A Randomized Clinical Trial of On-Demand Oral Pre-Exposure Prophylaxis shows no impact on lymphoid or myeloid HIV Target Cells in the Foreskin.

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38 **Abstract**

39 **Background**: Oral dosing of pre-exposure prophylaxis (PrEP) has shown efficacy in
40 preventing HIV acquisition in men-who-have-sex-with-men, but less evidence in heterosexual
41 men in Africa. As a secondary objective of a clinical trial in South Africa and Uganda, we
42 investigated the immunologic safety of PrEP in the foreskin.

43 **Methods:** HIV-negative males (n=144) were enrolled in an open-label randomized controlled
44 trial to receive high or low doses of emtricitabine-tenofovir disoproxil fumarate (F/TDF) or
45 emtricitabine-tenofovir alafenamide (F/TAF) 5h or 21h before voluntary medical male
46 circumcision (VMMC), or to a control arm with no PrEP prior to circumcision. Foreskins were
47 analyzed, blinded to trial allocation, to determine numbers of $CD4^+CCR5^+$ cells and $CD1a^+$
48 cells using fluorescence microscopy and correlated with tissue-bound metabolites and p24
49 production after *ex vivo* foreskin challenge with HIV-1_{bal}.

50 **Results:** There was no significant difference in $CD4^+CCR5^+$ or $CD1a^+$ cell numbers in
51 foreskins between treatment arms compared with the control arm. Claudin-1 expression was
52 34% higher (95% confidence interval (CI):11%-62%, p=0.003) in foreskin tissue from
53 participants who received PrEP relative to controls with no difference between F/TDF and
54 F/TAF observed. This association was no longer statistically significant after controlling for
55 multiple comparisons. There was no correlation of $CD4^+CCR5^+$, $CD1a^+$ cell numbers, or
56 claudin-1 expression with tissue-bound drug metabolites, nor with p24 production after *ex vivo*
57 viral challenge.

58 **Conclusion**: Oral doses and timing of on-demand PrEP and *in situ* drug metabolite levels in
59 tissue have no effect on numbers or anatomical location of lymphoid or myeloid HIV target
60 cells in foreskin tissue.

61

62 **Clinical trials Registration**: NCT03986970

Keywords: Claudin-1, Foreskin, HIV target cells, Pre-exposure Prophylaxis (PrEP), F/TDF (tenofovir disoproxil fumarate), F/TAF (tenofovir alafenamide), Voluntary Medical Male Circumcision (VMMC)

Introduction:

Due to a combination of antiretroviral (ARV) availability, increased HIV testing and introduction of a range of HIV prevention tools, HIV incidence and mortality in African have declined [1]. However, HIV remains a global burden, with approximately 320,000 people newly infected in Africa in 2022 [1]. The most common mode of transmission is by heterosexual intercourse with HIV incidence highest among women of reproductive age[2]. Most HIV prevention strategies have focused on preventing male-to-female HIV transmission, whereas preventing HIV acquisition in heterosexual men is relatively understudied.

Voluntary Medical Male Circumcision (VMMC) has been shown to reduce HIV infection in heterosexual men by 50–60% [3; 4; 5], demonstrating that the foreskin plays an important role in HIV susceptibility in males. As a result, VMMC has been rolled out as a standard of preventative care in Africa, with more than 26 million circumcisions performed between 2008-2019. Although effective, VMMC uptake is unevenly distributed across countries [6], and additional prevention strategies are required, including Pre-exposure prophylaxis (PrEP). Daily TDF/FTC PrEP has been shown to be highly effective in MSM and heterosexual HIV-serodiscordant couples [8; 9; 10]. Furthermore, on demand F/TDF and daily Tenofovir alafenamide (F/TAF) are also highly effective in MSM [7]. Tenofovir gel has been evaluated for vaginal and rectal PrEP [11; 12], with the CAPRISA 004 study [12] showing up to 54% lower HIV-incidence in women in the gel arm. However, women receiving 1% tenofovir gel had an accumulation of activated endocervical $CD4^+$ T cells, which were associated with increased drug metabolite levels in the tissue [13]. Although these studies focused on topically applied PrEP, their findings raise the issue of host immune activation in response to drug which may undermine the potential efficacy of PrEP. In an effort to identify the impact of oral PrEP in young men on immune status, we undertook a randomized clinical trial [14] to test the

93 secondary outcome of immunological safety by measuring numbers of $CD4^+CCR5^+$ and $CD1a^+$
94 cells in relation to claudin-1 barrier integrity in the foreskin.
95
96 **Methods:**
97 ***Study Design and Participants***
98 HIV-negative males aged 13-24 years were enrolled from South Africa and Uganda (72 per
99 country) into an open-label controlled trial (NCT03986970) and randomized in a
100 1:1:1:1:1:1:1:1:1 ratio to control arm (no PrEP) or one of eight arms receiving F/TDF or F/TAF
101 at one of two different doses (double dose on day 1 only, or double dose on day 1 plus single
102 dose on day 2) before undergoing VMMC 5 or 21 hours after receiving drug [14].
103
104 ***Sampling***
105 VMMC was performed using the dorsal slit method. Foreskin tissue was placed immediately
106 in cold DMEM and transported to the laboratory on ice within 30 minutes. All tissues were
107 immediately dissected into 8 x 2.5 mm^2 pieces (4 for inner and 4 for outer) and snap-frozen in
108 Optimal Cutting Temperature (OCT) compound as previously described [15].
109
110 ***Immunohistochemistry imaging and analysis***
111 The density of $CD4^+CCR5^+$ cells was assessed from 72 participants from South Africa and 40
112 from Uganda. A more limited number of samples were assessed for CD1a and claudin-1
113 expression including all control arm participants (n=16) and 4 randomly selected from each of
114 the 8 treatment trial arms (n=40 from South Africa and 40 from Uganda = 80 in total). OCT
115 snap frozen tissues were sectioned (5–10 μm thick) using a Leica CM1850 Cryostat (IL, USA)
116 and processed as described [15]. Tissues was stained with primary anti-human CD4 antibody
117 (1:50 dilution; Sigma Missouri, US); secondary Cy5 antibody (1:500; AEC Amersham,
118 Johannesburg, SA); anti-human primary MC-5 CCR5 antibody (1:10; courtesy of Prof.

Mathias Mack) and secondary Cy3 antibody (1:1000; AEC Amersham Johannesburg, SA); primary anti-human claudin-1 antibody (1:200; Thermofisher, Waltham, Massachusetts, US) and secondary DarB antibody (1:1000, AEC Amersham Johannesburg, SA); primary anti-human CD1a antibody (Ltc Tech SA, Randburg) and secondary Cy5 antibody (1:500; AEC Amersham Johannesburg, SA). Cell nuclei were stained with DAPI (Hoescht). Negative controls were stained with isotype mouse or rabbit and kidney tubular tissue and positive controls for CCR5 on tonsular tissue. Ten images for each section were collected using DeltaVision RT systems and softWoRx software (Applied Precision Instruments) and quantified using Image J and Pipsqueak. Percentage of claudin-1 expression was calculated by dividing the number of pixels taken up by claudin-1 staining (Figure 2D and E) by the total epidermal area for each foreskin section. We also delineated between $CD1a^+$ cells inside the claudin-1 staining (intrinsic, iCD1a) and $CD1a^+$ cells outside of the claudin-1 stain (extrinsic, eCD1a) and expressed this as a ratio: eCD1a/iCD1a.

Ex vivo challenge of foreskin tissue

Foreskin tissue was cut into $2mm^2$ explants [16] and cultured by adding an outer and an inner explant per well, in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 2.5µg of amphotericin B/mL, and antibiotics (100U of penicillin/mL, 100µg of streptomycin /mL) (Sigma, St. Louis, MO, USA). Explants were immediately challenged with HIV-1_{BaL} at either a high titre (10^4 $TCID_{50}$/mL [median tissue culture infective dose/mL]) or low titre ($2x10^2$ $TCID_{50}$/mL), being more physiologically relevant. Control explant tissues received no virus. Foreskin explants were cultured for 15 days with approximately two-thirds of culture supernatant harvested at days 3, 7, 11 and 15, and cultures replenished with fresh medium. p24 was measured in the supernates by ELISA (Innotest HIV antigen mAb ELISA, Fujirebio

Europe, Belgium). The lower limit of quantification (LLQ) for the assay was 0.02998 and p24 concentrations that were below the LLQ of each assay were expressed as half-LLQ values.

Tissue drug metabolites

Concentrations of the active phosphorylated intracellular metabolites – tenofovir-diphosphate (TFV-DP) and emtricitabine-triphosphate (FTC-TP) were determined in foreskin tissue as previously described [17].

RNA sequencing of foreskin tissue

The details of the procedure used for RNA sequencing of foreskin tissue have been previously described [18]. Transcriptomes were derived from combined inner and outer foreskin from each participant.

Statistical Analysis

The following outcomes were summarized both overall and by trial arm: $CD4^+$ cell density, CCR5 density, $CD4^+CCR5^+$, proportion of CCR5 expression on $CD4^+$ cells, distance from the epidermis, eCD1a/iCD1a ratio, $CD1a/cm^2$, % claudin expression. Except for the proportion of CCR5 expression on $CD4^+$ cells, outcomes were positively skewed and were therefore log-transformed for analysis. The relative effect of trial interventions was assessed through the following comparisons: (i) any PrEP *versus* control arm, (ii) F/TAF *versus* F/TDF, (iii) 2+1 tablets *versus* 2 tablets, (iv) 21h between PrEP and VMMC *versus* 5h. Further comparisons also assessed the effect of dosage separately for each drug, and the effect of the interval, separately for each drug and dosage. Mixed effects linear regression models, allowing for clustering within participants since several sections of tissue were imaged for each participant, were used to determine the mean difference and 95% CI for each comparison, with p-values determined by likelihood ratio tests. For all outcomes other than the proportion of CCR5

expression on $CD4^+$ cells, parameters were back transformed and reported as geometric mean ratios (GMR). Correlations between each study outcome and PK/PD parameters were assessed by Pearson's correlation coefficient, using the mean of the imaging outcome from the different sections for each participant.

Ethics Approvals

Written informed consent was obtained from all participants aged ≥18 years and emancipated minors (in Uganda); for those <18 years and not emancipated minors, their assent with parental consent was obtained. The trial was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice and approved in the South African Health Products Regulatory Authority (20181004). Ethical approval was granted from University of Cape Town (290/2018), University of the Witwatersrand (180906B, M1811148 and 180108), Uganda Virus Research Institute research ethics committee (GC/127/18/12/680), Uganda National Council of Science and Technology (HS2534), Uganda National Drug Authority (618/NDA/DPS/09/2019), and London School of Hygiene and Tropical Medicine research ethics committee (17403). The Swedish Ethics Review Authority approved the laboratory studies of the collected specimens at the Karolinska Institute (2020-00941).

Results

Impact of F/TDF and F/TAF on the density of $CD4^+CCR5^+$ and CCR5 expression on $CD4^+$ cells in the foreskin

As CCR5 is the main HIV co-receptor for most transmitted isolates [19; 20], we assessed the density of double-expressing CD4+CCR5+ cells along with the proportion of CCR5 staining on $CD4^+$ cells. Figures 1A-C show representative images of single- (A and B) and double-expressing $CD4^+CCR5^+$ cells (C) in foreskin tissue in relation to the outer epidermis. Figure 1D shows the distribution of double-expressing $CD4^+CCR5^+$ cell density by treatment arm,

where no significant differences were identified for any comparisons (supplementary Table 1). Timing and dose of F/TDF and F/TAF had no significant impact on CCR5 expression on $CD4^+$ cells (Figure 1E, supplementary Table 1). This finding was consistent at the tissue gene expression level, where there was no significant difference in the expression of CD4 or CCR5 genes between treatment and control arms (supplementary Figures 3A and B). Collectively, these data show that the density of HIV target cells and CCR5 expression was not modulated by drug dose or timing of PrEP prior to VMMC.

On a subset of samples (South Africa participants), we compared the impact of the drug dose and timing on the density of $CD4^+CCR5^+$ cells separately for inner and outer foreskin samples. Supplementary Figures 4A and B show that PrEP doses from the different arms had no impact on the density of $CD4^+CCCR5^+$ cells in either the inner or outer foreskin. There was also no significant difference ($p=0.14$) in the densities of $CD4^+CCR5^+$ cells between the inner and outer foreskin. However, there was 5.8 times greater expression of CCR5 on $CD4^+$ cells in the outer foreskin ($p=0.01$), inferring that the outer foreskin would be more susceptible to HIV-1 infection.

We then tested the hypothesis that drug may influence the anatomical location of $CD4^+CCR5^+$ cells and possibly drive them either deeper into the tissue or further to the apical layer of the epidermis. Figure 1F and supplementary Table 1 shows that the distance of potential HIV-1 target cells from the epidermis was not significantly different between treatment arms or relative to the control arm.

Impact of F/TDF and F/TAF on the density of $CD1a^+$ cells and claudin-1 expression in the foreskin.

CD1a is one of the unique proteins that is expressed on either dendritic cells [21] or epidermal Langerhans cells [22; 23]. Furthermore, this marker represents an important cell type as either an HIV target [24] or for delivery of HIV away from sites of infection [24]. We sought to identify whether either of the PrEP regimens, doses, or time of administration prior to VMMC impacted upon the density and location of $CD1a^+$ cells from the outer epithelial layer, as measured by claudin-1, one of the tight junction proteins found in the foreskin epidermis [25; 26]. Figure 2A shows a representative image of $CD1a^+$ cell staining, illustrating the presence of these cells in both epidermis and dermis. There was a significantly lower density of $CD1a^+$ cells in all combined samples from Uganda (p=0.03) compared with South Africa, although there remained no impact of PrEP dosing when stratifying by recruitment site (supplementary Figure 5A & B).

In addition to using claudin-1 to delineate the outer epithelial surface, we quantified expression as a measure of barrier integrity [27]. Table 1 shows the geometric mean of percent claudin-1 expression was 34% higher (p=0.003) in combined inner and outer foreskin tissue from all participants receiving drug, compared to the control arm. After allowing for multiple comparisons, this was no longer significant (p=0.288). Comparing F/TAF double dose given 5 hours before VMMC versus F/TAF double dose given 21 hours prior to circumcision, resulted in 28% lower expression of claudin-1 (p=0.007, Figure 2C), which was no longer significant after allowing for multiple comparisons.

Impact of F/TDF and F/TAF on the anatomical location of $CD1a^+$ cells in the foreskin

To better gauge the location of $CD1a^+$ cells in relation to the epidermis, we measured the ratio of $CD1a^+$ cells between those located within the area of claudin-1 staining (intrinsic, i), being exclusively expressed in the epidermis, or located outside claudin-1 expression (extrinsic, e). Figures 2D & E show representative images of iCD1a (D) and a mix of iCD1a and eCD1a

staining (E) respectively. Figure 1F shows that there were no differences in the anatomical location of $CD1a^+$ cells between the 8 treatment arms compared to the control arm, suggesting that drug exposure was not eliciting any migration of $CD1a^+$ cells within the foreskin.

No association between the density of $CD4^+CCR5^+$ and $CD1a^+$ cells and claudin-1 expression with foreskin tissue drug metabolite levels or p24 production after ex vivo viral challenge.

One of the secondary objectives of the clinical trial was to identify the immune safety profile of the different PrEP dosing and timing [14]. Additional evidence to show this was made by correlating the density of HIV target cells with drug metabolite levels in the tissue. Figure 3 shows a lack of correlation between drug metabolites (TFV-DP and FTC-TP) in whole foreskin tissue with densities of $CD4^+CCR5^+$, $CD1a^+$ cells, % CCR5 expression on $CD4^+$ cells and claudin-1 expression. This would suggest that the protective effect of PrEP is independent of the density of HIV target cells in the tissue. This was underscored by a lack of association between $CD4^+CCR5^+$, % CCR5 expression and $CD1a^+$ cell numbers with p24 production after *ex vivo* challenge with low or high viral titre (Figure 3). Likewise, there was no relationship between p24 production and the location of $CD1a^+$ cells in relation to claudin-1 (Table 1). There was a weak negative association between claudin-1 expression and p24 production after high titre viral challenge ($r=-0.28$, $p=0.01$, Figure 3). This would suggest a trend of higher claudin-1 expression and an association with lower viral replication upon *ex vivo* high dose challenge.

Discussion

We show, in a randomised clinical trial [14], that short-course oral dosing and scheduling of on-demand F/TAF or F/TDF PrEP had no impact on the density of HIV target cells in foreskin tissue when men were circumcised at 5h or 21h after taking drug. This was a novel trial where

we aimed to show, as a secondary objective, that different types of PrEP and dosing schedules were not associated with immune activation *in situ.* We showed that $CD4^+CCR5^+$ cells, as measured quantitively and by gene expression levels in the tissue, were similar across different dosing and timing of drug administration. Importantly, we show that the expression of the HIV co-receptor CCR5 [18; 28], well established to be increased upon cell activation [28; 29], was not impacted by drug dose. This was similarly the finding with $CD1a^+$ cells in the foreskin showing that epithelial dendritic cells [21; 30] and/or epidermal Langerhans cells [23] were consistently present across the different trial arms, but unchanged in numbers. In contrast to studies using topical PrEP whereby 1% tenofovir gel increased T cell densities in rectal tissue [11] and $CD4^+$ T cell activation in endocervical tissue [13], we show in our study that oral PrEP dosing has no effect on local foreskin T cell immunity. Previously we showed that the CHAPS study resulted in modulation of gene expression in the foreskin resulting in a potentially unfavourable environment for HIV replication [18]. Collectively, these findings show that short-term oral PrEP does not induce immunologic activity in the foreskin.

The foreskin protects the host from invasion of a myriad of pathogens and does so by employing physical barriers and an intricate network of resident immune cells [31] and can be regarded as a persistently "inflamed" tissue [32]. There is a fine distinction between Langerhans cells (LCs) and dermal dendritic cells. LC's have been shown to reside in the epidermis and can act as immune sentinels by actively sampling environmental antigens [33], whereas dermal dendritic cells have been found in the papillary dermis [34]. CD1a has been shown to mark both dendritic cells [21] and epidermal Langerhans cells [22; 23], and the epidermal location of $CD1a^+$ cells in our study would be consistent with staining for LCs. Although we could not differentiate between these two myeloid populations, their numbers were not modulated by PrEP dosage or timing and nor associated with drug metabolites in the tissue or p24 production after low or high dose *ex vivo* HIV challenge. Although the HIV

challenge model is a proxy for *in vivo* HIV transmission and acquisition in tissue, our finding suggests that the presence of *in situ* HIV targets cells in the tissue is independent of downstream viral replication.

Physical barrier function in the skin resides within the stratum corneum, which relies on the arrangement of epithelial cells and tight junction proteins. The importance of tight junction proteins, claudin-1 particularly, was demonstrated to be crucial for survival in mice [27], where deletion of claudin-1 resulted in death shortly after birth due to defects in epidermal barrier function. There was tantalizing evidence, albeit not significant after multiple comparisons, that claudin-1 expression may be enhanced with PrEP. Interestingly, the shorter exposure to drug (five hours) resulted in lower claudin-1 expression compared with the longer duration (21 hours) and infers that longer PrEP exposure may be more beneficial. This would suggest that barrier function may be enhanced with longer drug exposure and may represent a novel finding but needs to be explored in larger studies.

To our knowledge, this is the first time that the effects of oral PrEP has been assessed on immune cells in foreskin tissue. Although this study does not address the possible role of fibroblasts and epithelial cells in the foreskin accumulating F/TAF and F/TDF [35] and the consequent long-term impact on numbers of $CD4^{+}CCR5^{+}$ and $CD1a^{+}$ cells within the tissue, it does show that short term "on-demand" oral PrEP is immunologically safe and does not induce higher numbers of activated $CD4^{+}$ T cells in a vulnerable anatomical site for HIV acquisition in males.

Author's contributions.

CH, JS, PK, SK, NM, FC, JF, CMG conceived and designed the study. CLR, ELW, SM, LW, PN, GO, DO, ASS, LL contributed to the implementation of the study in South Africa and

Uganda. CLR, BA, AB, BM, TM, LL, SS, SP, SM, PN, GO, DO, AAPP, TBS contributed to the laboratory investigations and validation of the methods. CLR and CMG took the lead in writing the original draft and ELW conceptualised and performed all statistical tests used in the paper. All authors were involved in the review and editing of the manuscript. All authors had full access to all the study data and the final responsibility for the decision to submit for publication.

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Conflict of Interest

None of the authors have a conflict of interest.

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Figure Legends

Figure 1. Density of $CD4^+CCR5^+$ and CCR5 on $CD4^+$ cells in foreskins from the participants of the CHAPS trial. (A) Representative image of CD4+ cells in the foreskin; (B) Representative image of CCR5+ cells in the foreskin; (C) Representative image of dual stained CD4+CCR5+ cells in the foreskin showing the outline of the epidermis layer and representative distances (a, b, c, d) of cells from the surface; (D) Numbers of CD4+CCR5+ cells in the foreskin across the 9 trial arms. Box plots show median lines and interquartile ranges overlayed with each measurement. (E) Proportion of CCR5 expression on CD4+ cells in the foreskin across the 9 trial arms. Box plots show median lines and interquartile ranges overlayed with each measurement. (F) Distance of CD4+CCR5+ cells within the foreskin from the outer epidermis from participants across the 9 trial arms. Box plots show median lines and interquartile ranges overlayed with each measurement.

Figure 2. Density of CD1a+ and claudin-1 expression in the foreskins of men in different trial arms. (A) Representative image of CD1a+ cells located in the foreskin; (B) combined density of CD1a+ cells in foreskins (cells/cm^2) across the different trial arms; (C) combined expression of claudin-1 across the different trial arms; (D) Representative image showing the presence of CD1a cells within (intrinsic, i) claudin-1 expression; (E) representative image of CD1a+ cells within (intrinsic, i) claudin-1 expression and outside (extrinsic, e). The white circled cells delineate iCD1a from eCD1a; (F) Combined ratio of eCD1a/iCD1a across the trial arms.

Figure 3: Forest plots of correlations of TVF-DP, FTC-TP and p24 (high and low dose) with log_{10} cell densities, distance from the epidermis, % claudin and eCD1a/iCD1a ratio.

523 Pearson correlation coefficients with 95% confidence intervals are shown for each comparison.

524 The vertical line indicates a correlation coefficient of zero.

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