





Multimomics plasma effects of switching from triple antiretroviral regimens to dolutegravir plus lamivudine

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Introduction: The DOLAM trial revealed that switching from triple antiretroviral therapy (three-drug regimen; 3DR) to dolutegravir plus lamivudine (two-drug regimen; 2DR) was virologically non-inferior to continuing 3DR after 48 weeks of follow-up. Weight increased with 2DR relative to 3DR but it did not impact on metabolic parameters.

Methods: Multimomics plasma profile was performed to gain further insight into whether this therapy switch might affect specific biological pathways. DOLAM (EudraCT 201500027435) is a Phase 4, randomized, open-label, non-inferiority trial in which virologically suppressed persons with HIV treated with 3DR were assigned (1:1) to switch to 2DR or to continue 3DR for 48 weeks. Untargeted proteomics, metabolomics and lipidomics analyses were performed at baseline and at 48 weeks. Univariate and multivariate analyses were performed to identify changes in key molecules between both therapy arms.

Results: Switching from 3DR to 2DR showed a multimomic impact on circulating plasma concentration of N-acetylmuramoyl-L-alanine amidase (Q96PD5), insulin-like growth factor-binding protein 3 (A6XND0), alanine and triglyceride (TG) (48:0). Correlation analyses identified an association among the up-regulation of these four molecules in persons treated with 2DR.

Conclusions: Untargeted multimomics profiling studies identified molecular changes potentially associated with inflammation immune pathways, and with lipid and glucose metabolism. Although these changes could be associated with potential metabolic or cardiovascular consequences, their clinical significance remains uncertain. Further work is needed to confirm these findings and to assess their long-term clinical consequences.

Introduction

Antiretroviral drugs have improved over time, becoming more effective, simpler and better tolerated. However, for some patients current ART may still be challenging.¹ Despite better tolerability,

some patients may experience direct toxicities with contemporary antiretrovirals. The risk for most potential toxicities is usually cumulative and may further depend on specific individual factors. Beyond direct short-term harm, antiretroviral toxicities may have a long-term impact on the development or progression

of comorbidities. Medications for comorbidities may further increase the risk of significant interactions with some antiretrovirals. Switching antiretrovirals for reasons other than virological failure is a common and evolving strategy in clinical practice.²

The effects of switching to dolutegravir plus lamivudine (a two-drug regimen; 2DR) in adults suppressed on triple therapy have been assessed in several observational studies or single-arm trials and three large randomized clinical trials.^{3–9} The results of all these studies support that switching to 2DR in selected adults with HIV is virologically non-inferior and as safe as continuing triple ART (three-drug regimen; 3DR).

Whether switching from 3DR to 2DR might be associated with better long-term tolerability due to the reduction of antiretroviral drug burden has not been proven yet.¹⁰ Conversely, integrase inhibitors in general, including dolutegravir, have been associated with higher risk of weight gain, diabetes, hypertension and cardiovascular disease, although the not entirely consistent data leave many unknowns.¹¹

In the DOLAM (EudraCT 201500027435) study and SALSA (NCT00295620) study, switching to 2DR was associated with weight gain at 48 weeks compared with continuing 3DR but there were no differences in lipid parameters between arms.⁸ It was the discontinuation of tenofovir disoproxil fumarate that was associated with 48 week increases in total cholesterol and weight in persons switched to 2DR.⁸

Omics approaches have emerged as robust and powerful tools for a better understanding of immunometabolism in HIV pathogenesis. Proteomics-based approaches offer a high-throughput method not only to identify biomarkers for diagnostic antigens and therapeutic targets but also to investigate mechanisms of drug action in persons with HIV.^{12–14} Along with proteomics, metabolomics and lipidomics technologies have yielded new insights into the key role of cellular metabolism in the activity of immune cells and the treatment-induced metabolic derangements in the context of HIV infection.^{15–17}

An untargeted multiomics plasma profile, including proteomics, metabolomics and lipidomics data, was pre-planned as a substudy of the DOLAM study to gain insight on whether some immunometabolic pathways might be affected by this therapy switch. The untargeted approach was selected for a more comprehensive and systemic analysis of both unknown and known plasma compounds to discover new biomarkers to be selected for more accurate quantitation and annotation in future studies (targeted).

Methods

Study design

The DOLAM study is a Phase 4, multicentre, randomized, controlled with active treatment, open-label, parallel clinical trial. The study was done at six major HIV clinics in Catalonia (Spain). Institutional review boards from all participating centres and the Spanish Agency of Medicines and Medical Devices approved the study. Participants were randomly assigned to continue their current 3DR (control arm) or to switch to 2DR once daily (dual-therapy arm). Major results of the DOLAM study have already been published.⁸

Study participants and data collection

Consecutive asymptomatic adults (≥ 18 years old) with HIV on stable (defined as at least the previous 12 months) 3DR including two NRTIs plus

either a boosted PI, an NNRTI, or an integrase inhibitor with sustained viral suppression (defined by plasma HIV RNA < 50 copies/mL in two or more consecutive determinations during at least the 12 months before inclusion; blips up to 200 copies/mL were admissible) were invited to participate. For women of childbearing age, a negative pregnancy test within 10 days before randomization into the study was required. Exclusion criteria were: pregnancy, lactation or planned pregnancy during the study period; prior virological failure (defined as plasma HIV RNA ≥ 50 copies/mL in two consecutive tests or > 500 copies/mL in one test) to regimens containing lamivudine, emtricitabine or integrase inhibitors; any mutation conferring resistance to lamivudine, emtricitabine or integrase inhibitors; CD4 nadir < 200 cells/ μ L; any disease or history of disease that might confound the results of the study or pose additional risk to participant's treatment; and chronic hepatitis B, defined by a positive hepatitis B surface antigen (HBsAg) result at screening. Written informed consent was obtained from all eligible participants before randomization.

Participants were visited at baseline and every 12 weeks until completing at least 48 weeks of follow-up. At baseline and at 48 weeks, 10 mL EDTA blood samples were collected after at least 8 h of fasting and processed immediately; plasma samples were split into 1 mL aliquots and stored at -80°C for deferred multiomics studies.

Determination of the proteomic profile

Detailed information about protein extraction and identification can be found in the [Supplementary Methods](#) (available as [Supplementary data](#) at JAC Online). MS analyses were performed on an LTQ-Orbitrap Velos Pro from Thermo Fisher by an enhanced FT-resolution MS spectrum ($R = 30\,000$ FHMW) followed by a data-dependent FT-MS/MS acquisition ($R = 15\,000$ FHMW, 40% NCE HCD) from the 10 most intense parent ions with a charge state rejection of one and dynamic exclusion of 0.5 min. Protein identification/quantification was performed on Proteome Discoverer software v.1.4.0.288 (Thermo Fisher Scientific, CA, USA) by Multidimensional Protein Identification Technology (MudPIT) combining the two raw data files obtained from each sample. For protein identification, all MS and MS/MS spectra were analysed using the Mascot search engine (v.2.5). The workflow was set up using two different Mascot nodes combining a *Homo sapiens* database (74 449 entries) and a contaminants database (247 entries), both searches assuming trypsin digestion. Two missed cleavages were allowed and an error of 0.02 Da for FT-MS/MS fragmentation mass and 10.0 ppm for an FT-MS parent ion mass were allowed. TMT-10plex was set as quantification modification and oxidation of methionine and acetylation of N-termini were set as dynamic modifications, whereas carbamidomethylation of cysteine was set as static modification. The false discovery rate (FDR) and protein probabilities were calculated by Percolator. For protein quantification, the ratios between each TMT label against 126-TMT label were used and quantification results were normalized based on the protein median. The results are a ratio of reporter ion abundance and are dimensionless.

Determination of the metabolomic profile (analytical method)

Detailed information about the analytical method for metabolomic identification can be found in the [Supplementary Methods](#). Samples were analysed on a 7200 GC-qTOF from Agilent Technologies (Santa Clara, CA, USA). The chromatographic separation was based on the Fiehn method.¹⁸ Targeted compounds were identified using pure standards with a mass accuracy of 20 ppm and different internal standards were used to correct signal response. Chromatographic peaks were deconvoluted using Unknowns Analysis software (version B.09.00, from Agilent) based on the exact mass. Identification of compounds was tentatively made by comparing the mass spectra and retention time of all detected compounds with the Fiehn 2013 Mass Spectral RTL Library and the National Institute of Standards and Technology (NIST) library 11 (2014)

also using the Unknowns software. The identity of the main compounds was confirmed with commercial pure standards. After direct (with pure standards) or putative (with a library) identification of metabolites, these were semiquantified in terms of internal standard response ratio. For this relative quantification, the area of specific fragments for each metabolite was divided by the area of its specific internal standard to provide a reliable, accurate and reproducible relative concentration of metabolites.

Determination of the lipidomic profile (analytical method)

Lipid extraction procedure can be found in the [Supplementary Methods](#). The identification of lipid species was performed using the Agilent MassHunter Profinder B.08 software. First, a feature extraction deconvolution was made; accurate mass and tandem mass spectrum, when available, was then matched to Metlin-PCDL (2017) from Agilent containing more than 40 000 metabolites and lipids, allowing a mass error of 20 ppm and a score higher than 80 for isotopic distribution. To ensure the tentative characterization, the chromatographic behaviour of pure standards for each family and corroboration with the Lipid Maps database (www.lipidmaps.org) was used to ensure their putative identification. Afterwards, matched entities were selected to perform a targeted MS/MS acquisition on the LC-QTOF-MS instrument to corroborate the identification. Lipid species were then semiquantified in terms of internal standard response ratio using one internal standard for each lipid family.

Statistical analysis

To further delineate semiquantitative differences between groups due to the switching effect, a ratio between data from samples at 48 weeks and data from baseline timepoints was performed in each participant before statistical analyses. Only those proteins, metabolites and lipids that were present in >60% of the samples in at least one of the experimental groups were considered. In addition, \log_2 transformations were applied to the data (proteomics, lipidomics and metabolomics) for variance stabilization, data range compression and making the data more normally distributed. This transformation was performed by Mass Profiler Professional software v.15.1 from Agilent Technologies. Regression model such as partial least squares discriminant (PLSD) was performed from compounds in each comparison and random forest (RF) analyses were performed to determine those molecules with higher accuracy to differentiate both groups. An unpaired *t*-test (Mann–Whitney *U*-test) was then performed between ratios (48 weeks/baseline) from experimental groups (control and case). In each comparison, its significant level (*P* value) was corrected for multiple testing using the FDR with the Benjamin–Hochberg procedure and a *P* value cut-off of <0.05 was applied. Associations between quantitative variables were evaluated using the Spearman correlation test and receiver operating characteristic (ROC) curves were employed to confirm the statistical relevance of molecules in the switching from 3DR to 2DR. The protein network was constructed with the online String software (version 11.5).

Statistical analyses were performed using SPSS (version 21.0, SPSS Inc., Chicago, IL, USA), and graphical representations were generated with GraphPad Prism (version 5.0, GraphPad Inc., San Diego, CA, USA), MetaboAnalyst 5.0 software and Open Office software. The results were considered significant at *P* values <0.05.

Ethics

Institutional review boards from all participating centres and the Spanish Agency of Medicines and Medical Devices granted ethical approval of the study. Written informed consent was obtained from all eligible participants before randomization.

Table 1. Baseline demographic and clinical characteristics

	Triple therapy (3DR)	Dolutegravir plus lamivudine (2DR)
Age, years	47 (39–51)	44 (37–53)
Sex at birth		
Female	12 (14)	17 (16)
Male	72 (86)	86 (84)
BMI, kg/m ²	24 (23–26)	25 (23–27)
Suspected route of HIV transmission		
Male-to-male sex	62 (74)	71 (68)
Heterosexual sex	13 (15)	19 (18)
Injection drug	4 (5)	6 (6)
Other/unknown	3 (7)	7 (8)
CD4 and CD8 measurements		
CD4 cells/μL	746 (553–919)	698 (577–938)
CD4, %	35 (31–40)	36 (31–43)
CD8 cells/μL	795 (591–931)	731 (502–959)
CD8, %	36 (31–43)	35 (27–42)
CD4:CD8 ratio	0.88 (0.72–1.02)	0.92 (0.69–1.09)

Data are reported as *n* (%) or median (IQR).

Results

Patient characteristics

There were 198 (75%) out of 265 participants with valid samples for this substudy, 85 receiving 3DR and 113 receiving 2DR.^{8,9} Their baseline characteristics were like those of the main study. Participants were predominantly male (158; 84%) with a median (IQR) age of 45 (38–52) years. Roughly similar proportions were taking abacavir (35%), tenofovir disoproxil fumarate (37%) or tenofovir alafenamide (28%) before study entry (Table 1, Figure 1).

Switching from 3DR to 2DR is associated with the p53 signalling pathway

A total of 136 proteins were identified in plasma samples (Table S1) and the ratio between data from samples at 48 weeks and data from basal timepoint was compared between 3DR and 2DR groups. The PLSD regression model showed a group-average effect, although the two groups overlapped (Figure 2a). Case-control samples were then compared using a non-parametric test corrected by multiple test (FDR <0.05), and 3 of 136 proteins were found to be statistically different between 3DR and 2DR groups (Figure 2b). *N*-acetylmuramoyl-L-alanine amidase (Q96PD5) (*P*=0.035), insulin-like growth factor-binding protein 3 (IGFBP3) (A6XND0) (*P*=0.043) and LPS-binding protein (LBP) (P18428) (*P*=0.05) had elevated plasma concentrations in the 2DR group compared with the 3DR group. Furthermore, Q96PD5 and A6XND0 were consistent with those proteins that had the highest discriminatory power between groups (random forest analysis) (Figure 2c). Thus, by using a combination of univariate and multivariate approaches, Q96PD5 and A6XND0, which showed a positive correlation (ρ 0.247, *P*<0.001), were

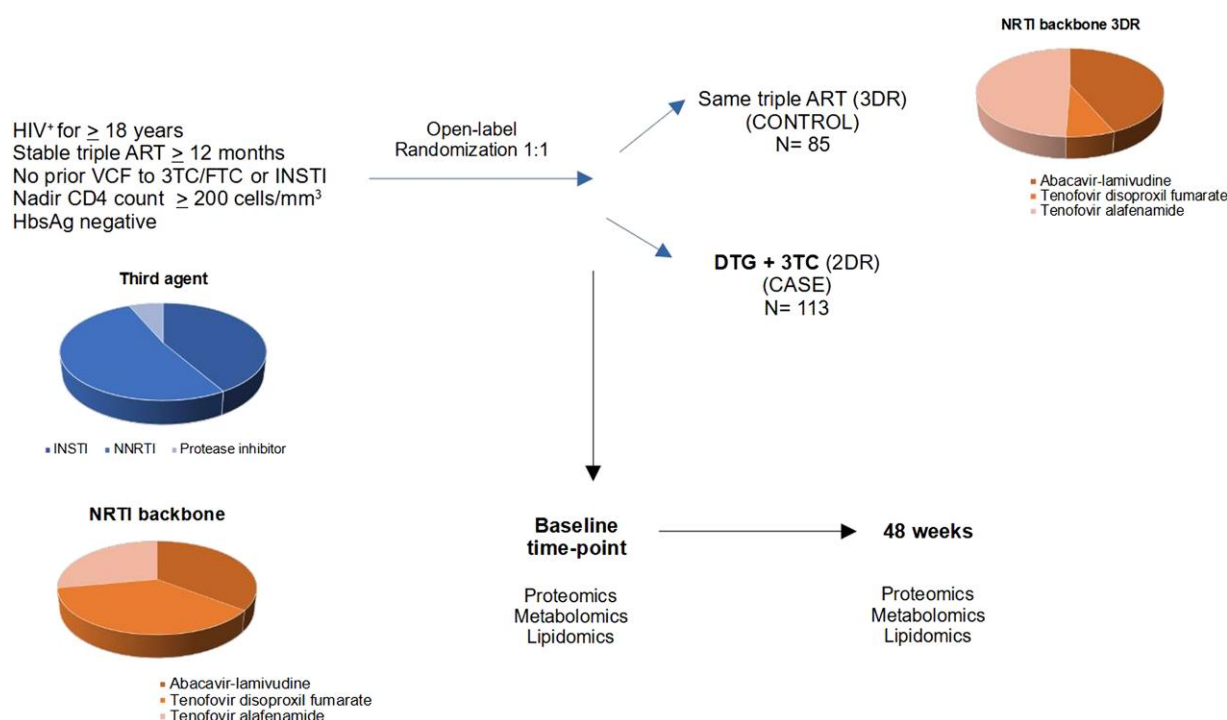


Figure 1. Study design. The study cohort comprised 198 PLHIV on stable triple ART for more than 12 months and with nadir CD4 T cell count greater or equal to 200 cells/mm³. Participants were assigned to a treatment group using computer-generated randomization. 3TC, lamivudine; DTG, dolutegravir; FTC, emtricitabine; INSTI, integrase strand transfer inhibitor; NRTI, nucleoside or nucleotide reverse transcriptase inhibitor; VCF, virological failure. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

discovered as the main proteins significantly increased in therapy simplification. These proteins were subjected to an ROC curve analysis to confirm their statistical relevance in the switching from 3DR to 2DR (Figure 2d). Both Q96PD5 and A6XND0 yielded significant AUC values (0.616, $P=0.005$ and 0.613, $P=0.04$, respectively) and the combination of both proteins resulted in an AUC of 0.624 (0.547–0.702, $P=0.003$). Network mapping identified an interconnecting cluster between LBP and A6XND0 (confidence score 0.4) and STRING database analysis confirmed the interaction between switching from 3DR to 2DR and proteins associated with the p53 signalling pathway (FDR=0.03, impact 0.05).

Switching from 3DR to 2DR is associated with increased alanine and TG 48:0

A total of 97 metabolites were identified in plasma samples (Table S2) and as previously performed with proteomics data, the ratio between metabolomics data from samples at 48 weeks and data from basal timepoint was compared between 3DR and 2DR groups. PLSD analysis also showed a group-average effect in the relative concentration of metabolites (Figure 3a). The univariate non-parametric test corrected by multiple test identified five metabolites statistically different between 3DR and 2DR groups; relative to 3DR, two were significantly decreased (2-hydroxybutyric acid, $P<0.001$; and benzoic acid, $P=0.03$) and three were significantly increased (2-hydroxy isobutyric acid, $P<0.001$; nonanoic acid, $P=0.04$; and alanine, $P=0.05$) in 2DR. However, the multivariate analysis revealed alanine only as the

main metabolite significantly increased by therapy simplification (Figure 3b and c). Of interest, circulating alanine levels positively correlated with A6XND0 levels ($\rho=0.141$, $P=0.018$).

Regarding lipidomics analysis, a total of 117 lipid species were identified in plasma samples and the regression model (PLSD) also showed a group-average effect in the relative concentration of lipid species (Figure 3a). However, the univariate analysis corrected by multiple tests (FDR) only identified TG 48:0 as significantly increased in the 2DR group compared with the 3DR group ($P=0.018$) (Figure 3c). The first number indicates the acyl carbon atoms, and the second number indicates the number of unsaturations.

Thus, alanine and TG 48:0 were analysed in combination to evaluate their association in therapy simplification. Correlation analyses confirmed a positive association between circulating alanine and TG 48:0 levels ($\rho=0.2134$, $P=0.003$) (Figure 3d) and the combination of both features were evaluated using the ROC curve, obtaining an AUC of 0.601 (95% CI 0.503–0.706) (Figure 3e).

No significant association was found between proteins and metabolites identified in the switching from 3DR to 2DR.

Discussion

Similar to other randomized clinical trials such as TANGO or SALSA, the DOLAM study showed that switching from 3DR to 2DR in selected virologically suppressed adults with HIV is virologically non-inferior and as safe as continuing 3DR.^{7,9} However, dolutegravir has been associated with weight increase

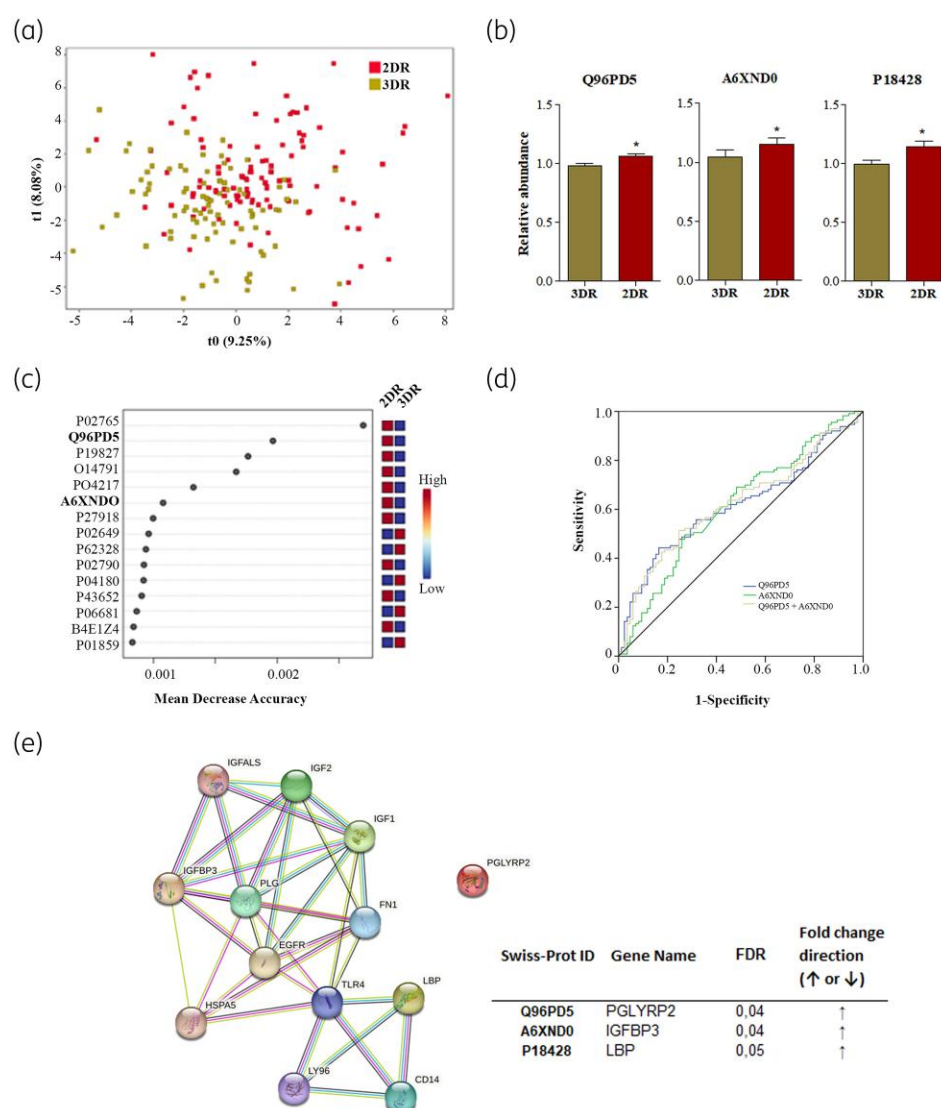


Figure 2. Changes in plasma proteins when switching from 3DR to 2DR. (a) A PLS regression model plot is shown based on relative protein levels. (b) Circulating levels of the three proteins with significant expression among groups by univariate analysis (FDR results for positive mode features ($FDR \leq 0.05$)). (c) RF analysis. The top 15 metabolites with the highest discriminatory power between both groups are listed. (d) ROC curve for proteins selected based on the combination of univariate and multivariate analyses. AUC for Q96PD5 was 0.616 (95% CI 0.538–0.694), AUC for A6XND0 was 0.613 (95% CI 0.534–0.693) and AUC for the combination of both proteins (Q96PD5+A6XND0) was 0.624 (95% CI 0.547–0.702). (e) STRING database analysis confirmed the interaction between switching from 3DR to 2DR and proteins associated with the p53 signalling pathway ($FDR=0.03$, impact 0.05). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

in both naive and treated persons with HIV and there are concerns that it could also be associated with diabetes and cardiovascular disease.^{19,20} In the DOLAM study, persons living with HIV (PLHIV) assigned to 2DR significantly gained more weight and there were more overweight or obese persons at 48 weeks relative to those continuing 3DR, but we were unable to detect significant changes in body fat, lean mass or bone mineral density (BMD) between arms.⁸ Taking advantage of omic approaches for a better understanding of cellular metabolism, we attempted to explore if there were any plasma molecular alterations affected by the switch from 3DR to 2DR.

The combination of untargeted proteomics, metabolomics (polar compounds) and lipidomics evidenced that switching

from 3DR to 2DR was related to a few circulating metabolomic perturbations. Specifically, the multiomic approach identified two proteins, one metabolite and one lipid differentially expressed in the 2DR group compared with 3DR. Correlation analyses identified an association among the up-regulation of these four molecules in the 2DR arm.

At 48 weeks of follow-up, participants receiving 2DR showed increased plasma concentration of Q96PD5 and A6XND0 compared with 3DR. Q96PD5 is a protein encoded by the peptidoglycan recognition protein 2 (PGLYRP2) gene, a gene from the peptidoglycan recognition proteins (PGLYRPs) family. PGLYRPs are proteins that are conserved from insects to mammals, acting in inflammation and immune responses independently of their

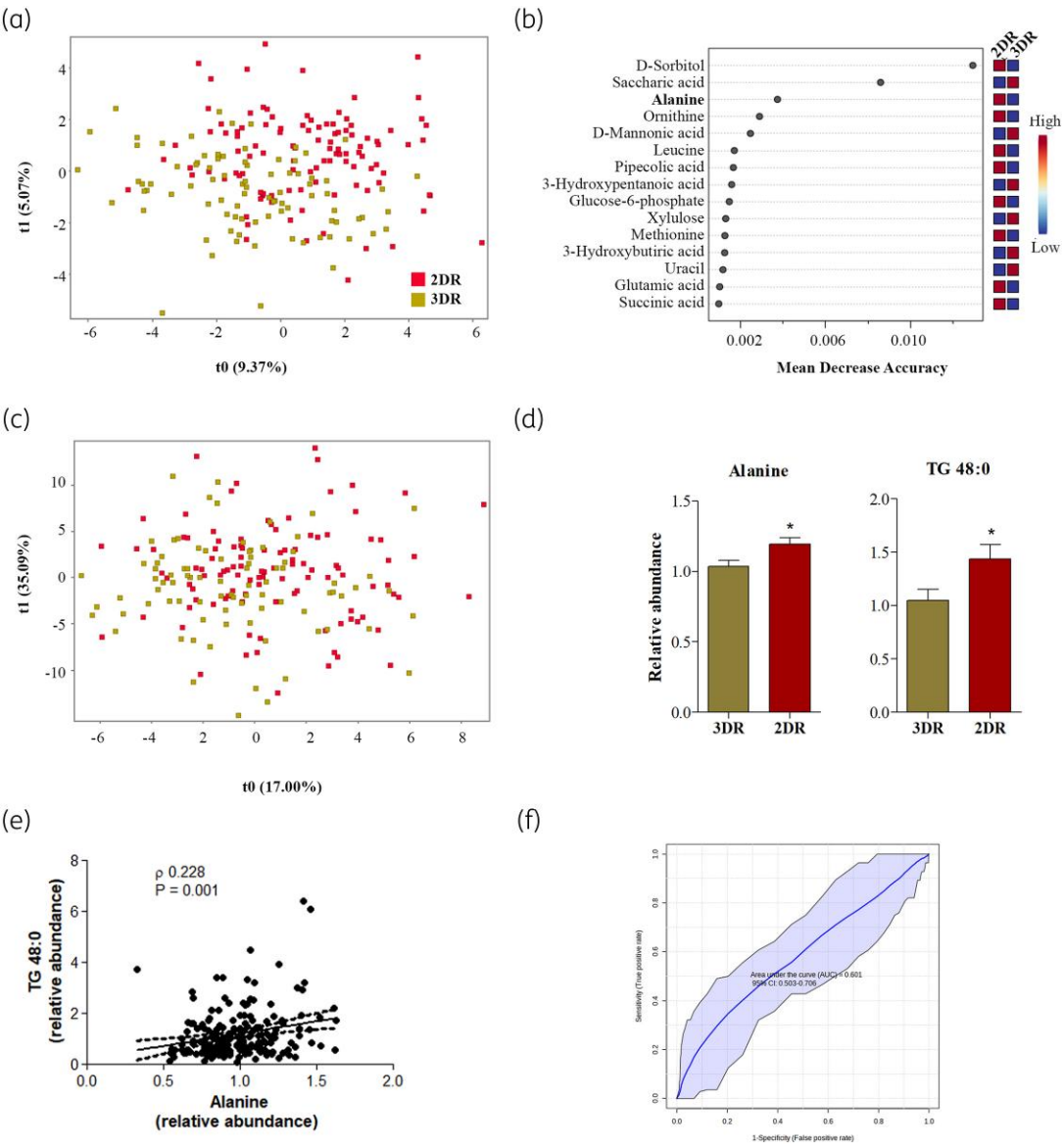


Figure 3. Changes in plasma metabolites and lipid species of switching from 3DR to 2DR. (a) A PLSD regression model plot is shown based on relative metabolite levels. (b) RF analysis. The top 15 metabolites with the highest discriminatory power between both groups are listed. (c) A PLSD regression model plot is shown based on relative lipid species levels. (d) Circulating differences between the significant metabolite and lipid species by univariate analysis corrected by multiple tests ($FDR \leq 0.05$). (e) Correlation analysis between alanine (metabolite) and TG 48:0 (lipid species). The Spearman (ρ) correlation coefficient and P value are indicated inside the graphical representation. (f) ROC curve model for the combination of alanine and TG 48:0 to evaluate the feature of these molecules in the therapy simplification. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

bactericidal and enzymatic activities.²¹ Specifically, PGLYRP2 has shown both anti-inflammatory and pro-inflammatory properties, probably related to its link to the transcription factor family nuclear factor κ B (NF- κ B).^{22–25} NF- κ B represents a family of inducible transcription factors involved in the regulation of genes belonging to immune and inflammatory pathways (including cytokines, chemokines and inflammasome) whose expression has been linked to the p53 signalling pathway.^{24,26} Our results suggested a possible association between the differentially expressed proteins associated with therapy simplification and the

p53 signalling pathway. Of interest, a recent study also showed a positive correlation between PGLYRP2 and ApoB/A1 in patients with systemic lupus erythematosus (SLE), which suggested a potential role of PGLYRP2 in the dyslipidaemia and cardiovascular disease risks in SLE patients.²⁷ In the present study, no association was found between Q96PD5 and the lipid species differentially identified among groups (TG 48:0), but Q96PD5 was shown to be significantly related to A6XND0, whose gene expression (IGFBP3) was previously related to lipid metabolism.²⁸ In serum, IGFBP-3 is the most abundant protein of the IGFBP family that

functions in an IGF-dependent (delivery of IGF and activation of IGF downstream signalling) manner, as well as in an IGF-independent (interaction with proteins) manner.²⁹ Although some studies unveiled its role in metabolic regulation, the exact role of IGFBP3 in glucose and lipid metabolism remains unclear, even in PLHIV.^{29,30} Some studies suggested that serum IGFBP3 could be involved in HIV disease progression and related to insulin resistance, but on the other hand, 3 months of treatment with IGF-I/IGFBP-3 improved whole-body glucose, glucose tolerance and fasting TGs in men living with HIV with excess abdominal adiposity and insulin resistance.^{31–33} Based on our results, the role of an increase in plasma IGFBP3 concentration in the 2DR arm remains uncertain as no significant metabolic alterations were clinically observed between 3DR and 2DR groups at 48 weeks of therapy randomization.⁸ However, it is important to highlight that our results suggested a positive relationship between A6XND0 and plasma alanine levels, and between plasma alanine levels and TG (48:0). At the molecular level, participants in the 2DR group showed increased plasma concentrations in both circulating plasma alanine and TG (48:0) compared with the 3DR group. Increased circulating alanine concentrations could indicate an alteration in the glucose-alanine cycle in which alanine can be synthesized from pyruvate deriving from skeletal muscle through the enzymatic reaction of ALT and then transported to the liver to be used for gluconeogenesis. High circulating alanine concentration has been related to the risk of incident type II diabetes while reduced rates of glucose-alanine cycling have been associated with the regulation of hepatic mitochondrial oxidation.^{34,35} Regarding the monoacid triglyceride TG (48:0), previous studies have considered the saturated TG (48:0) as a key lipid species in biomarker panels related to liver-fat content.^{36,37}

This study had limitations. The multiomic analysis was performed from baseline to 48 weeks after randomization, a period in which potential metabolic consequences of weight gain had not been apparent. Multiomics data integration can bring several problems and requires several attention to combine high-throughput data obtained from different molecular layers. To reduce the heterogeneity across the three different omics technologies applied and the difficulty of interpreting multilayered system models derived from the combination of omics data and non-omic data (clinical data), single analysis was preferred for the present work focused on the antiretroviral simplification. Performing a multiomic analysis after a longer follow-up would be of great interest. As a strength, this study provides novel and valuable information, as no multiomic analysis investigating changes after switching from 3DR to 2DR has been previously published to our knowledge.

Conclusions

In summary, 48 weeks of dolutegravir plus lamivudine (2DR) represents a reduction in antiretroviral drug burden that is virologically non-inferior, safe and without apparent clinical metabolic derangement compared with continuing triple ART (3DR). Switching from 3DR to 2DR, however, was related to some plasma metabolomics perturbations, in which four soluble compounds were involved. Plasma changes in Q96PD5, A6XND0, alanine and TG (48:0) might be associated with the activation of inflammation and immune pathways but also with an alteration

in lipid and glucose metabolism. Although these changes could be associated with potential metabolic or cardiovascular consequences, their clinical significance remains uncertain. Further work is needed to confirm these findings and to assess their long-term clinical consequences.

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Transparency declarations

Esteban Martínez has received funds for research from ViiV Healthcare. Jose M. Gatell is a full-time employee of and owns stock in ViiV as Senior Global Medical Director since 1 May 2018. All other authors: none to declare.

Author contributions

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Data availability

The datasets used and analysed during the current study may be made available by the corresponding author upon reasonable request.

Supplementary data

Supplementary Methods, Figures S1 to S3 and Tables S1 to S3 are available as Supplementary data at JAC Online.

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