATV) exhibited low concentrations in the FGT when compared to the blood (Dumond et al., 2007). These results indicate the heterogeneity of drug disposition and their respective metabolites within sub-compartments of the same anatomical surface or different compartments (Patterson et al., 2011). These studies suggested that certain ARVs may or may not be good PrEP candidates according to their ability to penetrate certain areas (Dumond et al., 2007). In addition, these data further underscore the importance of understanding factors affecting drug pharmacokinetics in tissues that are highly susceptible to HIV infections.

These variations show differential drug penetration levels, giving an insight into the varying levels of protection against HIV observed in some PrEP trials (Cottrell et al., 2015). These discrepancies may be due to the interplay between these PrEP drugs and various membrane-bound proteins that mediate drug transport and availability (Cottrell et al., 2015). For example ARVs such as TFV, FTC and ZDV have been previously shown to be substrates of drug transporters P-glycoprotein (P-pg), multi-drug resistance protein-1 (MRP-1) and organic anion transporters-1 (OAT-1), respectively (Kis et al., 2010; Hu et al., 2015). These data indicated that intracellular and extracellular ARV drug levels can be predominantly regulated by certain drug transporters (Kis et al., 2010; Hu et al., 2015). Therefore, understanding the distribution and biological characteristics of drug transporters may help further define their roles in affecting PrEP efficacy.

6 Drug transporters involved in PrEP pharmacokinetics

Drug transporters are types of transmembrane proteins that are ubiquitously expressed in the human body in areas such as the gastrointestinal tract, in epithelial cells in the FGT, lungs, blood-brain barrier, endothelial cells, and liver cells (Zhou et al., 2013; Arruda et al., 2016). Drug transporters comprise of two superfamilies: the ATP-binding cassette (ABC) and Solute Carrier (SLC) proteins (Hu et al., 2015). The ABC proteins are a large family of efflux pumps that bind ATP and utilize its hydrolysis energy to transport molecules across and out of the cell membrane. This family comprises seven subfamilies of which three are the most relevant in the efflux of PrEP drugs (Petzinger and Geyer, 2006; Hu et al., 2015). These include (Figure 1):

i) the *ABCB* subfamily that comprises P-glycoprotein (P-gp),
ii) the *ABCC* subfamily that comprises multidrug and toxin extrusion proteins (MATE) and MRPs, and,
iii) the *ABCG* subfamily that comprises breast cancer resistance protein (BCRP) (Petzinger and Geyer, 2006; Hu et al., 2015; Nicol et al., 2018). The SLC proteins influx or uptake molecules across and into the cell membrane *via* ATP energy dependant carriers or through an electrochemical gradient (Lin et al., 2015). Subfamilies that are the most relevant in the uptake or influx of PrEP drugs include (Figure 1):

i) the *SLC22* subfamily that comprises OAT and organic cation transporters (OCTs),
ii) the *SLC28* subfamily that comprises concentrative nucleoside transporters (CNTs),
iii) the *SLC29* subfamily that comprises equillibrative transporters (ENTs) and
iv) the *SLCO* subfamily that comprises organic anion-transporting polypeptides (OATPs) (Petzinger and Geyer, 2006; Hu et al., 2015; Nicol et al., 2018).

Most of these drug transporters are localised on polarized cells, and regulate substrate distribution on the apical or basolateral surfaces of cells, contributing to the pharmacokinetics of several ARVs (Sissung et al., 2012). In previous studies, TFV and FTC have been shown as substrates of these ABC and SLC drug transporters (Hu et al., 2015; Nicol et al., 2018). This data indicated that the delivery and absorption of these drugs in cells is facilitated by drug transporters, establishing their emerging role as critical determinants in drug pharmacokinetics. This interaction has been noted especially in HIV target cells such as the immune cells of the FGT that include macrophages, vaginal epithelial cells, T cells, and dendritic cells expressing CD4 receptors (Hu et al., 2015; Taneva et al., 2016), and also in peripheral blood mononuclear cells (PBMCs) and epithelial cells of the intestinal and renal system (Kerb, 2006).

6.1 Role of drug transporters in the FGT

The extracellular accumulation of TFV and FTC in cells overexpressing certain drug transporters has been demonstrated in various studies focused on the FGT. Findings from these studies suggested that the delivery of effective PrEP drug concentrations to cells and tissues in the FGT is highly associated with the mRNA expression level and functionality of drug transporters (Grammen et al., 2014; Nicol et al., 2014; Hijazi et al., 2015; Taneva et al., 2016). Zhou et al. (2013) showed varying drug transporter expression levels in the FGT (vaginal and ectocervix tissues) and liver. mRNA expression was defined as ≤2% (undetectable), 2%–10% (low expression), 10%–50% (moderate expression) and 50%–100% (high expression)

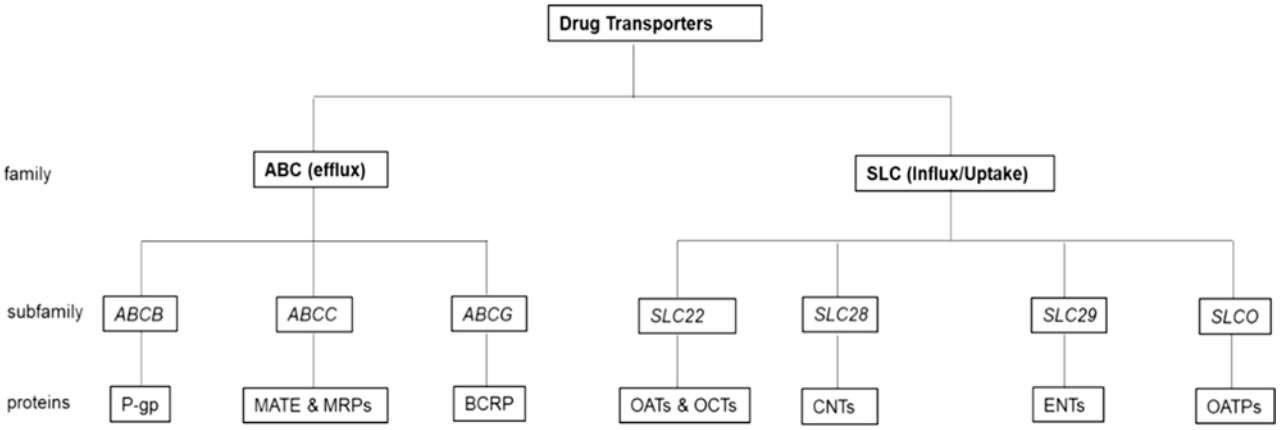

FIGURE 1
Drug transporters most relevant in the efflux and influx of PrEP drugs. In the ABC (efflux) family there are three-drug transporter subfamilies along with their respective proteins that are the most relevant in the efflux of PrEP drugs, while in the SLC (influx/uptake) family, there are four drug transporter subfamilies along with their respective proteins that are the most relevant in the uptake or influx of PrEP.

(Zhou et al., 2013). In the FGT, high mRNA expression of drug transporters (MRP-1, MRP-4, P-gp, BCRP, ENT-1 and OCT-2) was observed when compared to the liver, while MRP-2 and influx drug transporters OAT-1 and OAT-3 showed moderate and low mRNA expression as compared to the liver (Zhou et al., 2013). Similarly, a study by Taneva et al. (2016) also showed significantly low expression of uptake drug transporters OAT-1 and OAT-3 in vaginal epithelial cells and T-cells, which accounted for the poor permeability of TFV across the cell membranes and into the cells. Additionally, this study showed that the *in-vitro* transfection of T cells with the drug transporter OAT-1 increased TFV uptake, resulting in high intracellular drug accumulation (Taneva et al., 2016). These studies indicated that there is variability in drug transporter expression levels within different tissues. Therefore, analysing the expression levels of drug transporters in the FGT could aid in better understanding their role in the pharmacokinetics of drugs (Zhou et al., 2013; Taneva et al., 2016).

Nicol et al. (2014) showed high mRNA expression levels of efflux drug transporters P-gp and MRP-2 in vaginal tissues compared to colorectal tissue, while MRP-4 was only highly expressed in colorectal tissues. In contrast, uptake drug transporters OAT-1, OAT-3 and OATP1B1 exhibited extremely low to no expression in colorectal and vaginal tissues, respectively (Nicol et al., 2014). Additionally, immunohistochemistry that informed on the localisation of these drug transporters revealed high protein expression of P-gp and MRP-2 in vaginal epithelial cells compared to colorectal epithelial cells, while low to no protein expression of OAT-1 was observed in colorectal epithelial and vaginal cells, respectively (Nicol et al., 2014). Differences in protein localisation and expression suggested an increased expression of efflux drug transporters in vaginal tissues compared to colorectal tissues (Nicol et al., 2014). These data show that more drug is pumped out of cells in the vagina, while an increased expression of uptake drug transporters in colorectal tissues promoted an uptake of drugs (Nicol et al., 2014). These findings highlighted that inter-tissue variability in drug transporter expression may contribute to the greater intracellular accumulation of ARVs such as TFV and maraviroc in colorectal tissues compared to vaginal tissues (Nicol et al., 2014). High expression levels of efflux drug transporters P-gp, MRP-2 and BCRP in vaginal and endocervical tissues was also reported by Grammen et al. (2014). The study further established in intestinal cell lines-Caco-2 and vaginal epithelial cell lines-SiHa using specific drug transporter inhibitors, that ARV drugs darunavir, maraviroc and saquinavir are substrates of efflux drug transporters P-gp and MRP-2, which are likely to contribute to lower intracellular levels of these respective drugs (Grammen et al., 2014).

To further understand the role of drug transporters in the mucosal compartment, the relationship between the accumulation of topically applied PrEP drugs dapivirine, darunavir and TFV, and the expression of drug transporters was characterised in cervicovaginal cell lines (Hijazi et al., 2015). These included HeLa cell lines, VK2/E6E7, Ect1/E6E7 and End1/E6E7 derived from human cervical epithelial adenocarcinoma, primary vaginal, ectocervical and endocervical epithelial cells, respectively (Hijazi et al., 2015). Tenofovir significantly downregulated the mRNA expression of MPR5 in VK2/E6E7, while dapivirine significantly upregulated most MRP drug transporters in all cell lines. Darunavir stimulation also significantly upregulated the uptake drug transporter CNT3 in

all cells, while MRP3 was only significantly unregulated in VK2/E6E7 cell line (Hijazi et al., 2015). This characterisation by Hijazi et al. (2015) provided insight not only on the type of drug transporters present in the FGT but also how drug transporter disposition may be altered by the presence of certain drugs; which could assist in the assessment of ARV pharmacokinetics in the FGT. Furthermore, these findings could assist in the determination of suitable PrEP drug formulations that could provide sufficient drug concentrations to susceptible tissues and cells of the FGT (Hijazi et al., 2015).

6.2 Role of drug transporters in peripheral blood mononuclear cells

A study by Turriziani et al. (2008) determined the mRNA expression levels of drug transporters in PBMCs (isolated from buffy coats) from HIV infected individuals failing ARV therapy and HIV negative individuals. The mRNA expression levels of P-gp, MRP (-1,-4 and -5) was significantly higher in HIV infected individuals compared to HIV negative individuals (Turriziani et al., 2008). A higher inter-individual mRNA expression variability was also observed in HIV infected individuals, indicating a correlation between the presence of ARVs and drug transporter expression levels (Turriziani et al., 2008). Similarly, Bousquet et al. (2009) investigated if the singular or combined (dual or triple) use of TFV, FTC and EFV on PBMCs isolated from healthy donors disrupts mRNA drug transporter expression levels (Bousquet et al., 2009). Following a 20-h *in-vitro* incubation, a singular use of FTC induced MRP5, while TFV reduced MRP (-1,-5,-6) and P-gp mRNA expression in PBMCs (Bousquet et al., 2009). FTC was also shown to exhibit an inhibitory effect on the mRNA expression of efflux drug transporter MRP-1 in a dose-responsive manner. These findings suggest a correlation between the presence of FTC with MRP-1 expression (Bousquet et al., 2008). The use of ZDV was also previously shown to be associated with the upregulation of efflux drug transporters MRP-1 and MRP5 expressed on PBMCs (Jorajuria et al., 2004). Findings from these studies showed that an interaction between ARVs and drug transporters may alter drug transporter disposition by affecting mRNA expression levels; subsequently affecting intracellular drug accumulation (Jorajuria et al., 2004; Bousquet et al., 2009).

Contrary to these studies, Falasca et al. (2011) and Giraud et al. (2010) showed no correlations between the mRNA expression levels of efflux drug transporters and the presence of ARVs ritonavir (RTV), ATV, and LPV in PBMCs isolated from HIV infected patients (Giraud et al., 2010; Falasca et al., 2011). The study found no variation in the mRNA expression levels of P-gp and MRP drug transporters before and after ARV intake (Falasca et al., 2011). However, in a more recent study, a significant association was observed between ARVs and drug transporters P-gp, BCRP, MRP-1, ENT-2 and OCT-1 expressed on monocytes and monocyte-derived macrophages isolated from HIV negative individuals and HIV infected individuals receiving ARV therapy containing either abacavir, ATV, EFV, rilpivirine, TFV, 3TC, FTC, elvitegravir, dolutegravir, and cobicistat (Hoque et al., 2021). These findings showed that these associations could lead to sub-optimal intracellular drug concentrations, subsequently allowing HIV infections in HIV negative individuals or further HIV replication in HIV infected individuals (Hoque et al., 2021).

6.3 Role of drug transporters in the renal system

The entry of TFV into epithelial cells of the kidney tubule is mediated by the uptake drug transporters OAT-1 and OAT-3 expressed on its basolateral membrane (Cihlar et al., 2007), while the efflux of TFV into urine is mediated by efflux drug transporter MRP-4 expressed at the apical side of renal proximal tubules (Ray et al., 2006). These data together provide evidence that TFV is a substrate of OAT-1, OAT-3 and MRP-4 drug transporters expressed in renal tubules (Ray et al., 2006; Cihlar et al., 2007). TFV is also a substrate of the efflux drug transporter MRP-8 expressed in renal proximal tubules since higher cytotoxic concentrations of the drug were observed in cells overexpressing MRP-8 (Tun-Yhong et al., 2017). The uptake of ARVs cidofovir, adefovir and TFV was evaluated in human embryonic kidney (HEK293) cells transfected with uptake drug transporters OCT-2, OAT-1 and OAT-3 (Uwai et al., 2007). Results showed higher uptake of all ARVs through OAT-1 compared to OAT-3, while OCT-2 exhibited no uptake, indicating that OAT-1 plays a significant role in renal transport of these ARVs (Uwai et al., 2007). Similarly, renal secretion of FTC was mediated by MATE-1 which functionally acts as an efflux drug transporter, expressed on the apical side of renal proximal tubules (Reznicek et al., 2017).

These studies collectively provide insight that ARV drug levels are not only determined by drug adherence but also by other factors such as the presence of specific drug transporters and their expression levels. However, definitive conclusions on the full effects of drug transporters on ARV pharmacokinetics in at-risk groups such as young women especially in Africa have not been drawn. The paucity of data on African women warrants the need for new studies to fully understand:

i) the effect of ARVs on drug transporters expression,
ii) how varying drug transporter expression levels influence ARV penetration in vulnerable areas such as the FGT, and
iii) how different biological factors such as inflammation and polymorphisms may also affect drug transporter expression and function.

7 Biological factors modulating drug transporter expression and function

7.1 Genetic polymorphisms

Pharmacogenetic research has been used as a tool to determine individuals' susceptibility to certain diseases and for the customisation of drug therapies according to patient's genetic blueprint (Sissung et al., 2012; Castellanos-Rubio and Ghosh, 2019). As such, sequencing and genotyping technology have been widely used to identify and determine the effect of variants such as genetic polymorphisms in various genes. There are four types of genetic polymorphisms that have been shown to regulate genes (Ismail and Essawi, 2012). These include:

I. small insertions and deletions (InDels) which is a deletion or insertion in the DNA sequence (Boschiero et al., 2015),
II. interspaced or tandem repeat polymorphisms which are tandemly repeated nucleotides of approximately ≥2 base pairs (bp) in DNA sequences (Ismail and Essawi, 2012),
III. structure or copy-number variations (CNVs), polymorphisms which are various copies of differently sized segments of nucleotides in DNA sequences (Stankiewicz and Lupski, 2010) and
IV. single nucleotide polymorphisms (SNPs), which are point mutations of nucleotide bases within DNA sequences (Sissung et al., 2012; Yee et al., 2018).

Types of SNP variations include missense mutations or nonsynonymous substitutions which is a single nucleotide change within a codon, subsequently resulting in the coding of a different amino acid (Hunt et al., 2009). The presence of such mutations on protein binding sites may affect substrate binding, while those not found on protein binding sites may affect protein expression levels. For example, the missense mutation rs2273697 located on the efflux drug transporter gene *ABCC2* encoding MRP-2 results in a change from valine to isoleucine, on exon 417 (V417I), affecting its expression levels (Yee et al., 2018). Another type of SNP mutation is a silent mutation or synonymous substitutions, which are single nucleotide point mutations on a codon that do not result in an amino acid change (Hunt et al., 2009). However, these may still affect RNA transcription and stability that may affect mRNA expression levels and protein binding (Yee et al., 2018). For example, the SNP rs1045642 located on the efflux drug transporter *ABCB1* gene (3435C/T Ile1145Ile) encoding P-gp is a type of silent mutation that has been highly studied in drug pharmacokinetics (Sissung et al., 2012; Yee et al., 2018). This SNP has been also previously associated with low P-gp expression levels in the duodenum which correlated with an increase of digoxin plasma concentrations (Hoffmeyer et al., 2000). The presence of certain genetic variations in drug transporter genes has sparked a huge interest in further understanding their functional effect; especially since SNPs in certain drug transporter genes have been shown to modulate their function by affecting protein folding, expression levels, and their ability to bind substrates and regulate drug pharmacokinetics (Sissung et al., 2012; Yee et al., 2018).

SNPs involved in the pharmacokinetics of ARVs have led to adverse effects and varied ARV therapy outcomes amongst HIV infected patients (Shenfield, 2004; Arruda et al., 2016). Arruda et al. (2016) showed the association between SNPs in drug transporter genes and intolerance to ARVs in a cohort of HIV infected Brazilian participants (Arruda et al., 2016). Results showed an association between variations in *ABCC2* genes (rs3740066 and rs4148396) encoding MRP-2 and intolerance in patients taking regimens containing either LPV, RTV, indinavir or ATV PIs; while variations in *SLCO2B1* genes (rs2712816, rs12422149, rs1676885 and rs949069) encoding OATP2B1 caused intolerance in patients taking regimens containing stavudine or ZDV nucleotide reverse transcriptase inhibitors (NRTIs) (Arruda et al., 2016). The presence of the C allele on the *ABCC1* gene 198217C/T (rs212091) encoding MRP-1 and the TT genotype on the *ABCB1* gene 3435C/T (rs1045642) encoding P-gp; was also shown to be possibly associated with reduced gene expression in an HIV infected Brazilian participants receiving highly active antiretroviral therapy (HAART) regimens; subsequently affecting the efflux of ARV regimens containing PIs, leading to an increased risk of virological failure (Table 3) (Coelho et al., 2013).

Fellay et al. (2002) showed that the TT genotype on the *ABCB1* gene 3435C/T in an HIV infected Caucasian population was associated with low P-gp expression in PBMCs, affecting ARV concentrations (Fellay et al., 2002), However, a subsequent study showed that virological failure was associated with the CC genotype of the *ABCB1* gene 3435C/T instead of the TT genotype in HIV infected patients from the province of British Columbia in Canada (Brumme et al., 2003). To elucidate variations of the *ABCB1* 3435C/T SNP observed in these studies; prior results by Ameyaw et al. (2001) that assessed the frequency of this SNP in ten ethnic groups can be used (Ameyaw et al., 2001). Results showed noticeable differences in the SNP frequencies between African, Asian and European populations. The C allele was highly present in the African populations compared to Asian and European populations which exhibited high frequencies for the CT and TT genotypes (Ameyaw et al., 2001). Schaeffeler et al. (2001) also supported these findings by reporting a high frequency of the CC genotype in the *ABCB1* gene 3435C/T of West African and African American populations compared to the T allele (Schaeffeler et al., 2001). These findings could imply possible variations in drug transporter genes which could lead to varied ARV therapy outcomes in the African vs. Caucasian populations (Ameyaw et al., 2001; Schaeffeler et al., 2001).

Pharmacogenetic studies conducted with African populations have also shown high genetic diversity, which subsequently leads to varied drug transporter function and

TABLE 3 Effects of SNPs in drug transporter genes involved in the pharmacokinetics of ARVs in different ethnic groups.

SNPs	Ethnic group	ARVs	SNPs effect	Genotype causing effect	Genotype frequency Number of patients n (%)	References
ABCC2 224C/T (rs717620)	Thailand	TFV, Lamivudine, Efavirenz	Increased TFV plasma concentration	CC	CC 67 (57); CT 45 (39); TT 5 (4) n = 117	Manosuthi et al. (2014)
	Japanese	TFV, Emtricitabine, Darunavir, Ritonavir	TFV induced-KTD	CC	CC 18 (94.7); CT 1 (5.3); TT 0 (0) n = 19	Nishijima et al. (2012)
	Caucasian	TFV	TFV induced-KTD	CC	CC 9 (60.0); TT 1 (6.7); CT 5 (33.3) n = 15	Danjuma et al. (2018)
ABCC4 4131T/G (rs3742106)	Thailand	TFV	Increased TFV plasma concentration	TG/GG	TT 34 (22.7); TG 80 (53.3); GG 36 (24.0) n = 150	Rungtivasuwan et al. (2015)
ABCC1 198217C/T (rs212091)	Brazilian	Zidovudine, Lamivudine, Efavirenz/Nevirapine; Lopinavir/Ritonavir	Increased risk of virological failure	CC	CC 62 (84.9); TC 10 (13.7); CC 1 (1.4) n = 73	Coelho et al. (2013)
ABCB1 3435C/T (rs1045642)				TT	CC 37 (50.7); CT 25 (34.2); TT 11 (15.1) n = 73	
ABCC4 4976C/T (rs1059751)	Thailand	TFV	TFV induced-KTD	CC	CC 20 (37.0); TT 9 (16.7); CT 25 (46.3) n = 54	Likanonsakul et al. (2016)
ABCC4 3436A/G (rs1751034)	Caucasian	TFV	TFV induced-KTD	GG	AA 27 (64.3.); AG 9 (21.4); GG 6 (14.3) n = 42	Salvaggio et al. (2017)
SLCO1B1 463C/A (rs11045819)	African	Rifampin	Low plasma concentrations	CC	CC 30 (81); CA 7 (19) n = 37	Weiner et al. (2010)
	Ghanaian	Rifampin	High plasma concentrations	CC	CC 95 (84.1); CA 17 (15); AA 1 (0.09) n = 113	Dompreh et al. (2018)

expression levels; impacting drug pharmacokinetics differently as reviewed by Rajman et al. (2020). The presence of the *SLCO1B1* SNP 463C/A rs11045819 encoding the OATP1B1 protein was shown to impact rifampin pharmacokinetics differently in African populations (Weiner et al., 2010; Dompreh et al., 2018). A study by Weiner et al. (2010) showed that a high frequency of the CC genotype for the *SLCO1B1* 463C/A (rs11045819) gene was associated with low rifampin concentrations in African individuals during multidrug intensive therapy against TB (Weiner et al., 2010). However, in an African Ghanaian population also exhibiting a high frequency CC genotyping for the same gene taking standard first-line TB therapy; no effect on rifampin was observed (Dompreh et al., 2018) Table 3. Similarly studies by Chigutsa et al. (2011) and Gengiah et al. (2014) on the *SLCO1B1* (rs4149032) SNP both reported an associated between high SNP frequency and low rifampin plasma concentrations in TB and HIV-TB co-infected South African individuals taking rifampin (Chigutsa et al., 2011; Gengiah et al., 2014). This association was however, not observed in a TB infected Ghanaian population taking standard first-line TB therapy containing rifampin which also exhibited a high frequency for this SNP (Dompreh et al., 2018). The effect of the *ABCB1* SNP 4036G/G (rs3842) encoding P-gp on efavirenz was evaluated in different African populations. In an HIV infected South African population the AG and GG genotypes were significantly associated with decreased efavirenz plasma concentrations (Swart et al., 2012), however the GG genotypes in a healthy Ugandan population was associated with higher efavirenz plasma concentrations (Mukonzo et al., 2009). Similarly in Ethiopian and Tanzanian HIV infected populations the presence of the G allele was associated with higher efavirenz plasma concentrations, with higher frequency of the G allele observed in Tanzanians (Ngaimisi et al., 2013). These data indicated that the effects of SNPs may differ among African populations; therefore, in order to make definitive conclusions that a SNP affects the African population in a certain way, the functional or expressional effect SNPs should be tested among a wide range of different African populations as reviewed by Rajman et al. (2020). Despite the small sample size and sparsity of these data in various studies with African populations as reviewed in Rajman et al. (2020), these data do add to the understanding of how SNPs can impact drug pharmacokinetics in the African population. Together these studies could be used to adjust the standard recommended dose of ARV and TB drug for the African population that accounts for the presence of SNPs (Dandara et al., 2011; Rajman et al., 2020).

The effects of SNPs on drug transporter genes have also been associated with increased plasma concentrations of TFV. Studies on an HIV-infected cohort in Thailand showed higher TFV plasma concentrations in patients with the CC genotype on the *ABCC2* 224C/T gene (rs717620) encoding MRP-2 compared to patients with the *ABCC2* TT or CT genotypes (Table 3) (Manosuthi et al., 2014). Similarly, another Thailand

study by Rungtivasuwan et al. (2015) reported higher TFV plasma concentrations in HIV infected patients with the *ABCC4* 4131 (rs3742106) TG or GG genotypes (encoding MRP-4) compared to patients with the *ABCC4* TT genotype (Table 3) (Rungtivasuwan et al., 2015). These studies proposed that polymorphisms in these drug transporter genes may alter their gene expression or function in renal tubules leading to more effluxed drug and reduced glomerular filtration which is involved in TFV renal clearance; resulting in higher plasma concentrations (Manosuthi et al., 2014; Rungtivasuwan et al., 2015). A more recent study also showed in an HIV infected Caucasian population a significant association of the CC genotype in the *ABCC2* 224C/T gene with high TFV plasma concentrations, resulting in an increased risk of TFV induced-kidney tubular dysfunction (KTD) (Table 3) (Danjuma et al., 2018). Nishijima et al. (2012) previously confirmed that the CC genotype in the *ABCC2* 224C/T gene leads to high TFV plasma concentrations resulting in the induction of KTD or renal toxicity in Japanese patients (Table 3) (Nishijima et al., 2012). While the presence of the TT genotype in the *ABCC4* 4131T/G gene was not associated with TFV induced-KTD, the study attributed these findings to inter-individual variability in genetic backgrounds, which may cause patients to respond differently to the same drug (Kerb, 2006; Nishijima et al., 2012). Other SNPs on the *ABCC4* gene that have been associated with increased plasma TFV concentrations were evaluated in two studies; in an infected population from Thailand with the C allele on the ABCC4 4976C/T gene (Likanonsakul et al., 2016), and in a Caucasian population with GG genotype on the *ABCC4* 3436A/G gene (Salvaggio et al., 2017).

Reports obtained from these studies highlight the importance of understanding how the presence of SNPs may affect the efficacy of ARVs by affecting drug transporters' expression and function. Furthermore, these findings could be used to identify populations who are at a higher risk of developing adverse effects due to the presence of certain SNPs. However, most of these studies on SNPs in drug transporter genes affecting ARVs have been performed in non-African populations. Since SNP frequency differs significantly among different ethnic populations, more comprehensive investigations of SNPs in drug transporter genes are required, especially in the populations of African ethnicity. Data from populations of African descent will help us better understand how genetic diversity within these populations and SNPs influence drug transporter genes and subsequently lead to effective or ineffective therapy.

Pharmacogenetic research on polymorphisms present in drug transporter and drug-metabolizing genes is also vital in precision medicine, which enables the tailoring of effective therapies based on patients' genetic backgrounds (Hockings et al., 2020). The advantage of a precision medicine approach is the ability to predict putative ineffective therapies and possibly reduce adverse reactions (Hockings et al., 2020). Since there are reports of increased adverse reactions in patients in populations of African ethnicity taking ARVs, precision medicine is highly important in HIV prevention and treatment (Hockings et al., 2020). Patients taking ARV regimens containing EFV in SSA were predisposed to EFV-induced neuropsychiatric adverse reactions, due to specific genetic variants that reduced the functionality of cytochrome P450 2B6 (*CYP2B6*) the enzyme involved in EFV metabolism (Masimirembwa et al., 2016). One of the genetic variants of *CYP2B6* 516G>T (rs3745274) reported a frequency of 34%–50% in African populations compared to 15 and 20% in white populations (Masimirembwa et al., 2016). However, when the EFV dosages in ARV regimens were further titrated and reduced, there was improved EFV metabolism leading to significantly reduced neuropsychiatric adverse reactions (Gatanaga et al., 2007; Masimirembwa et al., 2016). These disparities in frequencies between the populations could lead to varied enzyme metabolism when similar drugs are used which may lead to ineffective drug metabolism and availability. This data highlights the importance of using pharmacogenetic research in guiding the development of precision medicine, especially in highly affected populations ensuring effective drug dosing, delivery, and metabolism.

7.2 Genital inflammation

Genital tract inflammation has been identified as an elevated profile of five of any of the nine pro-inflammatory cytokines (MIP-1α, MIP-1β, IP-10, IL-8, MCP-1, IL-1α, IL-1β, IL-6, and TNF-α) above the 75th percentile for each cytokine in a previous CAPRISA study (Abdool Karim et al., 2010; Masson et al., 2015) Genital inflammation creates an environment conducive for HIV infection and replication (McKinnon et al., 2018) increasing the risk for HIV by more than three-fold (Masson et al., 2015). The role of genital inflammation in undermining PrEP efficacy was demonstrated in a study by McKinnon et al. (2018). A 57% protective efficacy was found in women with no genital inflammation compared to 3% in women with genital inflammation (McKinnon et al., 2018). Although the mechanisms to explain why some people have comparatively high levels of genital inflammation while others do not are not fully understood, a likely driver of genital inflammation is bacterial vaginosis (BV), a microbial dysbiosis common in reproductively active women (Klatt et al., 2017). BV also plays a role in significantly modifying PrEP efficacy (Klatt et al., 2017). Klatt et al. (2017) showed that TFV gel reduced HIV incidence by 61% in women with a *Lactobacillus* dominant vaginal microbiome compared to only 18% in women with a *non-Lactobacillus* dominant vaginal microbiome (Klatt et al., 2017). Furthermore, sexually transmitted infections (STIs) (Masson et al., 2014) and exogenous hormonal contraceptives (HCs) (Deese et al., 2015) are also significantly associated with genital inflammation, through the secretion of pro-inflammatory

cytokines (Masson et al., 2014; Deese et al., 2015). The mechanisms by which all these factors individually or collectively interplay with drug transporter disposition, drug levels and in turn PrEP efficacy remains less well defined.

7.2.1 Role of inflammation-induced cytokines, in modulating drug transporter expression and function

The impact of inflammation on drug transporter expression and function has been examined in tissues of the intestines, kidneys, and blood-brain barrier (Petrovic et al., 2007; Saib and Delavenne, 2021). Despite the lack of data regarding the direct mechanisms involved; inflammation-mediated changes in drug transporter expression and function have been implicated in significantly impacting drug pharmacokinetics (Petrovic et al., 2007; Cressman et al., 2012). In an *in vitro* study, human brain cell lines (hCMEC/D3) treated with IL-6 and IL-1β, resulted in the downregulation of BCRP and P-gp expression levels (Poller et al., 2010). Additionally, the induction of IL-6 and IFN-γ on primary human hepatocytes was also shown to downregulate the mRNA expression levels of the efflux drug transporters BCRP, MRP-2, and MRP-3 and influx/uptake drug transporters OATP (-2B1,-1B1,-1B3) (Le Vee et al., 2009; Le Vee et al., 2011). Previous studies corroborated similar findings of inflammation IL-6 induced downregulation of P-gp expression on rat hepatocytes and human hepatoma cell lines (Sukhai et al., 2000). Similarly human cell line Caco-2 pre-treated with TNF-α significantly decreased intestinal P-gp expression, while IFN-γ had no effect (Belliard et al., 2004). In rats with endotoxemia, high levels of IL-6 and IL-1β reduced the mRNA expression levels of P-gp and MRP-2 in intestinal tissues (Arana et al., 2017). This lipopolysaccharide-induced endotoxemia in rats model showed that there was IL-1β induced downregulation of MRP-2 in enterocytes (Arana et al., 2020). These various cellular and small animal models demonstrate how infection and inflammation-induced cytokines can modulate drug transporter disposition. The caveat to the methods used in these models is that mRNA expression levels may not directly reflect functional proteins expressed. Future investigations are therefore required and should include both mRNA expression to its corresponding protein. There are also other biological factors related to inflammation that could also affect drug transporter disposition.

7.3 Role of toll-like receptors and pH in modulating drug transporter expression and function

7.3.1 Toll-like receptors-induced inflammation

TLRs are pattern recognition receptors, these receptors recognise pathogen-associated molecular patterns located on various microbes for example Pam3CSK-4 and lipopolysaccharide (LPS) which are TLR-2 and TLR-4 agonists, respectively (Cario, 2016; Suzuki et al., 2017). TLRs are activated by the binding to their respective agonists. This interaction causes the stimulation of appropriate signalling pathways in innate and adaptive immune cells which then regulate drug transporter expression levels (Cario, 2016; Suzuki et al., 2017). These TLR-mediated changes in drug transporter expression have been evaluated in the progression of atherosclerosis. To determine which downstream transcriptional signalling pathways were involved in this interaction; *in-vitro* testing using mouse macrophage cell lines (Raw 264.7) stimulated with TLR-2 and TLR-4 agonists Pam3CSK-4 and Lipid-A, respectively; were performed (Suzuki et al., 2017). Expression of myeloid differentiation primary-response protein 88 (MyD88), Toll/interleukin-1-domain-containing adapter-inducing interferon β (TRIF), liver X receptors (LXR), interferon regulatory factor 3 (IRF3), and the phosphorylation of nuclear factor kappa B (NF-kb) were determined with TLR-2 and TLR-4 activation. These results showed a differential pattern of significantly increased MyD88, LXR and NF-kb expression and low TRIF and IRF3 expression (Suzuki et al., 2017). This coincided with the significant upregulation of *ABCA1* expression levels, while ABCG1 expression levels were downregulated. TLR-2 stimulated cells pre-treated with NF-kb and p38 inhibitors MG-132 and SB203580, respectively suppressed the expression of *ABCA1*. These data provided evidence of the sensitivity of drug transporter expression to signal transduction–the MyD88, LXR, NF-kb and p38 pathways. These data provide support to the hypothesis that inflammation modulates the expression of drug transporters which can then lead to disease pathogenesis (Suzuki et al., 2017).

7.3.2 Sensitivity of drug transporter function to pH

The level of acidity or alkalinity (pH) in extracellular fluids is an additional factor that has been shown to modulate drug transporter function (Breedveld et al., 2007). The function of the efflux drug transporter BCRP was determined in Madin-Darby canine kidney (MDCK II) cells grown in pH adjusted media and exposed to methotrexate (MTX). At acidic pH levels, the efflux transporter BCRP pumped out MTX more efficiently when compared to physiological and basic pH levels. This data highlighted the possible clinical implications that the function of BCRP is pH-sensitive in the extracellular environment, thereby affecting intracellular concentrations and the effectiveness of MTX (Breedveld et al., 2007). These data suggest that pH is an additional factor that can also modulate drug transporter function which can then affect the effectiveness of drugs (Breedveld et al., 2007).

Collectively these studies demonstrate how inflammation-induced cytokines and TLRs are involved in regulating the expression of drug transporters in various tissues;

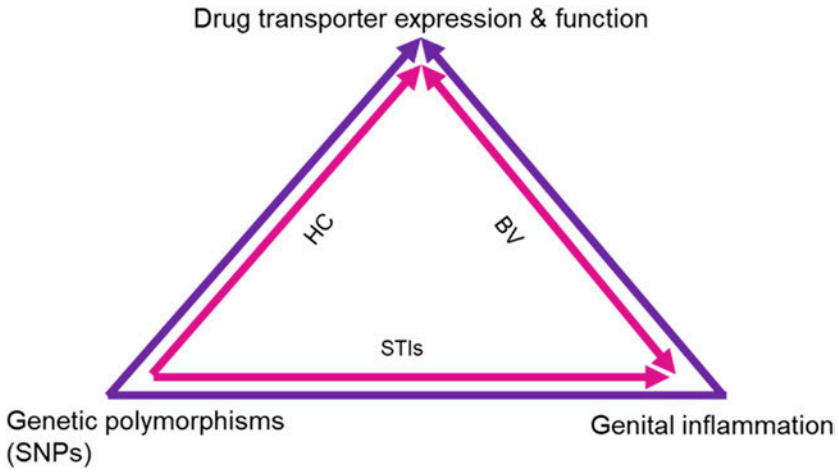

FIGURE 2
Proposed mechanism of effects on drug transporter expression and function. The schematic shows the intersection of different biological factors and SNPs in drug transporter genes that affect drug transporter expression in the FGT, renal system and blood, subsequently affecting PrEP efficacy. Genital inflammation and SNPs are known to directly affect drug transporter expression and functionality, while the combined use of HCs and ARVs also affects drug transporter expression and function. Additionally, the presence of STIs and BV are shown to contribute to genital inflammation which in turn affects drug transporter expression and function. HC, Hormonal contraceptives; BV, Bacterial vaginosis; STIs, Sexually transmitted infections.

subsequently altering intracellular and plasma drug concentrations, thereby affecting drug pharmacokinetics and efficacy. Inflammation mediated changes in drug transporter expression are however mostly based on animal models and cell lines (Cressman et al., 2012; Saib and Delavenne, 2021), thereby warranting the need for comparative *in-vivo* human studies. Future studies should also elucidate how BV, HC and STIs-induced genital inflammation contribute to drug transporter expression and function, subsequently, predisposing women to HIV infections, even during PrEP intake. Therefore, additional studies are needed to understand the interplay between inflammation and drug transporter expression, especially in sites highly susceptible to HIV such as the FGT and blood. Findings from such studies would provide a better understanding of how the presence of systemic and genital inflammation may alter drug transporters subsequently affecting ARV pharmacokinetics. Further elucidation of these factors either individually or collectively will aid in understanding disparities in PrEP efficacies observed in PrEP trials. This is especially important in highly susceptible groups such as African women from HIV endemic settings where PrEP is advocated as the standard of care for HIV prevention.

Together these studies show evidence that there may be an inextricable link between the expression and function of drug transporters with genetic polymorphisms, TLRs, pH and genital inflammation, which is further influenced by the presence of BV, STIs and HCs (Figure 2). Subsequently these factors may significantly affect drug concentrations and potentially drug efficacies.

8 Conclusion

The current review provides evidence that the FGT, renal system and blood are subject to a variety of host biological factors that may undermine PrEP efficacy by affecting drug transporter expression levels and function. These aforementioned studies show how drug transporters are increasingly recognised as key determinants in drug pharmacokinetics and response. However, their contributions to the inconsistent efficacies seen in PrEP clinical studies in African women from regions with high HIV infection rates such as South Africa, have not been elucidated. Characterising the expression level of drug transporters in the blood and FGT from a vulnerable population will better define the biological factors underlying compartment variation in drug exposure during oral PrEP in at-risk African women. In turn, we may be able to better understand why African women remain susceptible to HIV despite PrEP interventions. Additionally, findings from such studies will shed an important light on how the genetics and the biology of the mucosal environment may play a pivotal role in modifying drug transporter expression, subsequently modulating HIV risk. Understanding these data may also aid in the development of more effective, safe and optimal delivery systems that facilitate consistent effective dosage and usage of appropriate PrEP drugs.

Author contributions

Study conceptualisation was by DA, NZ, and PS. Manuscript writing (original draft) was conducted by NZ. Reviewing and edit suggestions for final manuscript was conducted by DA, PS, AS, SN, and VR. Final manuscript edited by NZ and accepted by all authors.

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Conflict of interest

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UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

07 March 2023

Ms Nomusa Margaret Zondo (213501374)
School of Laboratory Medicine & Medical Science
Medical School

Dear Ms Zondo,

Protocol reference number: BREC/00002195/2020
Project title: Drug transporter expression and genetic polymorphisms in women from HV endemic settings
Degree Purposes: PhD

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	**03 February 2023**
Expiration of Ethical Approval:	**02 February 2024**

I wish to advise you that your application for Recertification received on 27 February 2023 for the above protocol has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 11 April 2023.

Yours sincerely

……
Ms A Marimuthu
(for) Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

03 February 2021

Ms Nomusa Margaret Zondo (213501374)
School of Lab Med & Medical Sc
Medical School

Dear Ms Zondo,

Protocol reference number: BREC/00002195/2020
Project title: Drug transporter expression and genetic polymorphisms in women from HV endemic settings
Degree Purposes: PhD

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 03 February 2021. Please ensure that outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is subject to national and UKZN lockdown regulations, see (http://research.ukzn.ac.za/Libraries/BREC/BREC_Lockdown_Level_1_Guidelines.sflb.ashx). Based on feedback from some sites, we urge PIs to show sensitivity and exercise appropriate consideration at sites where personnel and service users appear stressed or overloaded.

This approval is valid for one year from 03 February 2021. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 09 March 2021.

Yours sincerely,

[redacted]

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

This is to certify that

NOMUSA ZONDO

has won the prize for

2nd Prize Oral Presentation

at the

School of Laboratory Medicine and Medical Sciences

Research Day 2022

On
30 November 2022
Nelson R Mandela Medical School

[redacted]

Prof Musa Mabandla
Dean and Head of School

[redacted]

Prof Bongani Nkambule
Academic Leader Research

Poster 1: IDA 2022

SNPs in drug transporter genes increase circulating plasma tenofovir drug levels in healthy South African women exposed to PrEP

Nomusa M. Zondo[1,2], Parveen Sobia[1,2], Aida Sivro[1,2], Lara Lewis[1], Leila E Mansoor[1,2], Veron Ramsuran[1,2], Derseree Archary[1,2]

[1]Centre for the AIDS Program of Research in South Africa, University of Kwa-Zulu Natal, Durban, South Africa.
[2]University of KwaZulu-Natal, Department of Medical Microbiology, School of Laboratory Medicine and Medical Sciences, Durban, South Africa.

Introduction

Pre-exposure prophylaxis (PrEP) as oral [Truvada®] or topical [1% tenofovir (TFV) gel] formulations in African women have produced inconsistent levels of protection against HIV infections. Transmembrane ATP-binding cassette (ABC) drug transporter proteins expressed in the female genital tract (FGT) and peripheral blood have been implicated as key regulators of PrEP pharmacokinetics. Genetic mutations such as single nucleotide polymorphisms (SNPs) in these drug transporter genes modulate their expression levels and function, subsequently affecting intracellular drug levels and efficacy. We therefore, investigated the association between SNPs in ABC drug transporter genes and circulating plasma tenofovir-diphosphate (TDF-DP) drug levels in n=402 healthy South African women taking oral Truvada® as PrEP from the CAPRISA 082 observational study.

Methods

Healthy South African women from the CAPRISA 082 observational study taking oral Truvada® n=402

Genomic DNA extracted using the QuickExtract™ DNA Extraction Solution

SNP genotyping conducted with Real-Time PCR - TaqMan™ Genotyping Master Mix and the TaqMan® SNP genotyping assays

Table 1: Selected SNPs in ABC drug transporter genes relevant in tenofovir pharmacokinetics

ABC drug transporter gene	SNPs
ABCC1 (MRP1)	198217C/T rs212091
ABCC2 (MRP2)	1249A/G rs2273697
ABCC4 (MRP4)	3463C/T rs1751034
ABCC4 (MRP4)	4131A/C rs3742106
ABCC4 (MRP4)	4976A/G rs1059751
ABCB1 (P-gp)	3435A/G rs1045642

Results

i. Single SNP analysis suggested an association between high plasma tenofovir drug levels and the AC genotypes *ABCC4 (*MRP4) 4131A/C SNP (p= 0.048) (**Figure 1**).

ii. Univariable analysis suggested two-fold more plasma tenofovir drug levels for the CT (p=0.020) and TT genotypes (p=0.024) compared to the CC genotype for the *ABCC4* 3463C/T SNP **(Table 2).**

iii. Multivariable analysis suggested:
- Two to three-fold more plasma tenofovir drug levels for the CT (p=0.002) and TT genotypes (p=0.014) compared to CC genotype for the *ABCC4* 3463C/T SNP.
- 46% less plasma tenofovir drug levels for the AA genotype (p=0.018) compared to the AG genotype for *ABCC4* 4976A/G SNP **(Table 2).**

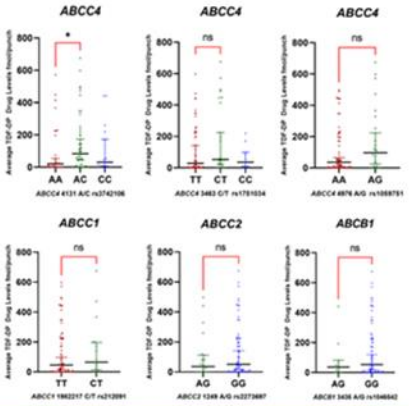

Figure 1: Association between average plasma tenofovir-diphosphate (TDF-DP) drug levels and SNPs in ABC drug transporter genes.

For single SNP analysis a significant association between high plasma tenofovir drug levels was only observed with the *ABCC4* (MRP4) rs3742106 SNP. No statistical significance was observed for all other SNPs. A Mann Whitney U-test was used to determine significance.(Asterisks p< 0.048).

Table 2: Association between genotype frequency and logged plasma tenofovir drug levels using linear mixed models

Gene	Genotype	Univariable adjusted exp(Estimate)[1]	Univariable adjusted p-value[1]	Multivariable adjusted exp(Estimate)[2]	Multivariable adjusted p-value[2]
ABCB1 (P-gp) 3535A/G rs1045642	AA	0.75	0.545	0.5	0.133
	AG	0.64	0.132	0.62	0.137
	GG	1		1	
ABCC1 (MRP1) 198217C/T rs212091	CT	1.09	0.736	0.89	0.611
	TT	1		1	
ABCC2 (MRP2) 1249A/G rs2273697	AG	0.88	0.647	0.92	0.784
	GG	1		1	
ABCC4 (MRP4) 3463C/T rs1751034	CT	2.21	0.020*	2.95	0.002*
	TT	2	0.024*	2.13	0.014*
	CC	1		1	
ABCC4 (MRP4) 4131A/C rs3742106	AA	1.11	0.761	0.69	0.329
	AC	1.25	0.469	0.98	0.952
	CC	1		1	
ABCC4 (MRP4) 4976A/G rs1059751	AA	0.68	0.121	0.54	0.018*
	GG	0.6	0.212	0.49	0.218
	AG	1		1	

[1]adjusted for time in study and BMI at enrolment, [2]adjusted for time in study and BMI at enrolment and all other polymorphisms, *p-value <0.05, significant. Linear mixed models were used (logged tenofovir drug levels). Model estimates were exponentiated for interpretation as logged drug levels were modelled.

Conclusion

High plasma tenofovir-diphosphate drug levels in healthy South African women taking oral PrEP were significantly associated with three SNPs in the *ABCC4* gene. These findings suggested that these SNPs may affect PrEP pharmacokinetics by increasing circulating tenofovir drug levels, consequently resulting in low intracellular drug levels of the active tenofovir-diphosphate metabolite . This study could inform on the genetic basis in African women for sub-optimal PrEP dosing measured by the intracellular levels of the active tenofovir-diphosphate metabolite which may undermine the effectiveness of PrEP.

Acknowledgements:
Special thanks to the Department of Science and Technology, the Centre for the AIDS Programme of Research in South Africa (CAPRISA), the CAPRISA mucosal immunology team and the University of KwaZulu-Natal Medical Microbiology Department for research support. This research was funded by the Department of Science and Innovation (DSI)-National Research Foundation (NRF) Centre of Excellence in HIV Prevention (Grant No. 96334) and the EDCTP fellowship (Grant No. TMA2017DF-1960).

Poster 2: ICHG 2023

CAPRISA
CENTRE FOR THE AIDS PROGRAMME OF RESEARCH IN SOUTH AFRICA

PRESENTER
Nomusa M. Zondo[1,2], Parveen Sobia[1,2], Aida Sivro[1,2,3], Sinaye Ngcapu[1,2], Leila E Mansoor[1], Lara Lewis[1], Veron Ramsuran[1,2], Benjamin Anthony[2]
[1]Centre for the AIDS Programme of Research in South Africa (CAPRISA), Mucosal Immunology Department, Durban, South Africa
[2]University of KwaZulu-Natal, Department of Medical Microbiology, School of Laboratory Medicine and Medical Sciences, Durban, South Africa
[3]JC Wilt Infectious Disease Research Centre, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada

SNPs ALTER GENE EXPRESSION & TENOFOVIR IN AFRICAN WOMEN ON PrEP

Background

Clinical trials testing pre-exposure prophylaxis (PrEP) as oral [Truvada®] or topical [1% tenofovir (TFV) gel] formulations in African women have produced inconsistent levels of protection against HIV infections.

- ❖ Single nucleotide polymorphisms (SNPs) in ATP-binding cassette (ABC) drug transporter proteins modulate their expression levels and function, subsequently affecting tenofovir pharmacokinetics and efficacy.
- ❖ We therefore, investigated the association between SNPs in ABC drug transporter genes, their mRNA expression levels and circulating plasma tenofovir in healthy South African women taking oral PrEP - Truvada®.

Methods

N=393
Sexually active black South African women from KZN 18-30 years taking oral PrEP Truvada® from the CAPRISA 082 observational study

Selected SNPs

Selected drug transporters for mRNA expression

ABC drug transporter genes
MRP2 (ABCC2)
MRP4 (ABCC4)
P-gp (ABCB1)

SNP Genotyping & mRNA expression
- Genomic DNA and RNA extracted from cytobrushes (FGT) and buffy coats (blood).
- TaqMan™ Genotyping Master Mix & SYBR™ Green Master Mix (mRNA expression) used.
- Quantitative-PCR method used.

Statistics
- Linear mixed models were used to determine the associations between SNPs and logged plasma tenofovir drug levels
- Kruskal-Wallis test was used to determine associations between SNP genotypes and mRNA expression levels

Conclusions

Our findings suggested that: *ABCC4* SNPs affect drug transporter mRNA expression and circulating tenofovir differently in African women on oral PrEP.

- ❖ The *ABCC4* 3463C/T SNP was associated with higher plasma tenofovir, suggesting reduced intracellular tenofovir, affecting PrEP efficacy.
- ❖ The *ABCC4* 4976A/G SNPs was associated with reduced plasma tenofovir, suggesting increased intracellular tenofovir. However increased *ABCC4* mRNA expression was also observed, which would result in higher plasma tenofovir. We hypothesise that haplotypes in this gene or influx/uptake drug transporters may counteract the effects of this SNP on expression.
- ❖ These pharmacogenetic findings could be used to identify clinically relevant SNPs in tenofovir pharmacokinetics and tailor PrEP dosage for African women towards implementing a precision medicine approach.

Results

SNPs in the *ABCC4* gene alter circulating tenofovir differently

Multivariable analysis suggested:
- ❖ Two to three-fold more plasma tenofovir in individuals with the CT (p=0.002) and TT genotypes (p=0.014) compared to CC genotype for the ***ABCC4* 3463C/T SNP**.
- ❖ 46% less plasma tenofovir for individuals with the AA genotype (p=0.018) compared to the AG genotype for the ***ABCC4* 4976A/G SNP**

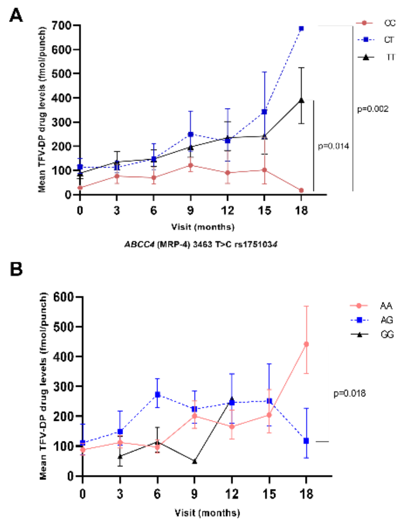

Figure 1: Mean tenofovir drug levels over time by genotype. A) *ABCC4* 3463T>C (CT p= 0.002 and TT p= 0.014) and **B)** *ABCC4* 4976A>G (p=0.018) SNPs were significant over time. Multivariant analyses used and tenofovir levels were measured from 3 months after PrEP initiation. Non-significant data - ns, significant data - p values <0.05. Mean standard error - error bars.

One *ABCC4* SNP is associated with altering *ABCC4* mRNA expression levels

Kruskal-Wallis tests suggested:
- ❖ In the female genital tract no significant associations between all SNPs and drug transporter mRNA expression levels were observed.
- ❖ In the blood only **the *ABCC4* 4976A>G SNP was** significantly associated with increased drug transporter mRNA expression. For this SNP *ABCC4* mRNA expression levels for individuals with the AA genotype were significantly higher when compared to individuals with the and AG genotypes (p=0.0132).

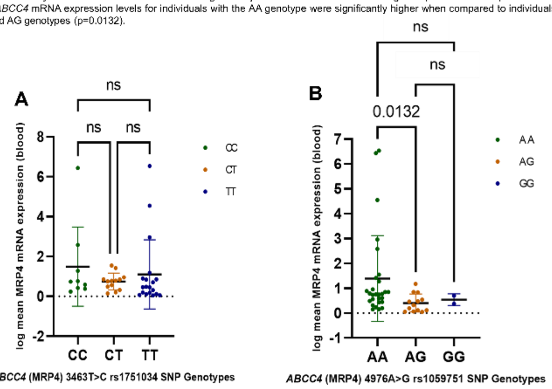

Figure 2: Mean *ABCC4* mRNA expression levels by genotype in the blood. A) the *ABCC4* 3463T>C SNP was not significant **B)** *ABCC4* 4976A>G SNP mRNA expression levels significantly different between AA and AG genotypes (p=0.0132). Analyses: Kruskal-Wallis test. Non-significant data - ns, significant data - p values <0.05. Mean standard error - error bars.

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