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Blood immune cells from people with HIV on antiviral regimens that contain tenofovir alafenamide (TAF) and tenofovir disoproxil fumarate (TDF) have differential metabolic signatures

Eleni Ritou [a], Sandro Satta [a], Anton Petcherski [b], Maria Daskou [a], Madhav Sharma [a], Hariclea Vasilopoulos [a], Eisuke Murakami [c], Orian S. Shirihai [a,d], Theodoros Kelesidis [a,*]

[a] Department of Medicine, Division of Infectious Diseases, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, USA
[b] Department of Medicine, Division of Endocrinology, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, USA
[c] Gilead Sciences, Inc., 333 Lakeside Drive, Foster City, CA 94404, USA
[d] Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California, Los Angeles, CA, USA

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ABSTRACT

Background: Mitochondria regulate immune and organ function. It is unknown whether higher intracellular drug levels observed in peripheral blood mononuclear cells (PBMCs) treated with tenofovir alafenamide (TAF) compared to tenofovir disoproxil fumarate (TDF) may alter mitochondrial function and energy production in immune cells in HIV$^{(+)}$ patients.

Methods: Cellular bioenergetics were determined in PBMCs from HIV-1$^{(-)}$ participants exposed to TAF *versus* TDF *in vitro*, at a comparable concentration to a clinically relevant plasma exposure. A decrease in cellular oxygen consumption rate (OCR) at baseline (basal-OCR) and under cellular stress (max-OCR) may suggest mitochondrial dysfunction. We also assessed the *in vivo* impact of TAF *vs* TDF on OCR in PBMCs from 26 people with HIV (PWH) interchanged from TDF-based to TAF-based antiretroviral therapy (ART) over a 9-month period in the setting of an open label clinical trial. The Wilcoxon and Mann Whitney tests were used for comparison of continuous variables.

Results: PBMCs from HIV-1$^{(-)}$ participants exposed *in vitro* to a concentration of 0.12–3.3 μM for TAF and TDF at 2 and 24 h, reduced basal and maximal OCR compared to vehicle control. Switch studies of antivirals (TAF *vs* TDF) within the same PWH showed that TAF-based ART was associated with reduced OCR compared to TDF-based ART in PBMCs. We observed that TAF-treated PBMCs selectively relied more on glucose/pyruvate supply rather than fatty acid to fuel their mitochondria.

Conclusions: Compared to TDF, TAF may alter bioenergetics in immune cells from PWH *in vitro* and *in vivo*. The clinical significance in terms of the differential impact caused by TAF *versus* TDF on mitochondrial function and energy production in immune cells, a regulator of immune function, requires further studied in HIV, preexposure prophylaxis and hepatitis B.

1. Introduction

Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) such as tenofovir alafenamide (TAF) and tenofovir disoproxil fumarate (TDF) are cornerstones of combination antiretroviral therapy (ART) used in people who live with HIV (PWH), and in patients with chronic Hepatitis B [1,2]. Both TAF and TDF are also used as preexposure prophylaxis (PrEP) for HIV prevention [1,2]. Given the prevalence of chronic HIV and Hepatitis B infections together with increased use of PrEP worldwide, it is essential to characterize the long-term impact of both TAF and TDF on human cells and tissues. Despite the benefits obtained from TAF and TDF compared to older antivirals [1,2], their exact cellular effects remain unclear.

Both TAF and TDF are prodrugs of tenofovir (TFV), and generate the active metabolite, tenofovir-diphosphate (TFV-DP) [1,2]. Compared to TDF, TAF achieves higher intracellular levels of TFV-DP in peripheral

* Corresponding author at: Department of Medicine, Division of Infectious Diseases, David Geffen School of Medicine at UCLA, 10833 Le Conte Ave. CHS 47-100, Los Angeles, CA 90095, USA.
E-mail address: tkelesidis@mednet.ucla.edu (T. Kelesidis).

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blood mononuclear cells (PBMCs), resulting in a more potent antiviral effect at lower doses [3]. Markedly lower plasma levels of TFV are thought to lead to the more favorable bone and renal safety profile of TAF compared to TDF [1,2]. However, in clinical trials increased fasting lipids were observed when TDF was switched to TAF at 48 weeks [1,2]. Importantly, the combination of TAF with integrase inhibitors has been associated with dyslipidemia and weight gain [4]. In addition, antivirals have been shown to have differential effects on immune activation and dysfunction [5], a key instigator of morbidity in treated chronic HIV. The mechanisms for these observations require further study.

Mitochondrial dysfunction likely plays a role in immune dysfunction, aging and end organ disease in humans and PWH [6–8]. Mitochondrial function in PBMCs has been described as a marker of overall cellular function in human disease during several pathological states [9–11]. It is unknown whether an increase in intracellular drug levels observed in PBMCs and other cells treated with TAF [3] may impact mitochondria, alter cellular bioenergetics, or contribute to alterations in lipid metabolism and immune function. There is also limited data on the impact of ART on mitochondrial function using gold standard direct assessment of the major function of the mitochondria which is energy production. Indeed, most prior work that assessed the impact of ART on mitochondria utilized PCR measuring mitochondrial DNA which is not a direct measure of mitochondrial function [12]. Mitochondria oxygen consumption (oxygen consumption rate; OCR) is coupled to adenosine triphosphate (ATP) production and has increasingly been identified as a key measure of mitochondrial function [13]. Notably, measurement of OCR in accessible blood PBMCs can be a meaningful surrogate of cardiometabolic dysfunction [10]. Blood immune cells also have distinct bioenergetic profiles that regulate energy production and their function or activation status [14].

Herein, we explored the differential impact of commonly used NRTIs (TAF, TDF) on mitochondrial health in accessible PBMCs as a surrogate measure for differential systemic effects of TAF *vs* TDF on metabolism for less accessible tissues (such as bone and kidney) in uninfected adults and PWH. For this reason, we utilized a robust experimental approach to study NRTI-related alterations in mitochondrial function using PBMC from HIV-$1^{(-)}$ people and PWH switching antiretrovirals. To evaluate the relative mitochondrial toxicity of TDF *vs.* TAF *in vivo*, we conducted an open-label switch study in virologically suppressed HIV-$1^{(+)}$ participants treated with TAF-based *versus* TDF-based ART. We determined mitochondrial function in PBMCs by measuring cellular bioenergetics and the ability of mitochondria to produce energy (OCR).

2. Methods

2.1. Materials

Elvitegravir 150 mg/cobicistat 150 mg/emtricitabine 200 mg/TDF 300 mg (E/C/F/TDF), E/C/F//TAF (10 mg) and F/TAF (25 mg) (F/TAF) single tablet regimens (STR), TAF and TDF were a generous gift from Gilead Sciences (Foster City, CA). Other chemicals were purchased as indicated in the Supplemental Material.

2.2. Study design

2.2.1. PBMCs from HIV-$1^{(-)}$ participants (Group A)

To characterize the direct effect of TAF *vs* TDF on mitochondria in human PBMCs independently of HIV-1 infection, we recruited 30 HIV-$1^{(-)}$ participants without any underlying medical conditions (18 males). Blood samples from HIV-$1^{(-)}$ donors were obtained from the UCLA Virology Core.

2.2.2. Open-label clinical trial of switch of antiretrovirals

First, we cross-sectionally characterized respirometry in PBMCs of ART-treated PWH with suppressed plasma viremia (<50 copies/ml) on TDF-based ART (group B) and TAF-based ART (group C) for at least 3 months prior to study entry (baseline). Seven study participants were on TDF-based ART with E/C/F/TDF, three were on rilpivirine (R)/F/TDF and one person was on dolutegravir (D)/F/TDF. Twelve participants were on TAF-based ART with bictegravir (B)/F/TAF and three were on R/F/TAF. Second, we characterized the switch from TAF to TDF and then back to TAF on mitochondria in PBMCs of ART-treated PWH. The fifteen study participants on TAF-based ART at baseline with B/F/TAF ($n = 12$) and R/F/TAF ($n = 3$) were switched to TDF-based ART with E/C/F/TDF ($n = 12$) and R/FTC/TDF ($n = 3$), respectively, for 2 months. These fifteen study participants were ultimately switched back to TAF-based ART with E/C/F/TAF (n = 12) and R/FTC/TAF (n = 3), respectively, for 6 months. Blood was collected at each timepoint for measurements of mitochondrial function in PBMCs and HIV-1 viral load. The study design is shown in Fig. 1. The primary outcome was determination of cellular bioenergetics as measured by oxygen consumption rate (OCR). All participants were recruited within the UCLA Center for AIDS Education and Research (UCLA CARE) Center in Los Angeles after written informed consent following institutional approvals. The clinical trial was registered at ClinicalTrias.gov (NCT03251144).

2.2.3. Processing of PBMCs

PBMCs were isolated using standard density gradient centrifugation and were then cryopreserved as previously established [15].

2.2.4. In vitro treatment of PBMCs with NRTIs

We evaluated the effects of 2-hour and 24-hour treatment of 3 NRTIs (TAF, TDF and dideoxycytidine or ddC) on mitochondria in non-activated PBMCs from HIV-$1^{(-)}$ donors using pharmacologically relevant validated *in vitro* concentrations of antiretrovirals that exceed the

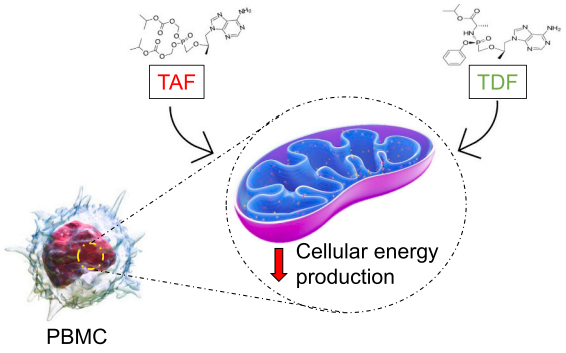

Fig. 1. Overall study design. The study included a complementary translational approach with both preclinical *in vitro* studies and clinical studies using accessible PBMCs from HIV-$1^{(-)}$ participants (men and women) and people with HIV (PWH) with suppressed plasma viremia on TAF-based *versus* TDF-based ART. Preclinical studies included *in vitro* exposures of PBMCs from HIV-$1^{(-)}$ people over 2 h (acute effect of antiviral) and 24 h (subacute effect of antiviral) to physiologically (0.12 μM for TAF and TDF and 1.1 μM for TAF) and supraphysiologic concentrations (3.3 μM) of TAF *versus* TDF. *In vivo* studies included cross-sectional comparisons of cellular bioenergetic profiles in HIV-$1^{(-)}$ PBMCs ($n = 30$), PWH on chronic exposure (for at least 3 months) to TDF-based ART prior to study enrollment ($n = 11$) and PWH on chronic exposure (for at least 3 months) to TAF-based ART prior to study enrollment ($n = 15$). In addition, we also included an open label clinical trial of switching from a TDF-based regimen to TAF-based ART regimen over 6 months (Switch group 1: direct switch TDF to TAF; n = 11). ART was also interchanged in 15 independent PWH from a TAF-based regimen of ART to TDF-based ART regimen over 2 months. Another crossover switch was made in the same participants from TDF-based regimen to TAF-based regimen of ART over 6 months (switch group 2: crossover switch TAF to TDF and then to TAF). This combined preclinical and clinical study design together with the comparison of parameters of cellular bioenergetics in PBMCs within the same person among study participants, accounted for heterogeneity in mitochondrial function between different individuals and dissected the impact of TAF *versus* TDF on mitochondrial functionality in human PBMCs.

clinically relevant intracellular drug exposures in humans [16]. Micromolar concentrations of intracellular levels of TFV-DP in PBMCs was calculated based on the number of cells (10^6) and a PBMC intracellular volume of 0.2 pL/cell as previously described [16]. See Supplemental material for details.

2.2.5. Seahorse respirometry assays

Mitochondrial respirometry was performed using a Seahorse XF96 Extracellular Flux Analyzer (Agilent Santa Clara, CA) to estimate the OCR and the Extracellular Acidification rate and using the Mito Stress, the Substrate Oxidation Stress Test protocols according to manufacturer's protocol and as previously described [17,18].

2.3. Statistical analysis

Data are presented as either mean ± standard error of mean (SEM) or as median and interquartile range (IQR) as indicated. Statistical significance was assessed with non-parametric Kruskal-Wallis, Mann-Whitney U and Wilcoxon matched-pairs tests, as appropriate. The software package GraphPad Prism 8.0 (GraphPad Software, San Diego, California) was used for data and statistical analyses. The study was exploratory and was not powered for assessment of specific exploratory outcomes [19]. To our knowledge, the impact of TAF and TDF on our specific measures of cellular bioenergetics has not previously been determined. Thus, the effect size on specific measures of mitochondrial function was not predetermined. This study will set the foundation for future larger studies to assess the impact of ART on mitochondrial function. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Characteristics of study participants

Twenty-six participants met the enrollment criteria and completed the study. The characteristics of all study participants are shown in Table 1. There were no differences in baseline demographics (age, gender, race, comorbidities) between compared groups.

3.2. TAF and TDF alter in vitro cellular bioenergetics in HIV-$1^{(-)}$ PBMCs

First, to confirm that our *in vitro* exposures of antivirals simulate pharmacologically relevant *in vivo*, we measured intracellular TFV-DP concentrations in PBMCs from 5 HIV-$1^{(-)}$ uninfected men (age 18–40 years old) after 2-hour treatments with 1.1 μM TAF. Intracellular levels of TFV-DP in PBMCs were 1.25 ± 0.46 μM (range 0.52–2.84 μM) and like previously validated TFV-DP levels in immune cells after treatment with 1.1 μM TAF [16].

Table 1
Baseline characteristics of study participants by experimental group.

	Group A HIV-1 uninfected persons (n = 30)	Group B HIV-1 infected persons on a TAF-based ART regimen (n = 15)	Group C HIV-1 infected persons on a TDF-based ART regimen (n = 11)
Age, median years (IQR)	45 (34,55)	42 (32,48)	45 (34,55)
Male sex	n = 18 (60 %)	n = 15 (100 %)	n = 11 (100 %)
Race/ethnicity, %			
White	n = 26 (100 %)	n = 10 (66.7 %)	n = 8 (72.3 %)
Black	0	2 (13.3 %)	2 (13.3 %)
Hispanic	n = 4 (13.3 %)	3 (20 %)	1 (6.7 %)
CD4+ cell count (cells/mm^3) median (IQR)	(−)	639 (461, 767)	546 (300, 948)
HIV-1 RNA (copies/mL)	(−)	<50	<50
Body mass index (kg/m^2)	N/A	25 (22,28)	24 (21, 26)
Hyperlipidemia	n = 0	n = 5 (33.3 %)	n = 3 (27.2 %)
Current smoking, %	n = 0 (0 %)	n = 0 (0 %)	n = 0 (0 %)
Hypertension, %	(−)	n = 2 (13.3 %)	n = 1 (9 %)

To simulate the *in vivo* acute impact of TAF *vs* TDF on mitochondrial function in PBMCs, we assessed the effect of a 2-hour incubation of TAF, TDF (10 min pulse) on mitochondrial function in non-activated PBMCs isolated from HIV-$1^{(-)}$ people 18–40 years old with no underlying medical conditions (n = 30, 18 males). A known mitochondrial toxin, ddC was used as a positive control for mitochondrial toxicity in all *in vitro* studies. We determined oxygen consumption rate (OCR), by measuring oxidative phosphorylation at baseline, before injection of electron transport chain (ETC) modulators, and under cellular stress after injection of ETC modulators. Representative OCR measurements for PBMCs from healthy participants (Control) and HIV-$1^{(+)}$ people on TAF-based and TDF-based ART regimens are shown in Fig. S1. Compared to the Dimethyl Sulfoxide (DMSO) vehicle control, TDF (0.12 and 3.3 μM), TAF (0.12, 1.1 and 3.3 μM) and ddC (3.3 μM) did not alter the basal and the maximal OCR (Fig. 2A-B), as well as the ATP-linked OCR (Fig. 2C) and the oligomycin-insensitive proton leak linked respiration (leak OCR) (Fig. S2A). Both 3.3 μM TAF and ddC reduced the spare respiratory capacity (SRC), a measure of reserve OCR capacity, (Fig. 2D) compared to vehicle control ($p < 0.05$ for both comparisons). A mean reduction of the SRC by 22 % was observed with 3.3 μM TAF compared to 3.3 μM TDF ($p < 0.001$).

By measuring the ATP in PBMCs, we would not be able to discriminate the ATP source (glycolytic *vs* mitochondrial). Also, since intracellular ATP levels are in steady state [20] fluctuations in the ATP demand would be undetected. Therefore, to further characterize the impact of TAF *vs* TDF on cellular bioenergetics in PBMCs, we assessed the partitioning of ATP generation between glycolysis (glyco-ATP) and oxidative phosphorylation (mito-ATP) by simultaneous measurements of extracellular acidification rate (ECAR), a measure of glycolysis, and OCR at baseline and under maximal respiration. A 2-hour treatment of TAF (0.12 and 3.3 μM) and ddC reduced basal glyco-ATP compared to vehicle control ($p < 0.01$ for all comparisons) (Fig. S3A). 3.3 μM TAF also reduced basal glyco-ATP by a mean of 33 % compared to TDF at the same concentration ($p < 0.001$), and this reduction was dose-dependent (Fig. S3A). ddC, TDF and TAF did not impact *in vitro* production rates of basal mitochondrial respiration at any concentration (Fig. S3B) and total ATP (Fig. S3C) in PBMCs over 2 h. Under cellular stress, 2-h treatment of TDF and ddC at 3.3 μM, increased and reduced, respectively, maximal glyco-ATP compared to vehicle control ($p < 0.01$ for all comparisons) (Fig. S3D). 3.3 μM TAF also reduced maximal glyco-ATP by a mean of 10 % compared to TDF at the same concentration ($p < 0.05$) (Fig. S3D). TDF and TAF at any concentration did not impact *in vitro* production rates of maximal mitochondrial respiration (Fig. S3E) and total ATP (Fig. S3F) in PBMCs. ddC also reduced maximal total ATP rate compared to vehicle control ($p < 0.001$) (Fig. S3F).

To simulate the subacute impact of TAF *vs* TDF *in vitro* at supraphysiological concentrations in mitochondrial function in PBMCs, we assessed the effect of a 24-h incubation of TAF, TDF and ddC on mitochondrial function of HIV-$1^{(-)}$ PBMCs (n = 30). Compared to the DMSO vehicle control, ddC (3.3 μM), TDF (0.12, 3.3 μM) and TAF (0.12, 1.1 and 3.3 μM) decreased the basal (Fig. 2E), the maximal (Fig. 2F), the ATP-linked (Fig. 2G), and the spare (Fig. 2H) OCRs ($p < 0.001$ for all comparisons). These observed effects were dose-dependent, since 3.3 μM TAF decreased the basal (Fig. 2E), the maximal (Fig. 2F), the ATP-linked (Fig. 2G), and the spare (Fig. 2H) OCRs compared to 0.12, 1.1 μM TAF and 1.1, 3.3 μM TDF ($p < 0.01$ for all comparisons). TDF (0.12, 3.3 μM) and TAF (0.12, 1.1 and 3.3 μM) but not ddC also decreased leak OCR compared to vehicle control ($p < 0.01$ for all comparisons) (Fig. S2B). 24-hour treatment of TAF (0.12, 1,1 and 3.3 μM) and ddC reduced production rates of basal and maximal glyco-ATP, Mito-ATP and total ATP compared to vehicle control ($p < 0.01$ for all comparisons)

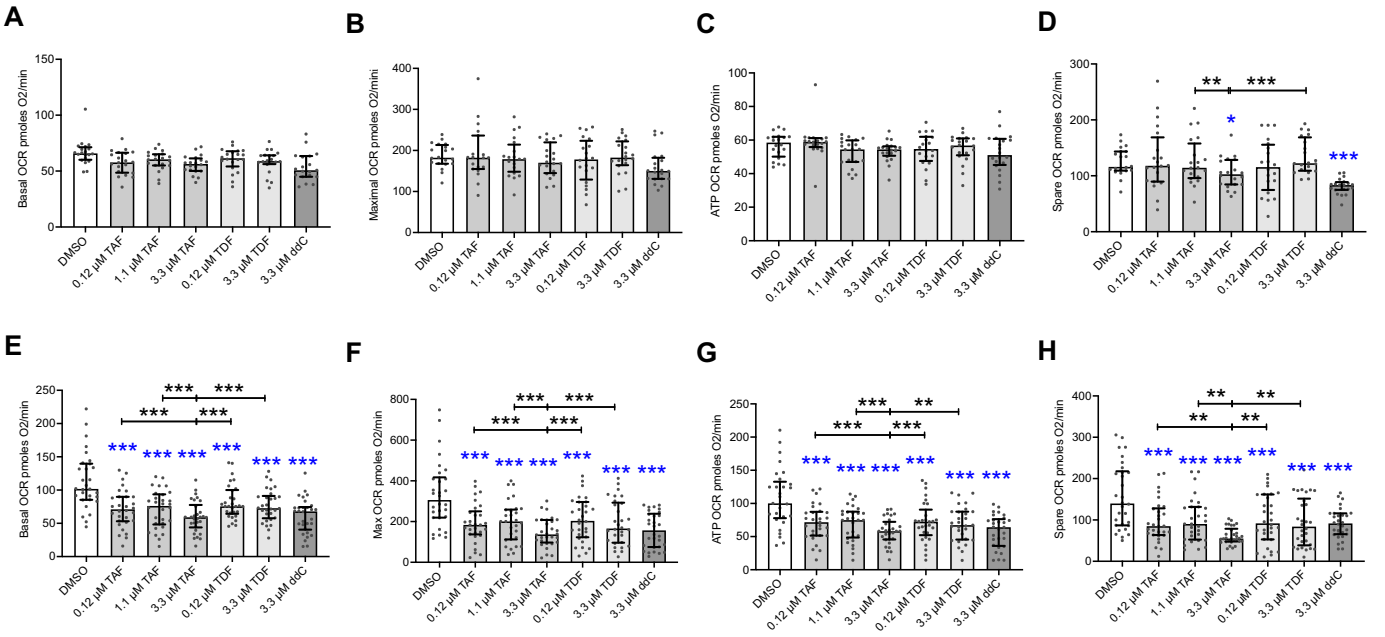

Fig. 2. *In vitro* impact of 2- and 24-hour treatment with antiretrovirals on cellular respiration in HIV-1$^{(-)}$ PBMCs cells. PBMCs cells from HIV-1$^{(-)}$ people ($n = 30$) were plated in XF96 microplates and incubated with antivirals *versus* DMSO vehicle control for 2 h (A–D) or 24 h (E–H) at 0.12 µM–3.3 µM concentrations as shown. Measurements of cellular bioenergetics parameters [measured by OCR in pmoles O2/min] were acquired using a XF96 Extracellular Flux analyzer as described in methods. A. Basal OCR (2 h); B. Maximal OCR (2 h); C. ATP linked OCR (2 h); D. Spare Respiratory Capacity or Spare OCR (2 h); E. Basal OCR (24 h); F. Maximal OCR (24 h); G. ATP linked OCR (24 h); H. Spare OCR (24 h). Data represent bars that display the medium and interquartile range (IQR). Each datapoint represents the average of replicates (≥5) of cells per person ($n = 30$). The Kruskal-Wallis test was used to compared multiple groups and the Wilcoxon matched-pairs signed rank test was used to compare 2 groups. Comparisons against the vehicle control are shown in blue (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

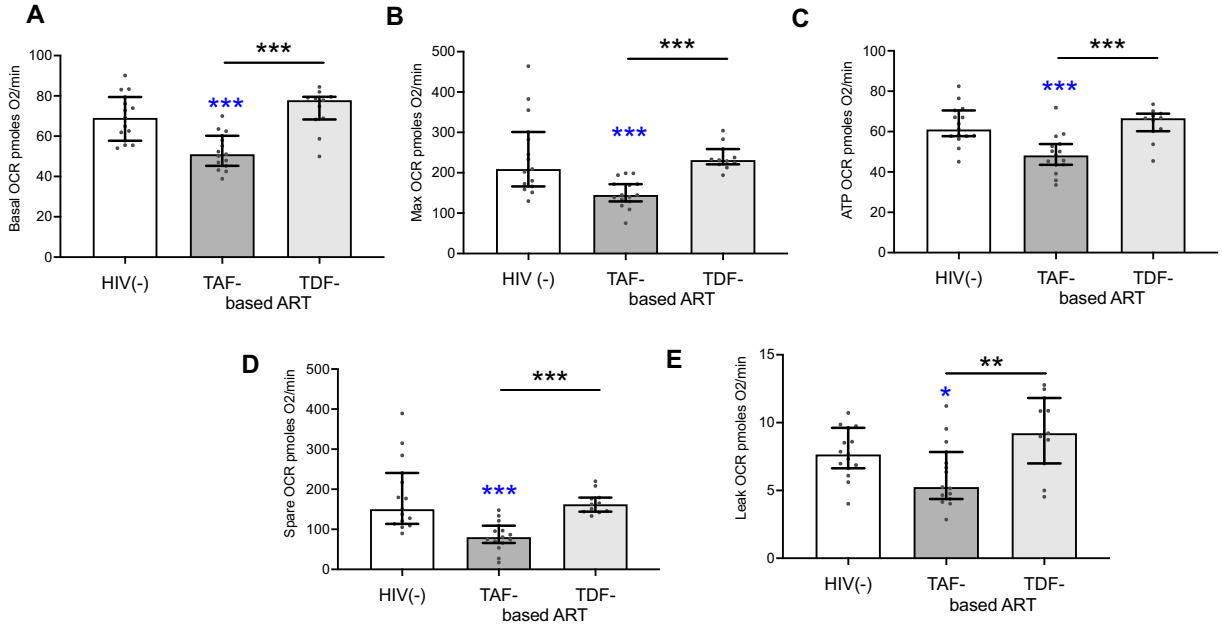

Fig. 3. Cross-sectional assessment of cellular bioenergetic profiles among PBMCs from HIV-1$^{(-)}$ and HIV-1$^{(+)}$ people on TAF-based *versus* TDF-based ART. PBMCs cells from HIV-1$^{(-)}$ persons ($n = 15$) and HIV-1$^{(+)}$ persons on TAF-based *versus* TDF-based antiretrovirals were plated in XF96 microplates and measurements of cellular bioenergetics parameters [measured by OCR in pmoles O2/min] were acquired using a XF96 Extracellular Flux analyzer as described in methods. TDF-based ART was emtricitabine, elvitegravir, cobicistat and tenofovir disoproxil fumarate (F/E/C/TDF) ($n = 7$), rilpirivine (R)/F/TDF ($n = 3$) and dolutegravir (D)/F/TDF (n = 1). TAF-based ART was bictegravir (B)/F/TAF ($n = 12$) and R/FTC/TAF (n = 3). A. Basal OCR; B. Maximal OCR; C. ATP linked OCR; D. Spare Respiratory Capacity or Spare OCR; E. Leak OCR. Data represent bars that display the medium and interquartile range (IQR). Each datapoint represents the average of replicates (at least 5) of cells per person. The Kruskal-Wallis test was used to compared multiple groups and the Mann Whitney test was used to compare 2 groups. Comparisons against the vehicle control are shown in blue (*p < 0.05, **p < 0.01, ***p < 0.001). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Figs. S4A–F). 24-hour treatment of TDF (0.12 and 3.3 μM) also reduced production rates of maximal (but not basal) glyco-ATP and both basal and maximal mito-ATP and total ATP compared to vehicle control ($p < 0.01$ for all comparisons) (Figs. S3A–F). 3.3 μM TAF also reduced basal and maximal Glyco-ATP, Mito-ATP and total ATP by at least 22 % compared to 3.3 μM TDF ($p < 0.001$), and this reduction was dose-dependent compared to 0.12 μM and 1.1 μM TAF (Figs. S4A–F). Both TAF and TDF did not induce any cell cytotoxicity in treated PBMCs over 2 and 24 h at any concentration based on cell viability assessed through Trypan Blue staining and seeding of identical number of viable cells for respirometry experiments. Collectively, our data suggests that all studied NRTIs (TAF, TDF, ddC), including the established mitochondrial toxin ddC, alter *in vitro* cellular bioenergetics in non-activated HIV-$1^{(-)}$ PBMCs both over 2 h and 24 h.

3.3. TAF-based ART is associated with differential cellular bioenergetics compared to TDF-based ART in PBMCs from PWH

To further investigate the *in vivo* impact of TAF *vs* TDF on mitochondrial function of PBMCs from PWH, we cross-sectionally explored differences in cellular bioenergetics between HIV-$1^{(-)}$ men (group A; $n = 15$) and PWH on a TAF-based ART regimen (group B; $n = 15$) *versus* a TDF-based ART regimen (group C; $n = 11$). Similarly, and consistently to our *in vitro* studies, PBMCs from study participants in group B exposed *in vivo* to TAF had lower basal, maximal, ATP, spare, and leak OCR compared to PBMCs from study participants in group C exposed *in vivo* to TDF and compared to PBMCs from HIV-$1^{(-)}$ study participants ($p < 0.05$ for all comparisons) (Fig. 3A-E). These relative differences in OCR measures in PBMCs between group B *versus* groups A and C were modest (mean range 20–54 % for all comparisons). The largest reduction in OCR in the PBMCs of the TAF-based compared to the TDF-based ART group was seen with maximal (mean 38 %) and spare (mean 50 %) OCR ($p < 0.001$ for all comparisons).

PBMCs from the TDF-based ART group had higher baseline production rates of glyco, mito and total ATP compared to PBMCs from the TAF-based group ($p < 0.01$ for all comparisons) (Fig. S5A–C). These differences were consistent under high energy demand conditions (maximal OCR) ($p < 0.05$ for all comparisons) (Fig. S5D–F). Additionally, in contrast to PBMCs from the TDF-based group, PBMCs from the TAF-based group had lower maximal production rates of glyco, mito and total ATP compared to PBMCs from the uninfected group ($p < 0.05$ for all comparisons) (Fig. S5D–F). The largest relative reduction in ATP production rates in the PBMC of the TAF-based compared to the TDF-based ART group was seen with mito (mean 41 %) and total (mean 45 %) ATP production rates ($p < 0.001$ for all comparisons). Collectively, these cross-sectional *in vivo* observational data corroborated our *in vitro* studies that TAF-based ART use is associated with reduced cellular respiration in PBMCs.

3.4. A switch of ART from a TAF-based to a TDF-based ART regimen directly increases cellular bioenergetics compared to TAF-based ART in PBMCs from PWH

To further investigate the *in vivo* impact of TAF *vs* TDF in PBMCs from PWH, we performed an open-label ART switch study with identical or similar backbone of other antiretrovirals (emtricitabine, integrase inhibitors, rilpivirine) so that any changes in cellular bioenergetics within the same person after the ART switch can be reasonably attributed to TAF *vs* TDF. Fifteen PWH with suppressed plasma viremia on TAF-based ART were switched to TDF-based ART over 2 months as described in the Methods. Consistent with our cross-sectional baseline data, a switch from a TAF-based ART regimen to a TDF-based ART over 2 months increased basal, maximal, ATP and spare OCR (Fig. 4A–D) and increased production rates of basal and maximal glyco-, mito- and total ATP

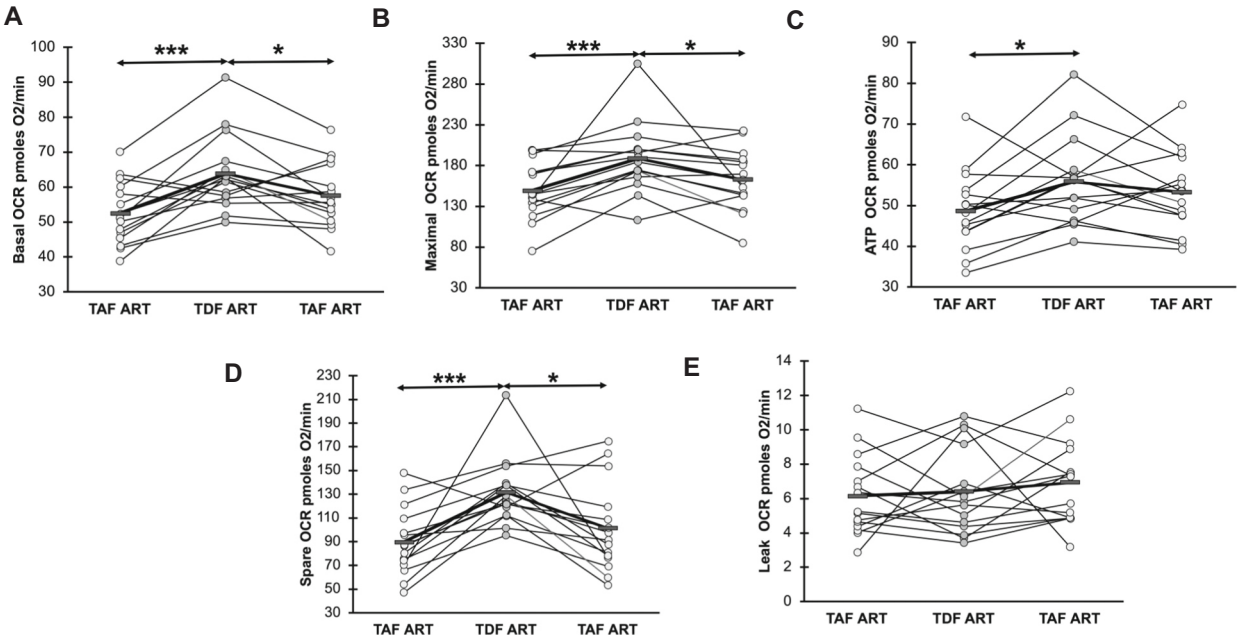

Fig. 4. Prospective assessment of cellular bioenergetic profiles among PBMCs from HIV-$1^{(+)}$ people switching between TAF-based and TDF-based ART. HIV-$1^{(+)}$ people ($n = 15$) on TAF-based ART [bictegravir (B)/emtricitabine (F)/TAF ($n = 12$) and rilpirivine (R)/F/TAF ($n = 3$)] for at least 3 months (baseline) were switched to TDF-based ART [elvitegravir (E), cobicistat (C), F/TDF] ($n = 12$) and R/F/TDF ($n = 3$)] for 2 months (switch 1; comparisons between first timepoint t1 (baseline) and second timepoint t2 (2-month post switch). The same individuals were then switched back to TAF-based ART [(E, C, F/TAF) ($n = 12$) and R/F/TAF ($n = 3$)] for another 6 months (switch 2; comparisons between second timepoint t2 (2-month post switch) and third timepoint t3 (6-month post switch). PBMCs were plated in XF96 microplates and measurements of cellular bioenergetics parameters [measured by OCR in pmoles O2/min] were acquired using a XF96 Extracellular Flux analyzer. A. Basal OCR; B. Maximal OCR; C. ATP linked OCR; D. Spare OCR; E. Leak OCR. Each datapoint represents the average of replicates (≥ 5) of cells from per person and timepoint. The mean in each group is shown in grey rectangular and thick black line. The Wilcoxon matched-pairs signed rank test was used for paired comparison between 2 timepoints (within the same person) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

compared to baseline (Fig. S6A–F) but did not impact the leak OCR (Fig. 4E).

3.5. A switch of ART from a TDF-based to a TAF-based ART regimen directly decreases cellular bioenergetics compared to TAF-based ART in PBMCs from PWH

A further switch in the same 15 individuals from a TDF-based ART regimen back to TAF-based ART over 6 months decreased basal, maximal, and spare OCR (Fig. 4) and decreased production rates of basal glyco-ATP and basal and maximal mito- and total ATP compared to TDF-based ART regimen (Fig. S6A, B, C, E, F) but did not impact the leak OCR (Fig. 4E) and maximal glyco-ATP (Fig. S6D). To further confirm the direct impact of switch from a TDF-based to a TAF-based regimen on cellular bioenergetics without prior ART switch, we also switched 11 independent PWH with suppressed plasma viremia on TDF-based to TAF-based ART over 6 months as described in the Methods. Similar to data shown in Figs. 3 and 4, a switch from TDF-based to TAF-based ART over 6 months consistently reduced basal, ATP, maximal and spare OCR (Fig. 5A–D) and production rates of both basal and maximal glyco-, mito- and total ATP (Fig. S7 A-F) ($p < 0.01$ for all comparisons) but did not impact the leak OCR (Fig. 5E). Overall, these data from our switch studies of antivirals corroborates our cross-sectional observational data and *in vitro* studies and provides direct evidence in PWH that TAF use is associated with reduced cellular respiration in PBMCs compared to TDF use.

3.6. A switch of ART from a TAF-based to a TDF-based ART regimen does not show cytotoxicity in specific immune cell subtypes or alter the number of immune cell subpopulations in PBMCs from PWH

To determine whether the *in vivo* impact of TAF *vs* TDF on cellular bioenergetics in PBMCs from PWH could be attributed to selective cytotoxicity in specific immune cell subtypes, we determined the frequency of immune cell subpopulations in PBMCs from PWH before and after each ART switch.

A switch from a TAF-based ART regimen to a TDF-based ART over 2 months in 15 PWH on TAF-based ART did not impact the total white blood cell (WBC) count (Fig. S8A), CD4 T cell count (Fig. S8B), CD4/CD8 T cell ratio (Fig. S8C) and monocyte cell count (Fig. S8D). A further switch in the same 15 individuals from a TDF-based ART regimen back to TAF-based ART over 6 months did not impact the total WBC count (Fig. S8A), CD4 T cell count (Fig. S8B), CD4/CD8 T cell ratio (Fig. S8C) and monocyte cell count (Fig. S8D). A switch from TDF-based to TAF-based ART over 6 months in 11 independent PWH on TDF-based ART did not impact the total WBC count (Fig. S9A), CD4 T cell count (Fig. S9B), CD4/CD8 T cell ratio (Fig. S9C) and monocyte cell count (Fig. S9D). Thus, the *in vivo* impact of TAF *vs* TDF on cellular bioenergetics in PBMCs from PWH cannot be attributed to selective cytotoxicity in specific immune cell subtypes and reflects changes in the quality rather than the quantity of immune cells.

3.7. TAF induces differential substrate utilization for ATP production compared to TDF in PBMCs from PWH

Increased aerobic glycolysis, decreased mitochondrial substrate oxidation, and increased *de novo* synthesis of fatty acids and accumulation of lipids is essential for the activation and function of myeloid cells and for macrophage activation [21,22], a hallmark of HIV-1 immunopathogenesis in treated chronic HIV. Given the differential impact of TAF *vs* TDF on ATP production in PBMCs, we investigated the effect of TAF *vs* TDF on substrate utilization for energy production. We used 3 substrate inhibitors utilized for energy production prior to mitochondrial respirometry experiments of PBMCs; 1) UK5099, a mitochondrial pyruvate carrier [MPC] inhibitor that reduces glucose and pyruvate oxidation [21], 2) etomoxir, an inhibitor of long chain fatty acid (FA) oxidation [23] and 3) BPTES, an inhibitor of glutaminase that generates glutamate from glutamine [24]. We utilized both *non-*

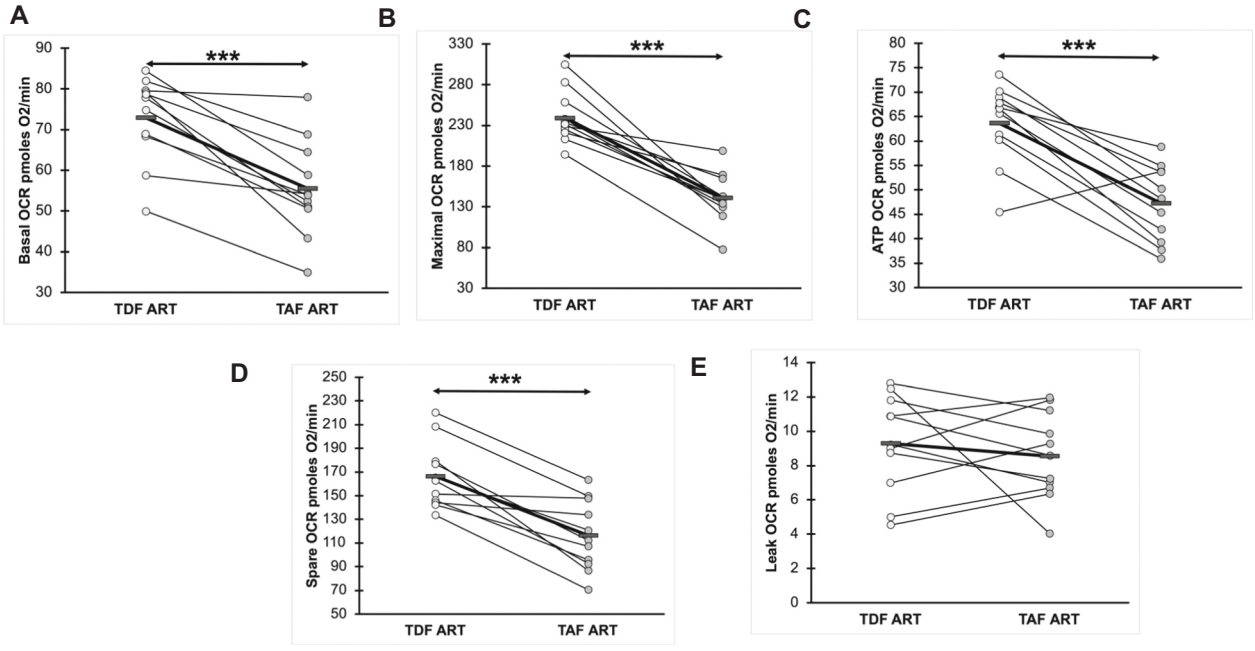

Fig. 5. Prospective assessment of cellular bioenergetic profiles among PBMCs from HIV-$1^{(+)}$ people switching from TDF-based to TAF-based ART. HIV-$1^{(+)}$ people ($n = 11$) on TDF-based ART for at least 3 months (baseline) were switched to TAF-based ART for 6 months. TDF-based ART was emtricitabine, elvitegravir, cobicistat and tenofovir disoproxil fumarate (F/E/C/TDF) ($n = 7$), rilpirivine (R)/F/TDF (n = 3) and dolutegravir (D)/FTC/TDF (n = 1). TAF-based ART was F/E/C/TAF ($n = 8$) and R/F/TAF (n = 3). PBMCs were plated in XF96 microplates and measurements of cellular bioenergetics parameters [measured by OCR in pmoles O2/min] were acquired using a XF96 Extracellular Flux analyzer. A. Basal OCR; B. Maximal OCR; C. ATP linked OCR; D. Spare OCR; E. Leak OCR. Each datapoint represents the average of replicates (≥5) of cells from per person and timepoint. The mean in each group is shown in grey rectangular and thick black line. The Wilcoxon matched-pairs signed rank test was used for paired comparison between 2 timepoints (within the same person) (*p < 0.05, **p < 0.01, ***p < 0.001).

activated PBMCs from HIV-1$^{(-)}$ participants and *activated* PBMCs from PWH on TAF *vs* TDF.

As expected, treatment with all substrate inhibitors reduced both basal and maximal OCR compared to DMSO vehicle control in all PBMC groups for 2 h (Fig. 6). UK5099, Etomoxir and BPTES treatment did not have a differential effect on basal OCR (Fig. 6A–C) and maximal OCR (Fig. 6F) in PBMCs from uninfected people and PWH on TDF- and TAF-based ART regimens. The degree of maximal OCR inhibition by UK5099 was similar in PBMCs from HIV-1$^{(-)}$ participants and PWH on TDF-based ART regimens but less in PBMCs from patients on TAF-based ART regimens (Fig. 6D) ($p < 0.001$).

The degree of maximal OCR inhibition by etomoxir was similar in PBMCs from PWH on TDF-based and TAF-based ART regimens but less than PBMCs from HIV-1$^{(-)}$ participants (Fig. 6E) ($p < 0.001$ for all comparisons). The largest relative effect in inhibition in maximal OCR in the PBMCs exposed to TAF-based ART compared to PBMCs from HIV-1$^{(-)}$ people was seen with UK5099 (mean 20 %) and etomoxir (mean 36 %) ($p < 0.001$ for all comparisons).

To further investigate the possible subacute impact of treatment of TAF *vs* TDF at supraphysiological concentrations on substrate utilization for energy production in PBMCs, *non-activated* HIV-1$^{(-)}$ PBMCs were exposed to a 24-hour *in vitro* incubation of TAF and TDF (3.3 μM) before addition of substrate inhibitors. We focused on the effect of these inhibitors on the reduction in maximum OCR, since the majority of circulating PBMCs are in a quiescent state characterized by a relatively low energy demand (mainly covered by oxidation of FA and glucose/pyruvate) [25]. Addition of all 3 inhibitors in HIV-1$^{(-)}$ PBMCs for 2 h decreased maximal OCR compared to PBMCs treated with vehicle (DMSO) without inhibitors, and the largest reduction was observed with etomoxir (mean 72 %). Notably, pretreatment of HIV-1$^{(-)}$ PBMCs with 3.3 μM TAF abolished the inhibitory effect of both etomoxir (by a mean of ~60 %) and UK5099 (by a mean of ~15 %), but not BPTES, on maximal OCR (Fig. S10A-C). Pretreatment of HIV-1$^{(-)}$ PBMCs with 3.3 μM TDF also abolished the inhibitory effect of etomoxir (by a mean of ~43 %), but not of UK5099 or BPTES, on maximal OCR (Fig. S10A-C). Collectively, these data suggest that both TAF and TDF selectively alter FA utilization for energy production in PBMCs, but that TAF treated PBMCs rely more on glucose/pyruvate rather than FA to fuel their mitochondria under cellular stress.

4. Discussion

To our knowledge, this is the first study that dissects the differential impact of TAF *versus* TDF on metabolic signatures of PBMCs from HIV-1$^{(-)}$ men and women and from PWH on TAF- *versus* TDF-based ART regimens, using detailed assessment of cellular bioenergetics. We found that all studied NRTIs (TAF, TDF, and the established mitochondrial toxin ddC), reduced *in vitro* (at pharmacologically relevant concentrations over both 2- and 24-h treatments), cellular bioenergetics (basal, ATP linked and maximal respirations and the SRC) in *non-activated* PBMCs from HIV-1$^{(-)}$ patients. Our detailed switch studies of antivirals (TAF and TDF) within the same PWH further corroborated our cross-sectional observational data and *in vitro* studies and provided direct evidence in PWH that TAF use is associated with reduced cellular respiration and specifically reduced mitochondrial ATP production rate in PBMCs compared to TDF. Mechanistic *in vitro* studies using established inhibitors of substrate utilization for energy production showed that both TAF and TDF selectively alter FA consumption for mitochondrial energy production in PBMCs. We observed that TAF treated PBMCs selectively rely more on glucose/pyruvate rather than FAs to fuel their

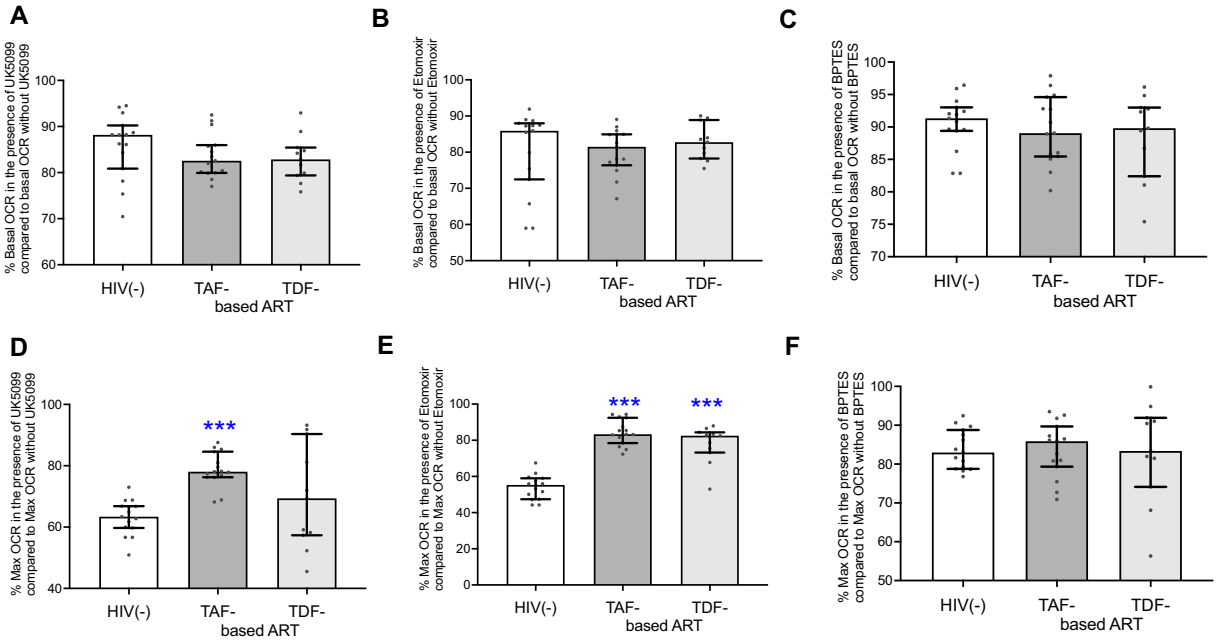

Fig. 6. Cross-sectional assessment of the *ex vivo* impacts of inhibitors of substrate utilization for energy production on cellular bioenergetic profiles in PBMCs from HIV-1$^{(-)}$ and HIV-1$^{(+)}$ people on TAF-based *versus* TDF-based ART. PBMCs cells from HIV-1$^{(-)}$ ($n = 15$) and HIV-1$^{(+)}$ people on TAF-based *versus* TDF-based antiretrovirals (see Fig. 3) were plated in XF96 microplates in the presence of inhibitors of substrate utilization for energy production. The inhibitors used were etomoxir (4 μM) to inhibit oxidation of long chain fatty acids, BPTES (3 μM) to inhibit oxidation of glutamine and UK5099 (2 μM) to inhibit oxidation of glucose/pyruvate. Measurements of cellular bioenergetics [OCR in pmoles O2/min] parameters were acquired at baseline (basal) and under cellular stress (maximal measures after FCCP injection) using a XF96 Extracellular Flux analyzer. OCR data were expressed as % of mean OCR data in the presence of each inhibitor relative to the mean OCR without each inhibitor. A. Basal OCR in the presence of UK5099; B. Basal OCR in the presence of etomoxir; C. Basal OCR in the presence of BPTES; D. Maximal OCR in the presence of UK5099; E. Maximal OCR in the presence of etomoxir; F. Maximal OCR in the presence of BPTES. Data represent bars that display the medium and interquartile range (IQR). Each datapoint represents the average of replicates (≥5) of cells per person. The Kruskal-Wallis test was used to compared multiple groups and the Mann Whitney test was used to compare 2 groups. Comparisons against the vehicle control are shown in blue (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mitochondria under cellular stress. Given that mitochondrial function in PBMCs has been described as a marker of overall cellular function in human disease [10,11,26] our study provides a unique insight into how of commonly used ART regimens differentially impact cellular bioenergetics in blood immune cells, that may contribute to alterations in immune function which are important for triggering immune cell dysfunction in treated chronic HIV. We found that compared to TDF, TAF induced *in vitro* and *in vivo* differential impact on several measures of cellular bioenergetics. The effect of TAF on cellular bioenergetics in treated chronic HIV should be further explored in patients switching from TDF to TAF regimens.

Given that mitochondria are heterogeneous organelles with complex functions, gold standard approaches are needed to investigate their main function and energy production, in a complex viral infection such as HBV and HIV. Observational cross-sectional human studies cannot prove causality between measures that are associated with each other and cannot differentiate complex effects of age, gender, viruses (HIV-1, HBV) *per se*, antivirals, complex biological confounding inherent to studies of mitochondrial function and other (un)known confounders on primary cells (such as immune cells). This is one of the main reasons why prior data on mitochondrial dysfunction in chronic HIV infection are controversial and limited. In addition, many prior observational cross-sectional human studies focused on the use of PCR to study the total content of mitochondrial DNA, which is not a robust readout for mitochondrial function in heterogeneous cell populations such as PBMCs [6–8]. To address these major limitations, we utilized a translational approach with detailed characterization of mitochondrial health using gold standard assessment of cellular bioenergetics in PBMCs in both *in vitro* and *in vivo* human studies. Our robust experimental design, in the setting of a clinical trial, with interchanged antivirals within the same person accounted for heterogeneity of mitochondria in accessible PBMCs.

Immune cells adopt distinct metabolic configurations that allow them to balance their requirements for energy, molecular biosynthesis, and survival. Changes in their metabolic programs were shown to affect their immune function which may contribute to disease [27]. Non-activated PBMCs isolated from uninfected people and exposed *in vitro* to TAF within 2 h, had different substrate dependency on fatty acids to produce energy compared to PBMCs not exposed to TAF. When we examined the substrate dependency of PBMCs from HIV$^{(+)}$ people exposed to a TAF- or TDF-containing ART regimen, we observed that their PBMCs did not have the same dependency on FA oxidation as the PBMCs from HIV-1$^{(-)}$ people. In addition, the PBMCs from PWH exposed to TAF did not display the same substrate reliance on pyruvate to produce energy compared to those exposed to TDF. Although the differential effects of TAF *vs* TDF on the studied metabolic signatures in PBMCs were small or modest, it is important to note that the majority of circulating PBMCs are in a quiescent state characterized by a relatively low energy demand (mainly covered by oxidation of FA and glucose/pyruvate) [25]. Given, the established role of metabolic reprogramming on immune function [27], it is possible that the observed differential effects of TAF *vs* TDF on the studied metabolic signatures in immune cells may be more prominent in states of high immune activation in treated chronic HIV or in metabolically more active cells and tissues from these patients. Differential substrate utilization for ATP production, such as induced glutaminolysis and altered cellular bioenergetics have previously been shown to have an important role in immunometabolism, autophagy and functions of HIV-1 specific T cells such as antiviral immune responses in naturally protected elite controllers (EC) [28,29]. Thus, the clinical relevance of our findings across a wide spectrum of PWH with variable levels of immune activation and metabolic disease will require further long-term studies.

It is important to note that the clinical trial was carefully designed to switch antivirals (TAF *vs* TDF) in PWH who were taking an identical or similar regimen of other antivirals so that any observed changes in the phenotype of cellular bioenergetics in PBMCs within the same person can be reasonably attributed to TAF or TDF alone. However, complex interactions between antiretrovirals cannot easily be dissected in humans. For example, use of TAF in combination with integrase inhibitors is associated with worse metabolic alterations, including weight gain and dyslipidemia, compared to TAF alone [4,30]. For this reason, further evidence is needed to understand whether any differential metabolic effects of TAF may be the direct effects of TAF or reflect the loss of the effects of TDF. Our study elucidates this important question and suggests that TAF can directly alter metabolic signatures in cells. Importantly our *in vitro* treatments with TAF *versus* TDF *vs* vehicle control were done in PBMCs from the same HIV-1$^{(-)}$ donors. Directionality and magnitude of observed changes in measures were *consistent* among independent experiments and study designs (*in vitro* studies, cross-sectional comparisons, clinical trial switch). Thus, our data strongly suggest that the metabolic phenotypes seen with TAF-based regimen are directly caused by TAF.

To fully understand the clinical relevance of our data, further long-term studies in humans are needed to determine whether the TAF- and TDF- induced metabolic alterations have any irreversible impact on cells. Tenofovir has been used for decades in millions of people with chronic viral infections like HIV and HBV. No known irreversible long-term toxicity has been reported. TDF-induced nephrotoxicity is mostly reversible in humans once TDF is stopped [31]. Unlike other NRTIs, tenofovir diphosphate is a weak inhibitor of mammalian DNA polymerases α, γ, δ and ε *in vitro* [32]. Tenofovir has not been shown to decrease mtDNA levels and shows low cytotoxicity [32]. However, prior studies have shown that physiologically relevant concentrations of TDF induced *in vitro* changes to mitochondrial morphology and cristae structure in kidney epithelial cells [33]. Our studies showed that the effects of TAF on cellular bioenergetics in immune cells were reversible since a switch of ART from a TAF-based to a TDF-based ART regimen directly increased cellular bioenergetics compared to TAF-based ART in PBMCs from PWH. Notably, our switch studies in PWH showed that although TAF gives higher intracellular concentrations of tenofovir diphosphate compared TDF, the use of TAF over a period of at least 3 months was not associated with any selective cytotoxicity to specific immune cell populations since the leukocyte composition within the same person remained stable over time.

Given the established role of metabolic reprogramming in immune function, these different metabolic signatures in blood immune cells with different ART regimens may contribute to altered immune function in treated chronic HIV. On the molecular level, emtricitabine/tenofovir has been linked to mitochondrial dysfunction, increased oxidative stress, and cellular senescence [34,35], suggesting a possible role for trained immunity, epigenetic imprinting and rewiring of cellular metabolism in response to pathogens such as *mycobacterium tuberculosis* [36]. Thus, dose-dependent alterations in intracellular levels of tenofovir diphosphate in monocytes and T cells may induce differential immune responses in both adaptive and innate immunity. Whether systemic differential effects of TAF *versus* TDF in accessible blood immune cells are *also* biologically and clinically relevant to other metabolically active cells and tissues that produce lipids (such as hepatocytes) remains to be shown. Our findings are also relevant to PWH who take preexposure prophylaxis with TAF- or TDF- based ART regimens and persons with chronic hepatitis B infection on TAF or TDF.

Despite our cautious study design, our study presents several limitations. We did not perform detailed studies of metabolomics and immune dysfunction (T cell activation, macrophage activation, immune senescence). These studies will be needed to further elucidate the cross-talk between metabolic effects by ARTs and immune effects. We did not perform a detailed assessment of the metabolic status of the study participants (such as measurement of Homeostatic Model Assessment for Insulin Resistance). Detailed clinical assessments of end organ disease and cardiometabolic toxicity with regards to measurements of cellular bioenergetics are necessary to further elucidate the clinical relevance of our findings but these studies were beyond the scope of this study.

Extensive real-world clinical experience with the use of both TAF and TDF in millions of people worldwide has not shown any major clinical evidence of long term cardiometabolic toxicity. Although PBMCs from uninfected women were included in our study, all our HIV infected study participants were men in order to dissect the direct impact of ART switch on cellular bioenergetics regardless of the complex effects of gender on mitochondrial function and metabolism [37]. Future studies using PBMCs from women such as the Women's Interagency HIV Study can further enhance our knowledge in terms of the differential impact of TAF and TDF on cellular bioenergetics in men compared to women [38]. Other limitations include limited power to detect effect sizes with adjustment for multiple biomarker comparisons, and selection bias of study participants when restricting the cohort for virologically suppressed individuals who remained on specific ART regimens, all of which may limit generalizability of our findings. We utilized cryopreserved rather than fresh samples so that all PBMCs can be processed simultaneously. We did not perform studies of cellular bioenergetics in homogeneous cell populations (*e.g.*, monocytes, lymphocytes) because cell sorting of immune cells can induce a major impact on the metabolome which could affect measures of cellular bioenergetics [39]. Finally, the relatively younger age of our study population with no comorbidities may have limited our ability to study the cross-talk between immune dysfunction and metabolic perturbations in the use of antivirals. It is also recognized that the metabolic signatures reported in this analysis are not limited to discrete signatures but are part of a broader and interrelated group of biological systems that consist of multiple intermediary complex cellular pathways that may be a result of not only the antivirals but also several other mechanisms in treated chronic HIV infection.

Despite the above limitations, this is the most comprehensive study describing changes in metabolic signatures of PBMCs using cellular bioenergetics and interrogation of substrates for energy utilization with regards to use of TAF- *vs* TDF-based ART regimens. Our data support the hypothesis that blood immune cells from PWH on antiviral regimens that contain TAF and TDF have differential metabolic signatures. A deeper understanding of the specific mechanisms mediating effects of antivirals on immune cells is crucial to allow selection of antivirals that may prevent drug-associated morbidity and immune dysfunction in treated chronic HIV infection.

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CRediT authorship contribution statement

Eleni Ritou: Data curation, Investigation, Methodology. **Sandro Satta:** Data curation, Investigation, Methodology. **Anton Petcherski:** Data curation, Investigation, Methodology, Writing – review & editing. **Maria Daskou:** Data curation, Investigation, Methodology. **Madhav Sharma:** Data curation, Investigation, Methodology. **Hariclea Vasilopoulos:** Data curation, Investigation, Methodology. **Eisuke Murakami:** Data curation, Investigation, Methodology, Writing – review & editing. **Orian S. Shirihai:** Data curation, Investigation, Methodology, Writing – review & editing. **Theodoros Kelesidis:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing.

Declaration of competing interest

Eisuke Murakami was an employee of Gilead during this research and performed the measurement of intracellular drug levels in PBMCs. All other authors have declared no conflict of interest.

Data availability

All data needed to understand and assess the conclusions of this research are available in the main text and supplementary materials. Raw datasets supporting the findings of this study are available from the corresponding author on reasonable request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2022.155395.

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