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Drug transporter mRNA expression and genital inflammation in South African women on oral pre-exposure prophylaxis (PrEP)

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Abstract

Globally HIV remains a major public health problem. In sub-Saharan Africa most new HIV infections occur in adolescent girls and young women. Previously tested antiretroviral drugs as different pre-exposure prophylaxis (PrEP) formulations have shown inconsistent levels of protection against HIV in African women. Besides adherence, biological factors such as drug transporter proteins are increasingly recognized as key modulators of PrEP levels. Drug transporter mRNA expression levels has been significantly correlated to altered PrEP levels in-vitro in different tissues, with inflammation identified as a further modifier of drug transporters mRNA expression and thus PrEP levels. We therefore, aimed to determine possible concordance between drug transporter mRNA expression in the female genital tract (FGT) and blood of N = 45 South African women taking oral PrEP-Truvada® [TDF/FTC)] over 6 months for HIV prevention. Additionally, we determined associations between drug transporter mRNA expression, genital inflammation, and blood-tenofovir diphosphate (TFV-DP). mRNA-expression of four efflux P-gp; MATE-1; MRP-2; MRP-4 and two influx OAT-1 and OAT-3 drug transporters were determined by qRT-PCR. Multiplexed technology was used to measure 27 cytokines to define genital inflammation. Significant positive correlations of mRNA expression for P-gp, MATE-1, MRP-2, and MRP-4 were observed between the FGT and blood at 3- and 6-months post-PrEP initiation ($p < 0.05$). For OAT-1 however, significant positive correlations were observed pre- and post-PrEP exposure ($p < 0.05$). Linear-mixed models showed moderate associations between FGT cytokines and drug transporter mRNA expression, with a direct relationship observed between MIP-1β concentration and MATE-1 mRNA expression. Similarly, PLS-DA showed that in women with genital inflammation, consistently higher mRNA expression of MATE-1 was observed compared to women without genital inflammation. No significant associations were observed between drug transporter mRNA expression and blood TFV-DP. Our results suggest that drug transporters may be similarly expressed in the FGT and blood. Furthermore, genital inflammation may modify PrEP levels by altering drug transporter mRNA expression. Collectively, our data may be used to better understand biological factors that may affect PrEP efficacy in African women who remain vulnerable to HIV.

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Introduction

Antiretrovirals (ARVs) as oral pre-exposure prophylaxis (PrEP) Truvada® [a combination of tenofovir disoproxil fumarate (TDF) and emtricitabine (FTC)] or topical tenofovir gels are some of the most effective strategies used to prevent HIV infections [1–3]. 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 sites of sexual transmission [4–7]. The CAPRISA 004 1% tenofovir gel trial that conferred 39% protection against HIV in African women [8] and the Partners PrEP trial in HIV-serodiscordant heterosexual couples in Kenya and Uganda showed significant protection of 75 and 67% respectively, with oral TDF-FTC and with TDF alone [9]. 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 [1].

Drug transporters are transmembrane proteins expressed in various cells of the body and comprise two superfamilies: the ATP-binding cassette (ABC) efflux proteins and Solute Carrier (SLC) influx or uptake proteins [10, 11]. PrEP candidates such as tenofovir and emtricitabine are substrates of various drug transporters such as the efflux drug transporters multidrug resistance associated protein (MRP)−1, MRP-2, MRP-3, MRP-4, P-glycoprotein (P-gp), multi-drug and toxin extrusion protein (MATE)−1 and the influx drug transporters organic anion transporters (OAT)−1 and OAT-3 [10, 12]. This interaction between ARVs and drug transporters has underscored drug transporter expression and function as critical in the delivery and availability of ARVs in HIV-vulnerable sites such as the FGT and peripheral blood [13].

The FGT offers a unique anatomy and physiology where locally expressed drug transporters are likely impacted by the milieu to support all necessary functions [14]. This is important since the sufficient delivery and absorption of tenofovir and emtricitabine is dependent on drug transporters. However, varying levels in the mRNA or protein expression of drug transporters relevant in tenofovir and emtricitabine pharmacokinetics, have been shown to impact drug efficacy [15–18]. Studies comparing drug transporter mRNA expression levels between the liver, colorectal tissues, and FGT showed moderate to low mRNA expression of influx drug transporters OAT-1 and OAT-3 and moderate to high expression of efflux drug transporters P-gp, breast cancer resistance protein (BCRP), MRP-4 and MRP-2 in the FGT [15, 16, 18–20]. 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 [18]. 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 [15–18]. 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 [17] and non-human primates [21].

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 equilibrative nucleoside transporter (ENT-2) and organic cation transporters (OCT-1) [22, 23]. These significant associations suggest that the correlations between ARVs and drug transporter mRNA expression levels could undermine therapy through sub-optimal intracellular drug concentrations. Sub-optimal drug levels would permit HIV replication in HIV infected individuals [22]. Similarly, the mRNA expression levels of BCRP in CD8 T cells [24] and P-gp in PBMCs [25] were also significantly increased in HIV infected individuals on ARVs compared to healthy controls [24, 25]. 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 were found [26], while emtricitabine reduced mRNA expression of MRP-1 in a dose-responsive manner [27]. Collectively these studies illustrate 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 in HIV-infected individuals, and not in healthy individuals taking PrEP. Furthermore, all of the above-mentioned studies evaluated drug transporter mRNA expression in either Caucasian and Asian [15–19] or in people living with HIV [20–26, 28]. This makes it difficult to extrapolate their findings to African populations to determine if drug transporter mRNA expression is altered upon PrEP exposure in the absence of HIV or in people living with HIV. Additionally, the impact of other biological factors remains less well defined for example how the local milieu affects the mRNA expression and function of drug transporter proteins.

Besides ARVs, the effect of inflammatory cytokines on drug transporter mRNA expression levels and function

have been evaluated and implicated in significantly impacting drug pharmacokinetics [29, 30]. In studies undertaken to find associations between cytokines and drug transporter mRNA expression, conflicting results have been observed, most likely due to differences in experiment designs and cell types [31]. Cytokines affect cells and tissues differently, therefore cytokine-mediated inflammation can modify mRNA gene expression and lead to increased or decreased expression of drug transporter proteins, such as P-gp, OAT-1 and a wide range of MRPs [32–35]. Genital inflammation has been previously defined as the elevation of any of these five of nine inflammatory cytokines and chemokines—macrophage inflammatory proteins (MIP)−1α, MIP-1β, interferon gamma-induced protein (IP)−10, interleukin (IL)−8,, IL-1α, IL-1β, IL-6, monocyte chemoattractant protein (MCP)−1 and tumor necrosis factor (TNF)-α above the 75th percentile [36]. Data from the CAPRISA 004 1% tenofovir gel trial underscores the key role of genital inflammation as a significant modifier for both HIV risk and for undermining PrEP efficacy [37]. The impact of genital inflammation in the cervicovaginal compartment on drug transporter mRNA expression remains less well defined. Different in-vitro studies have shown that the treatment of human cell lines derived from brain, colorectal adenocarcinoma cells and hepatocytes 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 drug transporters organic anion transporting polypeptides (OATP)−2B1, OATP-1B1 and OATP-1B3 [32, 33, 35, 38]. These data suggest that inflammation affects the mRNA expression levels of various efflux and influx drug transporters in different tissues. However, studies have shown no correlations between inflammation in the systemic compared to the genital compartment [31]. Therefore, there is a need to further elucidate if there are inflammation-mediated effects in the FGT on drug transporter mRNA expression levels and if they can have a direct impact on circulating and local drug levels, toxicity, and possibly PrEP efficacy.

Currently, there is a paucity of studies evaluating drug transporter 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 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 aims to therefore determine the mRNA expression levels of efflux and influx drug transporter genes relevant in tenofovir pharmacokinetic disposition in the FGT and blood. Additionally, we wanted to determine two associations: firstly, between drug transporter mRNA expression profiles and circulating tenofovir-diphosphate (TFV-DP) drug levels; and secondly between drug transporter mRNA expression profiles and inflammatory cytokines in the genital tracts of healthy South African cisgender women offered oral PrEP (TDF/FTC).

Materials and methods

Study population and design

The current study used previously collected blood and genital tract samples from the CAPRISA 082 observational study [39]. The CAPRISA 082 study aimed to determine oral PrEP initiation and adherence in young, sexually active women from South Africa. The inclusion criteria were as follows: the participants had to be female, aged 18 to 30 years old, have negative HIV and pregnancy tests at enrolment, be sexually active "(sexual intercourse at least three times in the last 3 months)" and be able to provide written informed consent, locator information for retention in the study and agree to adhere to the study procedures and visits. Exclusion criteria were as follows: an HIV-positive status and any medical or mental health condition that could prevent study participation and informed consent. This study recruited black cisgender women (who will further be denoted as women in this manuscript) from two high HIV incident CAPRISA Clinical Research Sites—urban eThekwini and rural Vulindlela in KwaZulu-Natal, South Africa between March 2016 and February 2018. 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 [300 mg TDF and 200 mg FTC]. Three sets of specimens were collected and stored every three months for all women who enrolled into this study. These specimens included buffycoats from the blood and cytobrushes and soft-cups specimens from the female genital tract. For any of the visits in the study, if the women were menstruating, we deferred them to a later time to take the required genital and blood samples. Tenofovir-diphosphate (the active form of tenofovir or TFV-DP) drug levels (fmol/punch) were measured from dried blood spots for a subset of participants who had 6 months or more of oral PrEP use and no missed study visits while on oral PrEP. This was done using a modified liquid chromatography-tandem mass spectrometry assay (Division of Clinical Pharmacology, University of Cape Town, SA) [40]. 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.

[39]. This observational study was approved by the Biomedical Research Ethics Committee (BREC) at the University of KwaZulu-Natal (BREC/0002195/2020).

RNA extraction and cDNA synthesis

Previous literature was used to select ABC and SLC drug transporter proteins, which are relevant in the pharmacokinetics of tenofovir and emtricitabine [10, 12, 18]. 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. The resulting cDNA was diluted 1 in 5 and used to determine drug transporter mRNA expression levels.

Quantitative real-time PCR (qRT-PCR)

Quantitative RT-PCR was performed to compare the mRNA expression levels of specific drug transporters in the 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 ATGCTGTTTCCC ACCTCTTTG; reverse TCCAACCTTCTGATTTCC ACTC); MRP-2 (forward TAATGGTCCTAGACAACG GG, reverse GGGCCTTCTGCTAGAATTT); MRP-4 (forward GGACAAAGACAACTGGTGTGCC, reverse AATGGTTAGCAC-GGTGCAGTGG); OAT-1 (forward GGGCACCTTGATTGGCTATGTC, reverse GATGAC AAGGAAGCCCACAAGC); OAT-3 (forward ACTCGG GTACTGCTACACCT, reverse CAGGTCACTTGCGGT GTACT); P-gp (forward CCCATCATTGCAA-TAGCAG G, reverse TGTTCAAACTTCTGCTCCTGA) and the housekeeping gene Beta-actin (forward TCCTTCCTG GGCATGGAGT, reverse AGCACTGTGTTGGCG-TACAG). Optimal conditions included 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. Negative controls used nuclease free water instead of the template and a melting curve (at 95 °C) were performed after each run, to determine non-specific PCR products. Only expressions with CT < 35 values were considered positive in the expression data. Relative mRNA expression levels of target genes were calculated using the comparative CT (2^ΔΔ Ct) method [41], and expression was normalized using the endogenous reference gene Beta-Actin as described previously by Brugè et al. [42]. This method calculates the difference initially between the housekeeping gene and the gene of interest and also does a further calculation using a calibrator or reference sample (calibrators are the same samples run in each plate). The results are shown as fold change which is the gene expression of the gene of interest relative to the gene expression of the calibrator or reference sample, normalized to the housekeeping gene. This method also accounts for negative or positive differences between the housekeeping gene and the gene of interest [41].

Cytokine/chemokine measurements

Cytokine/chemokine concentrations were determined using soft-cup specimens derived from the FGT as previously described by Archary et al. [43] in 45 participants with matching samples at baseline, 3 and 6 months. Cytokine/chemokine concentrations were measured using the Bio-Plex Pro Human Cytokine/Chemokine Magnetic Bead 27-Plex Panel (Biorad, 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.

Statistics analyses

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 0.05 was considered significant. Linear mixed models were used to test for a linear association between the logged drug transporter mRNA expression values and cytokines/chemokines, accounting for correlation due to repeated measurements on participants over time. With the exception of IL-1Rα, cytokine values were categorized into quintiles for the models. IL-1Rα was categorized into terciles because the distribution was concentrated in only a few values. Regression was interpreted as a unit increase in X (X being logged drug transporter mRNA expression), and the percent change in Y (cytokine/chemokine quintiles) was equal

to exponential function (exp) (the negative or positive estimate value)−1. A multivariable model was not built because the cytokine/chemokine values are highly correlated. Linear mixed modelling was also used in measuring the association between the logged blood drug transporter mRNA expression values and logged blood TFV-DP values in the blood. These analyses included participants with matching soft-cup and cytobrush specimens (FGT drug transporter mRNA expression vs cytokine/chemokine expression in the FGT) and participants with detectable drug levels (blood drug transporter mRNA expression vs TFV-DP drug levels in the blood) at 3 and 6 months. Timepoints with undetectable drug levels were removed as it was assumed that participants had not taken oral PrEP recently at these time points. Linear mixed models were built using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). For these analyses p-value ≤ 0.1 was considered significant. GraphPad Prism was used for all graphical representations. A partial least-squares discriminant analysis (PLS-DA) using the R software was conducted to identify the clustering of drug transporter mRNA expression levels based on the genital tract inflammation status of women at baseline, 3 months, and 6 months. All variables were standardized to zero means and unit variances for the PLS-DA. In each PLS-DA, the outcome variable was the inflammation status (inflamed versus uninflamed), and the exposures were drug transporter mRNA expression levels (P-gp, MRP-2, MRP-4, MATE-1, OAT-1, OAT-3,). From each PLS-DA, we plotted the drug transporter loadings of the first two principal components (PC1 and PC2) which also referred to as the X-variates. To demonstrate the overall drug transporter mRNA expression of the samples and identify any clustering based on inflammation status, we plotted the sample scores of PC1 on the X-axis and PC2 on the Y-axis coloured or grouped by inflammation status (inflamed orange and uninflamed blue). We included the 95% confidence ellipses of the PC scores by inflammation status on each sample plot. The sample plots were complemented with a clustered image map (CIM) to show each sample's expression levels of the six drug transporters at 6 months. For the CIM, hierarchical clustering with Euclidean distance and complete linkage methods were applied to the drug transporters on the X-axis and the samples on the Y-axis. For all of the statistical analyses conducted no multiple comparison adjustments were performed.

Results

Sample description

A total of 429 participants enrolled in the study, 262 participants elected to take PrEP. Among these participants, 200 had matching stored soft-cup (from the FGT), cytobrush (from the FGT) and buffycoat (blood) samples at baseline, 3 and 6 months (Fig. 1A). Among these 200 participants, 108 were excluded due to not having measured drug levels. For the remaining 92 participants at baseline, 3 and 6 months, they had blood TFV-DP drug levels measured and they had matching soft-cups, cytobrush, and buffycoat specimens at these timepoints.

Of the 92 participants:

I. n = 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 buffycoat samples each and were included in mRNA expression correlation analyses between the two compartments.
II. n = 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/chemokine levels [36]. This provided a total of 134 cytobrush soft-cup and samples each.
III. n = 29 of the 92 participants (31.5%) who had detectable TFV-DP drug levels (from dried blood spots) at 3 months and 6 months were correlated to drug transporter mRNA expression in the blood.

Three different analyses are depicted in Fig. 1B which shows the interrelationship as follows; firstly, drug transporter mRNA expression was determined between buffycoat and cytobrush samples; secondly the drug transporter mRNA expression in buffycoat samples was compared to TFV-DP drug levels which was measured in dried blood spots and lastly drug transporter mRNA expression in cytobrush samples was compared to the concentration of cytokine/chemokines measured in soft-cup samples.

Table 1 describes the demographic and clinical data of 45 participants who volunteered to take PrEP. Most participants were aged 18 to 24 years (n = 31, 69%), were from the Vulindlela rural site (n = 26, 58%), had a matric education (n = 29, 64%), and BMI of > 30 (n = 19, 42%). At enrolment, the prevalence of *C. trachomatis* was (n = 9, 20%), for *N. gonorrhoea* (n = 3, 7%), and none of the participants presented with *T. vaginalis*. All 45 of the participants were included in drug transporter mRNA expression and genital inflammation analyses. Of the 45 participants, n = 18 (40%) had genital inflammation while n = 27 (60%) did not have genital

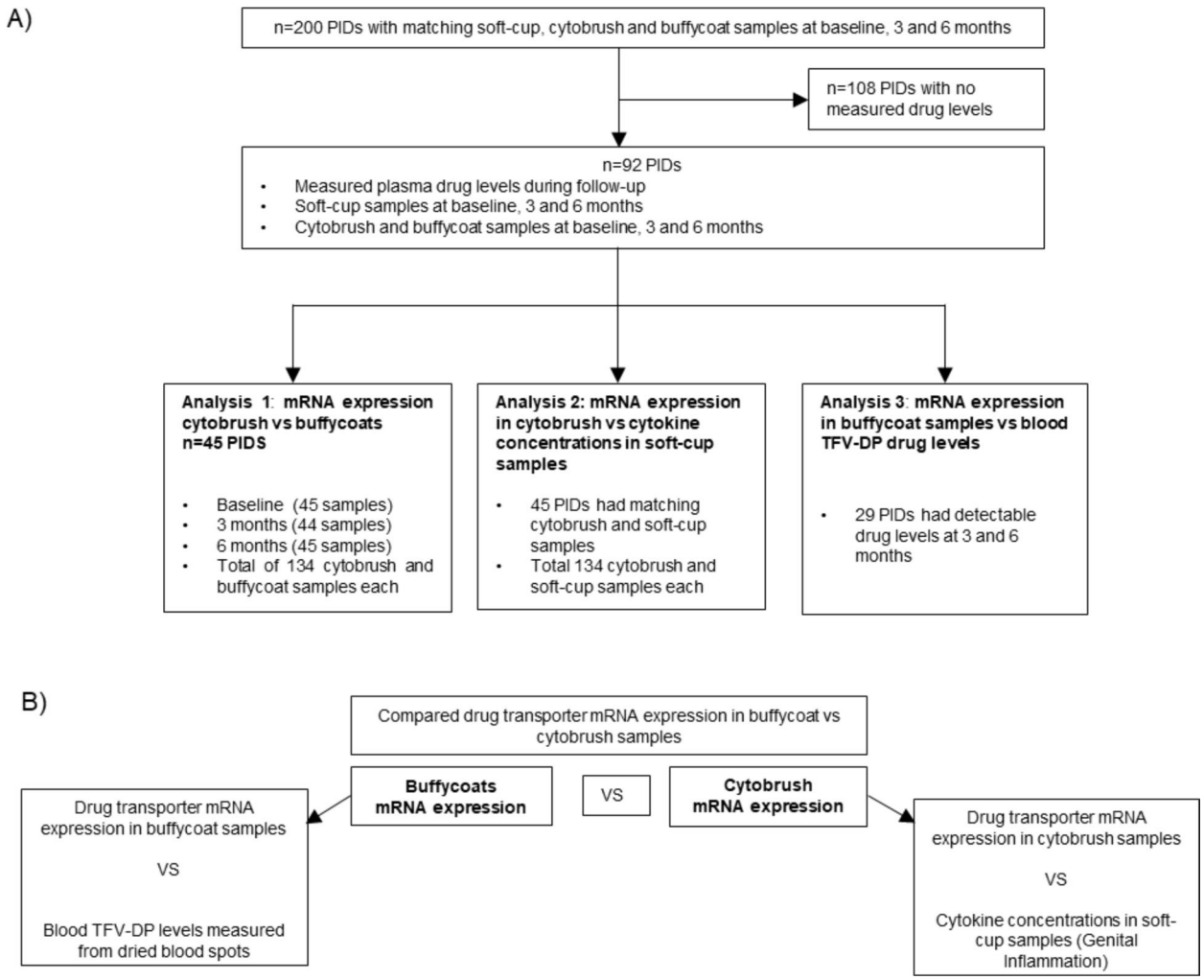

Fig. 1 **A** Description of the participants and sample allocation for three experimental and analysis plans in the study. Description of the number of soft-cup, buffycoat, and cytobrush samples included in this cohort study. A description of the analyses conducted, and the total number of samples included in each of the three analyses is shown. **B** Diagram detailing analyses between drug transporters mRNA expression (buffycoat and cytobrush) and measured TFV-DP drug levels in the blood and cytokine concentrations in the FGT. VS = versus

inflammation. However, n = 29 of the 45 participants (64%) were included in TFV-DP drug levels analyses.

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 Spearman's rank correlation tests were done to determine if there are associations between drug transporter mRNA expression levels in the FGT and blood. Table 2 shows significant correlations for OAT-1 at baseline [Spearman's rank correlation coefficient (rs)] (rs 0.50, p = 0.0022), at 3 months (rs 0.54, p = 0.0001) and at 6 months (rs 0.33, p = 0.048). A graphical representation of the correlations for OAT-1 mRNA expression between the FGT and blood are shown in Figs. 2A, 2B and 2C. mRNA expression values for P-gp were significantly correlated at 3 months (rs 0.56, p = 0.002). At 3 and 6 months, significant correlations for mRNA expression were found between the FGT and blood for MATE-1, MRP-2 and MRP-4, post PrEP exposure. No significant correlations were observed for OAT-3. Graphical representation for OAT-3, P-gp, MATE-1, MRP-2 and MRP-4 correlation analyses are shown in Additional file 1: Fig. 1S. In addition to correlation analyses, mean fold change mRNA expression between the two compartments FGT and blood was determined and compared. The mRNA expression profile plots showed that drug transporters P-gp, MRP-4 and OAT-1 had primarily higher mean

Table 1 Demographic and clinical characteristics of study participants

Characteristic	Number of participants n (%)
Age category (years)	
18–24	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* category	
0–18.5 (Underweight)	3 (7)
18.6–25 (Normal weight)	15 (33)
26–30 (Overweight)	8 (18)
> 30 (Obese)	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)
With Genital inflammation^{+++}	18 (40)
Without Genital inflammation^{+++}	27 (60)
TFV-DP drug levels (detectable drug levels)$^{++}$	29 (64)

*BMI- Body Mass Index. $^{+}$mRNA expression includes matching cytobrush (FGT) and buffycoat samples (blood). $^{++}$TFV-DP drug levels analyses only included in buffycoat samples (blood); 29 participants were included in TFV-DP drug levels analyses this included 4 participants that had HIV seroconverted. $^{+++}$Genital inflammation analyses only included cytobrush (FGT) and soft-cup (FGT) samples in n = 45 women in total

fold change mRNA expression in the blood, while drug transporters MATE-1, MRP-2, and OAT-3 had primarily higher mean fold change mRNA expression in the FGT. A graphical presentation of the individual mean fold expression of drug transporter mRNA for blood and the FGT is depicted in Additional file 2: Fig. 2S.

Association between pro-inflammatory cytokines/chemokines and drug transporter mRNA expression in the FGT

Linear mixed models were used to determine if there are associations between drug transporter mRNA expression and inflammation, which was measured by cytokines/chemokines in the FGT. Table 3 shows the associations between inflammatory cytokines/chemokines and mRNA expression of efflux drug transporters P-gp, MATE-1, MRP-2, MRP-4 and influx drug transporters OAT-1 and OAT-3. We observed significant associations between IL-1β and influx drug transporters OAT-1 (p = 0.06) and OAT-3 (p = 0.04). For both these drug transporters an inverse relationship was observed for OAT-1 [Est = − 0.22; 95%Cl (− 0.45 to 0.01)] and OAT-3 [Est = − 0.17; 95%Cl (0.34to − 0.01)]. The estimate (Est) values were interpreted as follows: for OAT-1 and OAT-3 for IL-1β the Est values were − 0.22 and − 0.17 respectively, therefore OAT-1 values decreased by 22% with every increase in one quintile of IL-1β similarity OAT-3 values decreased by 17% with every increase in one quintile of IL-1β. A similar trend was also observed between IL-1Rα and efflux drug transporters MRP-2 (p = 0.05) and MRP-4 (p = 0.03). Here too an inverse relationship between IL-1Rα concentration and MPR-2 [Est = − 0.20; 95%Cl (0.41–0.00)] and MRP-4 [Est = − 0.15; 95%Cl (− 0.29 to 0.01)] mRNA expression was observed. Similarly, for MCP-1, and TNF-α moderate associations with OAT-3 (p = 0.06), and MRP-2 (p = 0.07), respectively, were observed. An inverse relationship was observed between MCP-1 concentration and OAT-3 mRNA expression [Est = − 0.15; 95%Cl (− 0.31 to 0.01)], and for TNF-α and MRP-2 mRNA expression

Table 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%Cl	r_s	p	95%Cl	r_s	p	95%Cl
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	**0.50**	**0.0022***	0.19–0.72	**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%Cl- confidence intervals (lower to upper limits)

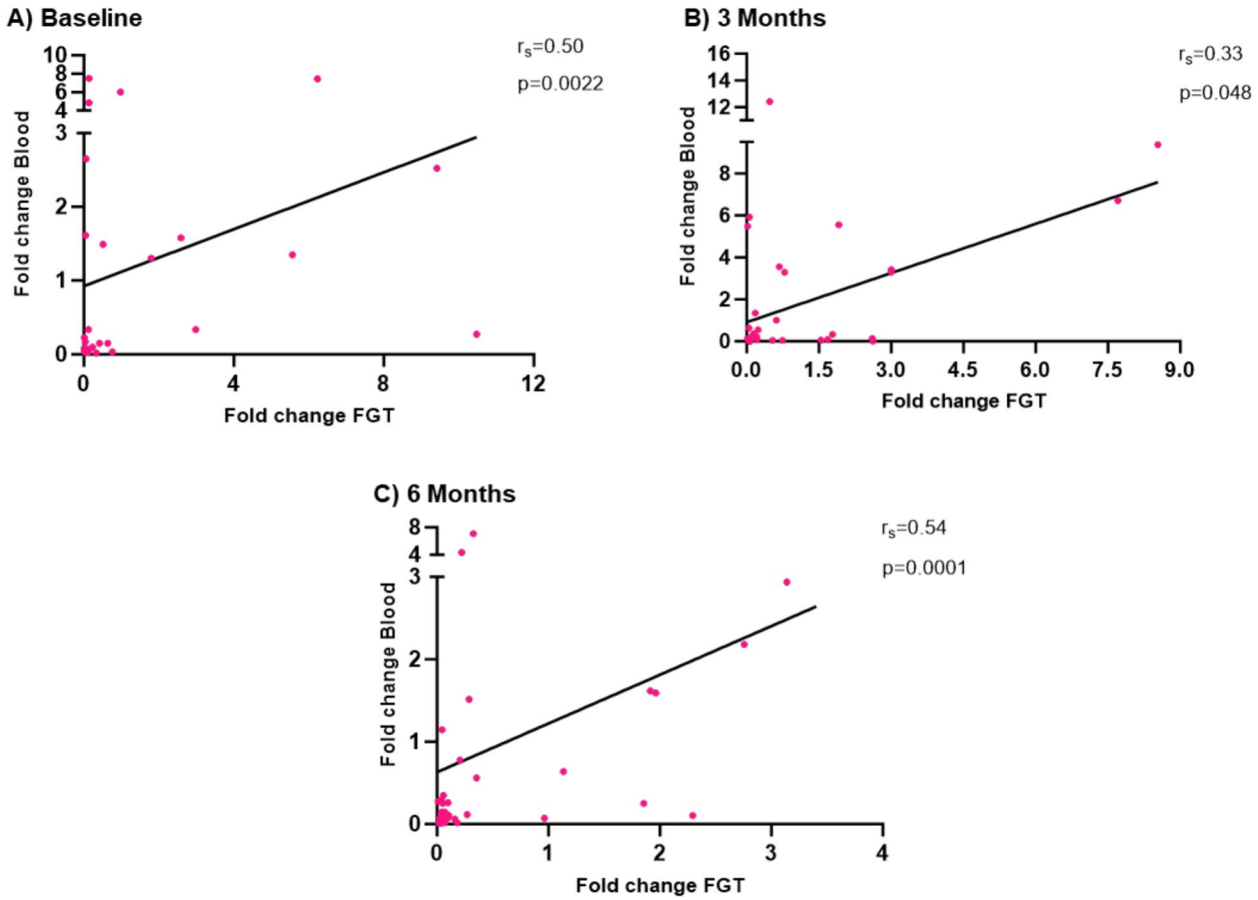

Fig. 2 Graphical representation of correlations for OAT-1 mRNA expression between the FGT and blood. Moderate and significant correlations are shown at three timepoints **A** baseline, **B** 3 months, and **C** 6 months for OAT-1 in cytobrushes (fold change FGT) and buffycoats (fold change blood). Analyses were done using the Spearman's rank correlation coefficient (rs) and a two-tailed p-value of ≤ 0.05 was considered significant

[Est = −0.10; 95%Cl (−0.20 to 0.01)]. MIP-1β was the only chemokine that showed a positive relationship with efflux drug transporter MATE-1 (p = 0.07). This indicated that as MIP-1β concentration increased so did MATE-1 mRNA expression [Est = 0.12; 95%Cl (−0.01 to 0.25)]. There were no associations observed for IL −6, IL-8, IP-10, and MIP-1α with any of the drug transporters.

Partial least squares discriminant analysis (PLS-DA) to determine associations between drug transporter mRNA expression in the FGT and genital inflammation

Partial least squares discriminant analysis was conducted to identify clustering of drug transporter mRNA expression levels based on inflammation status. This was conducted in N = 45 women at three timepoints baseline, 3 months, and 6 months. The outcome variable was the inflammation status, and the explanatory variables were the drug transporters. A PLS-DA is a supervised version of principal component analysis (PCA), which adjusts for the labels of the explanatory variables within the model [44]. In our PLS-DA model, scores were generated by maximising the co-variance between the drug transporter mRNA expression and inflammation status. Genital inflammation status was determined in these women as defined by Masson et al. [36], women that presented with concentrations above the 75th percentile for any five of nine inflammatory cytokines were considered inflamed, while those that did not were considered uninflamed. Figure 3 shows PC1 and PC2 loadings plots, higher scores are the farthest away and lower scores are the closest to the zero axes. The negative or positive signs indicate which drug transporters are influential for the scores based on the loadings. Based on the loadings, the more negative the scores, the lower the expression of the drug transporters correlations with that PC and vice versa (Fig. 3). Figure 4 shows scatter plots of PC1 (X variate1) and PC2 (X variate2) scores from the PLS-DA at each time point (baseline, 3 months, and 6 months). Each plot shows the 95% confidence ellipse by inflammation status. Scatter plots showed tighter drug transporter

Table 3 Associations between drug transporter mRNA expression and cytokines/chemokine

	Log P-gp			Log MATE-1			Log OAT-1			Log OAT-3			Log MRP-2			Log MRP-4		
Cytokine/ Chemokine	**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 (positive/negative association), *p ≤ 0.1 = significance, 95%CI: confidence intervals (lower to upper limits), values that showed associations highlighted in bold

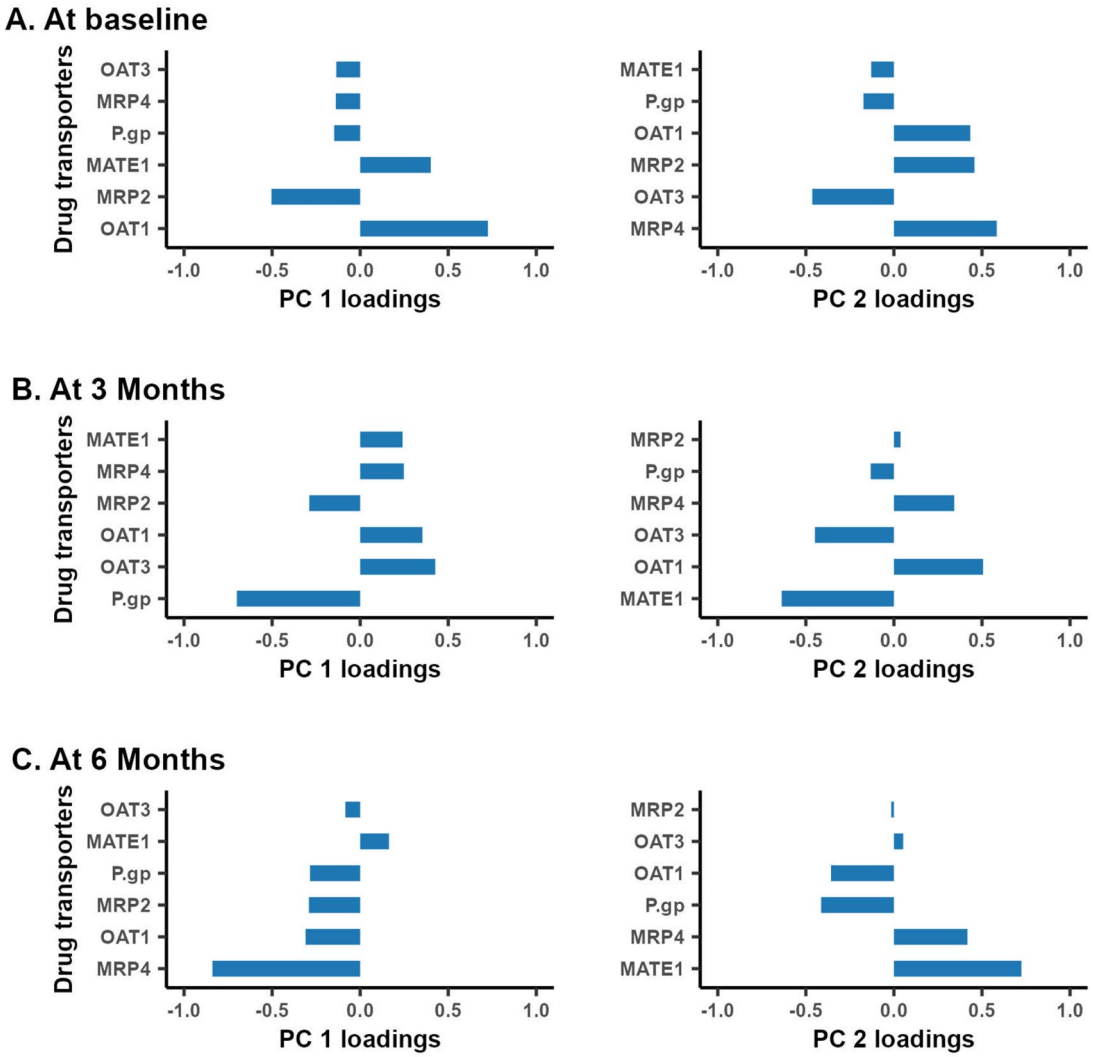

Fig. 3 Loadings plot showing the correlation coefficients between drug transporter mRNA expression levels and PC scores. The loading plots for PC1 and PC2 are shown at three timepoints. **A** Baseline: PC1 positive correlation coefficients were observed for MATE-1 and OAT-1 and for PC2 it was OAT-1, MRP-2 and MPR-4, while negative correlation coefficients for PC1 were observed for OAT-3, MRP-4, P-gp and MRP-2 and for PC2 it was MATE-1, P-gp and OAT-3. **B** 3 months: PC1 positive correlation coefficients were observed for MATE-1, MRP-4, OAT-1 and OAT-3 and for PC2 it was OAT-3, MRP-4 and OAT-1, negative correlation coefficients for PC1 were observed for MRP-2 and P-gp, and for PC2 it was OAT-3 and MATE-1. **C** 6 months: PC1 positive correlation coefficients were observed only for MATE-1 and for PC2 it was OAT-3, MRP-4 and MATE-1, negative correlation coefficients for PC1 were observed for OAT-3, P-gp, MRP-2, OAT-1 and MPR-4 and for PC2 it was MRP-2, OAT-1 and P-gp

clustering by inflammation status at 6 months (Fig. 4C) as compared to baseline (Fig. 4A) and 3 months (Fig. 4B). Inflamed women had on average, lower values of MRP-4 (PC1 score = −0.29) and consistently higher values of MATE-1 (PC1 score = 0.16) at 6 months than uninflamed women (see Additional file 3: Table 1S for PC1 and PC2 scores). At 6 months although there is no clear separation between the inflamed and uninflamed groups, the orange-coloured ellipse shows tighter clustering. This pattern of clustering is driven by higher MATE-1 expression levels with concomitant low MRP-4 expression (Figs. 4C and 5). The depiction of Fig. 4C is further expanded as a heatmap in Fig. 5 which highlights the higher MATE-1 expression at 6 months with lower MRP-4 expression in genital inflammation women compared to women with no genital inflammation (Fig. 5).

Associations between blood TFV-DP and drug transporter mRNA expression in the blood

Linear mixed models were also used to determine if there are associations between drug transporter mRNA expression and blood TFV-DP. These analyses were

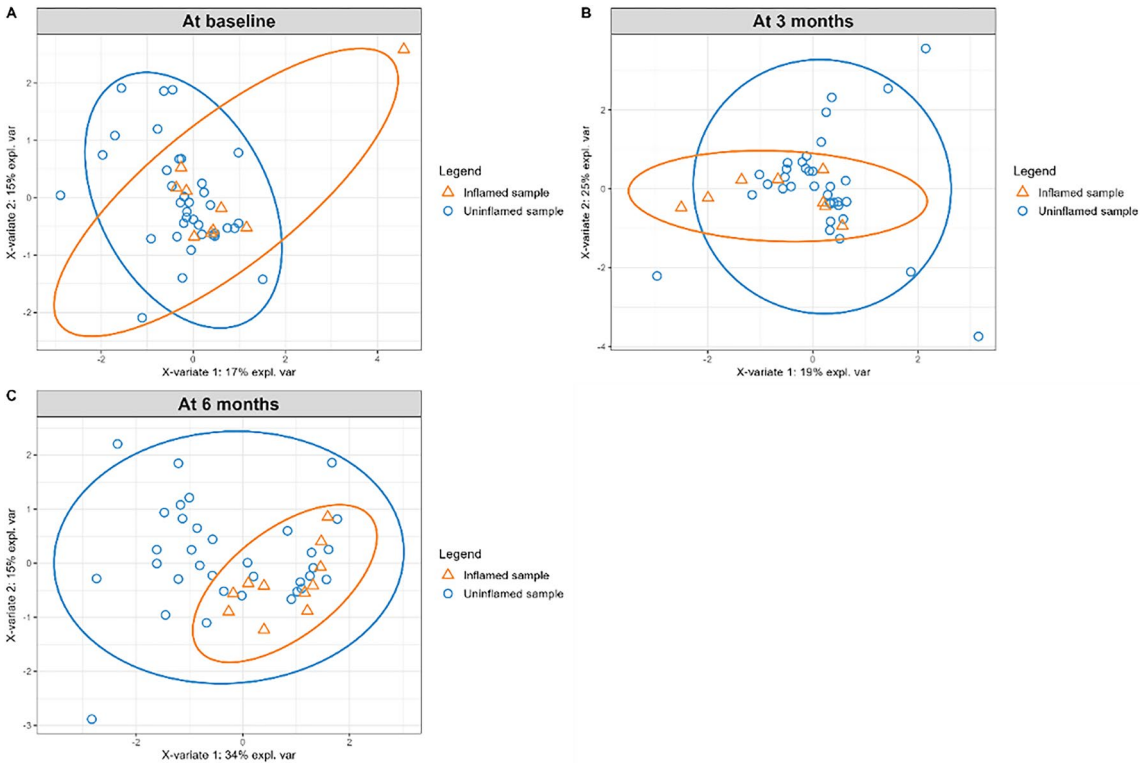

Fig. 4 Scatter plots of PC1 (X variate1) and PC2 (X variate2) scores from the PLS-DA comparing overall drug transporter mRNA expression levels in inflamed versus uninflamed women. No clear separation was observed at **A** baseline and **B** 3 months for drug transporter mRNA expression in inflamed women (orange) versus uninflamed women (blue). At **C** 6 months a slight separation was observed between inflamed and uninflamed women

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 mRNA expression and blood TFV-DP measured at 3 and 6 months (Table 4).

Discussion

In our study, we showed consistent and significant correlations between mRNA expression of ABC and SLC drug transporters in the FGT and blood. Linear-mixed models and PLS-DA analyses both showed a positive and significant associations between genital inflammation and MATE-1 efflux drug transporter mRNA expression. However, no significant associations were observed between any influx or efflux drug transporter mRNA expression levels and blood TFV-DP drug levels in healthy South African women offered oral PrEP (TDF/FTC).

The crosstalk or concordance between influx and efflux drug transporters in the blood to that of the genital tract remains relatively poorly characterized. In our study, we established that there is some degree of direct associations for four efflux drug transporters namely P-gp, MATE-1, MRP-2 and MRP-4, and one influx drug transporter OAT-1. The four efflux drug transporters showed no significant correlation at baseline before PrEP exposure, however a correlation was observed after 3- and 6-months post-PrEP exposure. 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 [19], no direct comparisons have been shown between the blood and the genital compartments in humans. Furthermore, the study by Zhou et al. [19] showed high baseline expression of these efflux drug transporters P-gp and MRP-4 in human and mouse vaginal tissue models [19].

Drug transporters have also shown heterogeneous expression profiles for vaginal tissues compared to colorectal tissues [16]. Whereas high mRNA expression of

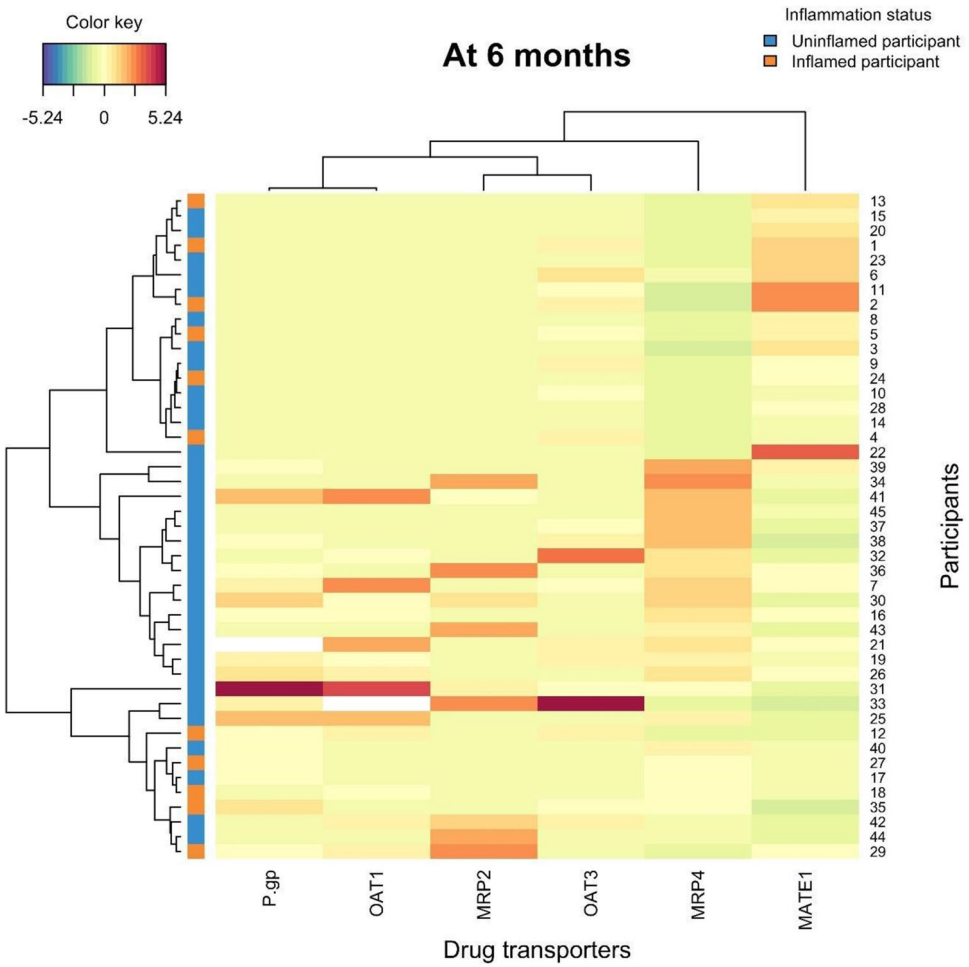

Fig. 5 Clustered image map demonstrating drug transporter mRNA expression levels by inflammation status at 6 months. Colour key: colours red to orange represents high drug transporter mRNA expression levels, while green to blue colours represent low drug transporter mRNA expression. On the right axis each row represents an individual participant's pattern of drug transporter mRNA expression. Dendrograms are used along the axes (top and left) to depict how each row and column clusters based on the hierarchical clustering method

Table 4 Associations between drug transporter mRNA expression and blood 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

Estimate (positive/negative association), p significance ≤ 0.1, 95%CI: confidence intervals (lower to upper limits)

P-gp and MRP-2 was found in human vaginal compared to colorectal tissues, the opposite was found for MRP-4. Taneva et al. [18] 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 tenofovir across the cell membranes and into the cells [18]. 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 [16, 18]. In contrast, our findings differ by the high baseline OAT-1 expression (influx drug transporter) even before PrEP exposure in the FGT and this pattern is preserved post drug exposure.

This data suggests that a direct relationship may exist between the expression of influx and efflux drug transporters after PrEP drug exposure. In addition, at the cellular level there may be certain internal control mechanisms yet to be determined that may lead to variations in the expression of influx and efflux drug transporters.

These data therefore indicate that more in-depth analyses are still required to fully elucidate correlations between drug transporters expressed in the blood and FGT, if they differ significantly or are affected by biological factors in the same manner. Understanding the impact that ARVs have on drug transporter mRNA expression levels is particularly important in women. In previous studies it was demonstrated that tenofovir levels even at the same dosage were significantly higher in colorectal compared to vaginal tissue, which directly impacted PrEP efficacy [45]. These data support why more research is needed in women to optimise PrEP and dosage to confer sufficient protection against HIV in the FGT [45–49].

We also found evidence of associations between drug transporter mRNA expression and pro-inflammatory cytokines/chemokines. Negative correlations were observed between: IL-1β with influx drug transporters OAT-1 and OAT-3; IL-1Rα with efflux drug transporters MRP-2 and MRP-4; MCP-1 with OAT-3 and TNF-α with MRP-2. This indicated an inverse relationship between cytokine/chemokine concentrations and drug transporter mRNA expression levels. Therefore, as IL-1β, IL-1Rα, MCP-1 and TNF-α concentrations increased, the mRNA expression of OAT-1, OAT-3, MRP-4 and MRP-2 drug transporters decreased. A positive relationship was however observed between MIP-1β with the efflux drug transporter MATE-1, indicating possibly a direct concentration effect of MIP-1β on MATE-1 expression. Although these associations were moderate, this led us to make certain inferences from these findings. MIP-1β and MCP-1 are two chemokines that in addition to elevating inflammation, have a dual role of trafficking cells to the genital tract. The inverse and direct associations between the influx-OAT-3 and efflux- MATE-1 drug transporters with these two chemokines suggests their differential effect on drug transporter mRNA expression. In addition, these data also show that elevated cytokines and chemokine may differentially affect drug transporters in these women. This may be significant for maintaining intracellular PrEP drug retention to prevent HIV infections in women with genital inflammation.

To further dissect the complex relationship between drug transporter mRNA expression and genital inflammation, PLS-DA was conducted. The PLS-DA showed that there was no clear separation between the inflamed versus the uninflamed women in terms of drug transporter mRNA expression levels at baseline and at 3 months. However, at 6 months a tighter pattern of clustering was observed for the inflamed than the uninflamed women. This data therefore suggested that inflamed women have, on average, lower values of the efflux drug transporter MRP-4 and higher values of MATE-1 than uninflamed women. This suggested that genital inflammation may affect the expression levels of efflux drug transporters MRP-4 and MATE-1 indicated by the subsequent decreased MRP-4 expression and increased MATE-1 expression levels in inflamed women. Emtricitabine and tenofovir are two constituents of the oral PrEP drug Truvada®, emtricitabine is a known substrate of the efflux drug transporter MATE-1 [12], while tenofovir is a known substrate of the efflux drug transporter MRP-4 [10, 18]. We therefore propose that, while low mRNA expression of MRP-4 may allow for adequate intracellular retention of tenofovir, high mRNA expression levels of MATE-1 may undermine this by affecting the optimal intracellular retention of emtricitabine, which could impact the overall effectiveness of PrEP in the FGT.

Our data suggest that pro-inflammatory cytokines/chemokines in the FGT can possibly shape drug transporter mRNA expression levels. In addition, our findings support our hypothesis that certain cytokines/chemokines can differentially affect the mRNA expression of drug transporter families. This is corroborated by studies in animal and human in-vitro and in-vivo models showing the effect of pro-inflammatory cytokines on efflux and influx drug transporters, which in turn affect drug levels [50]. These studies also showed that modulation of drug transporters by pro-inflammatory cytokines varies according to tissue type [50]. 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 [31]. 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 belonging to the OATP-B, OATP-C and OATP-8 subfamilies, while the mRNA expression of the efflux drug transporter MRP-2 was significantly downregulated [33, 38, 51]. These data highlight that further elucidation of pro-inflammatory cytokines relative to drug transporter mRNA expression in different tissues is needed. This is especially important considering genital inflammation despite topical PrEP is a significant HIV risk modifier [36, 37] and may also indirectly impact drug transporter mRNA expression, thereby affecting drug disposition [52] and ultimately PrEP efficacy.

In addition to inflammation, the effect of tenofovir and blood TFV-DP drug levels itself on drug transporter mRNA expression cannot be ignored. We, however, did not observe any significant associations between tenofovir drug levels and drug transporter mRNA expression. Most studies evaluating such associations for ARVs used as treatment rather than for prevention showed high mRNA and protein expression of efflux drug transporters

P-gp and MRP-4 in HIV infected individuals on ARVs compared to healthy controls [22, 53]. These results suggest that ARVs 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 mRNA expression [22, 53]. ARVs have been directly implicated as inducers and/or inhibitors of drug transporters affecting corresponding protein expression and function [19, 53]. Furthermore, in-vitro studies using cervicovaginal tissues from drug naïve macaques exposed to tenofovir and darunavir showed significantly increased efflux drug transporter MRP-2 expression [21]. However, human cervicovaginal cell lines VK2/E6E7 exposed to tenofovir, showed significantly reduced MRP-5 and not MRP-2 mRNA expression [17]. Such discrepancies suggest that the relationship between drug transporter mRNA 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.

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. Our findings could be further validated with more specific and sensitive assays such as probe-based PCR or digital droplet PCR [54, 55]. However, we were unable to perform these assays due to insufficient genital tract specimens which prevented direct comparisons between mRNA and protein expression. In addition to genetic variations and microbiome composition, other biological factors such as coinfections with other STIs and hormonal contraception use may also affect drug transporter mRNA expression and function. Conducting a validation study using different methods such as protein expression analysis or functional assays could help to validate the findings of this study and provide additional evidence on the impact of drug transporter proteins on PrEP efficacy. However, mRNA expression analyses alone as shown in previous studies still provide a basis for the selection of drug transporters [17, 19, 56]. We included only participants with detectable drug levels which further impacted our modest sample size and could possibly limit the statistical power of the findings. In addition, in the drug level analysis we did not have drug adherence data to correlate with tenofovir levels, this was a limitation since variations in adherence can confound the biological associations being investigated. In this study we also did not measure intracellular levels of TFV-DP drug levels in cells from the FGT and cannot account for how inflammation may affect drug levels. However, it may be plausible that the genital compartment is subject to external stimuli that may modulate local inflammation and immune cell activation which may then alter drug transporter expression profiles [10, 30, 31, 33, 35]. Furthermore, we did not adjust for multiple comparisons for any of the analyses conducted. Therefore, our findings remain largely exploratory and will require further confirmation in larger African cohort studies using PrEP for HIV prevention. 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 had the advantage of comparing drug transporter mRNA expression accordingly even though the [10]study included participants with samples up to 6 months. Examining whether blood and tissue expression of transporters is similar or dissimilar is crucial for understanding how drugs such as PrEP are absorbed, distributed, metabolized, and eliminated in the body. Correlated drug transporter mRNA expression patterns between the blood and the genital tract (which is the primary site for HIV exposure) for our purpose of preventing HIV in at-risk populations such as African women would largely simplify predictions and monitoring. Furthermore, the lack of correlations between these compartments would necessitate more complex approaches to ensure effective and safe PrEP for HIV prevention. Another 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/chemokines, and blood tenofovir associations were done in a limited sample size making it challenging to 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.

Conclusion

In conclusion, our study provided three 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/chemokines and drug transporter mRNA expression in the FGT. An increase in the chemokine MIP-1β concentration directly increased the mRNA

expression levels of the efflux drug transporter MATE-1. Lastly, the efflux drug transporter MATE-1 was shown to be highly expressed in women presenting with genital inflammation compared to those with no inflammation. 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/chemokines, respectively. Examining associations between drug transporter mRNA expression, genital inflammation and TFV-DP drug levels is a novel aspect that enhances the understanding of the complex interplay of factors influencing PrEP pharmacology and efficacy. 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. Overall, future research could build on these findings to optimize PrEP for women at high risk of acquiring HIV infection.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12981-025-00713-z.

Additional file 1

Additional file 2

Additional file 3

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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 and KA 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.

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Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval and consent to participate
This sub-study was approved by the Biomedical Research Ethics Committee (BREC) at the University of KwaZulu-Natal (BREC/0002195/2020).

Informed consent
Informed consent was obtained from all subjects involved in the study.

Competing interest
The authors declare no competing interests.

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