

# First-line dolutegravir/lamivudine in HIV-1 infection: lymph node penetration and reservoir decline in a randomized open-label clinical trial

Corresponding Author: Dr Jose Molto

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Comments and Recommendations

Major

1. Line 150. The authors state that the 32% of tissue covered by combined DTG and 3TC indicates that this regimen was penetrating to a “high” extent within the LN. To this reader, 32% doesn’t sound high. Can the authors explain why 32% is high; what data and comparisons support this is high? It may be better to just report the value and let the readers decide whether 32% is high or not.

2. The authors conclude that the magnitudes of reservoir decay by week 48 are comparable between dual and triple therapy (Lines 105 and 185) and that fold change estimates support the comparable reductions in vDNA and vRNA (Line 195). This reviewer isn’t convinced. At baseline, both vDNA+ and RNA+ were numerically higher in the 3DR group (Table 1). The week 48 fold change ratios (week 48/baseline) for both vDNA+ and RNA+ (Table 1) were lower for the 3DR group (Table 1). These data would indicate the 3DR group had a greater reduction quantitatively in both vDNA+ and vRNA+ cells. My question, acknowledging the very small sample size of this study, is could these data suggest the 3DR regimen is more potent/suppressive than the 2DR regimen?

3. TFV concentrations (in the 3DR regimen) were not measurable by MSI (Lines 136-141), and therefore the authors only reported the results of the spatial analysis for participants in the 2DR group (DTG and 3TC). An objective of the study, however, (Lines 78-79) was to investigate whether the 2DR regimen (DTG/3TC) achieves sufficient tissue penetration compared with triple therapy. This objective is not presently met.

I recommend the authors include the spatial analysis for participants in the 3DR group based on DTG and FTC. This would allow some comparison between the 2DR and 3DR groups and may (or may not) provide some insight into the question above of whether the 3DR regimen could be more potent than the 2DR regimen? It would also double the sample size for DTG in LN tissue by MSI, which is useful for comparison and rigor and reproducibility.

4. In Line 140, the authors state because the MSI LLOQ exceeded the relevant in vitro IC90 for DTG and 3TC that the locations where DTG and 3TC were measured had efficacious drug concentrations. In Table 2, the 3TC-TP reference value is 564 fmol/10<sup>6</sup> while the measured median 3TC-TP concentration in the LN was 527 fmol/10<sup>6</sup> cells with a range down to 5, indicating approximately 50% of concentrations were less than the reference value. This is also the case for FTC-TP in the LN. The authors need to explain this discrepancy. The authors should also provide a conversion for DTG in the LN (in Table 2) so that DTG values can be compared with the reference value of 64 ng/mL provided.

Finally, there is the question of whether just exceeding the in vitro IC90 in the LN enough, when, for example, the usual goal in plasma (for integrase inhibitors) is to exceed by 4-fold or greater? A little commentary on the “how much is enough” problem and how to assess regimen (vs. individual drug) activity would be appropriate.

Other

1. Line 51. Should modify the DTG/3TC recommendation to “certain people” or include exceptions (from the DHHS guidelines) of for persons with (1) HIV RNA > 500,000 copies/mL, (2) HBV coinfection or (3) before the results of HIV genotype resistance testing for RT or HBV testing are available, should be added.
2. Line 170, clarify that there was no change in collagen in both treatment groups.
3. Line 329, clarify that the tissue homogenate for LC-MS/MS analysis was prepared from the flash-frozen sample, and whether before or after tissue sectioning (line 284)? If after sectioning, how many sections and how was the final concentration for the homogenate determined?
4. Table 2. Add citations for the reference values (eg, IC90, IC50, etc).

#### Reviewer #2

##### (Remarks to the Author)

This is a huge international collaborative effort on an important topic relating to oral antiviral drug combinations (ARVs or cART), anchored by integrase inhibitor dolutegravir on residual virus in the cells presented as viral DNA and RNA located in the lymph nodes and blood circulation. There are some key information relating to the timing of initiating the cART for the first time in people who are verified HIV positive. The degree and extend of viral integration to host DNA within the lymph nodes and periphery may be related to the timing of HIV infection as it related to the timing of initiating treatment for the first time. While the attempt to probe drug distribution, in the node tissue, using Infrared Matrix-Assisted Laser Desorption Electrospray Ionization (IR-MALDESI) Mass Spectrometry Imaging (MSI) is laudable, the spatial resolution [voxel (volumetric pixel) ~100x100µm], may be insufficient to support some of the statement regarding drug-coregistration in the cells linking to reduction on HIV reservoir within the lymph nodes and related cellular viral load in the cells of periphery. Providing key critical information on estimated initial HIV positive confirmation before initiating treatment in this cohort will be helpful. While there appeared to be reduction in viral load within the cells, whether and mechanistic proposal of which specific drug within found within the cells may linked to the observed viral load reduction in cells is not fully supported by the data. With respect to the main point of 2 drugs (DTG/3TC) vs 3 drugs (DTG/FTC/TAF), the two cohort has different degree of median viral load at treatment initiation; and by the end of 48 weeks, the outcomes of viral load- vDNA or vRNA in the nodes and cells are reduced but again highly variable for a small number of participant in the n=4 per group. Thus it may be challenging to make a broad statement; but could suggest a comparable outcomes, with 3D potentially be more favorable when individuals are presented with higher viral load at the time of treatment initiation in ARV naive individuals. However a time-frame within which one would expect significant vDNA reduction can be expected in the nodes and cells could be helpful.

#### Reviewer #3

##### (Remarks to the Author)

Over the past decades there have been incredible advances in drug development for HIV-1 antiretroviral therapies. From the co-formulation of combination antiretroviral therapy into single-tablet regimens HIV treatment regimens have become easily administered, tolerable, minimal adverse events, strong efficacy, and simple to take longer term for patient use. An evolving regimen is 2-drugs that contain antiretroviral drugs with a strong therapeutic index. This includes, but is not limited to treatments for people living with HIV (PLWH) that include the integrase inhibitor dolutegravir (DTG) and the nucleoside reverse transcriptase inhibitor (NRTI) lamivudine (3TC). The preferable pharmacokinetic and pharmacodynamics profile, and clinical trial efficacy and safety of DTG/3TC affirm the importance as current treatment ARV options. Overall similar efficacy and tolerability were achieved with DTG within two-or three drug regimens in clinical trials. The effectiveness in people living with HIV-1 (PLHIV) switching to DTG with lamivudine (3TC) or rilpivirine (RPV) is now well established. Indeed, DTG and 3TC or DTG/RPV demonstrates low rate of virological failure and a high rate of viral suppression in virologically suppressed infected person. notably DTG/3TC is an effective treatment option for people with HIV with a viral load <50 copies/mL independent of age, sex, or race. In the present study peripheral HIV-1 reservoir markers declined similarly in patients treated with DTG/3TC or triple therapy. The present work evaluated 39 person undergoing inguinal LN excision at baseline or during the first year of treatment with DTG/3TC for HIV-1 DNA and RNA by DNA/RNAscope and antiretroviral spatial distribution by mass spectrometry imaging. HIV-1–infected cells declined after treatment with comparable reductions between each of the treatment groups. Antiretrovirals were detected in LN containing HIV-positive cells. The results provide spatial evidence that DTG/3TC achieves effective LN penetration and supports effective antiviral exposure within key anatomical HIV reservoirs. The investigators show that HIV-infected cells within LN decline rapidly after ART initiation and that the magnitude and kinetics of reservoir decay are comparable between dual and triple therapy with DTG/3TC and triple regimens. The findings extend the prior observations made by this group in peripheral blood and provide direct tissue-level evidence of antiretroviral responses in anatomical reservoirs. The data is well documents and affirmed. The impact, however, is more limited based on the fact that this report is supportive of a range of prior reports and provides only affirmation of what is already known clinically.

Version 1:

Reviewer comments:

#### Reviewer #1

##### (Remarks to the Author)

The authors have responded in a thoughtful manner to all of the comments by the reviewers, and have modified the manuscript accordingly. Thank you. I have no further comments or recommendations.

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## REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

### Comments and Recommendations

Major

1. Line 150. The authors state that the 32% of tissue covered by combined DTG and 3TC indicates that this regimen was penetrating to a “high” extent within the LN. To this reader, 32% doesn’t sound high. Can the authors explain why 32% is high; what data and comparisons support this is high? It may be better to just report the value and let the readers decide whether 32% is high or not.

**Response:** We thank the reviewer for this valuable suggestion. We agree that the term “high” may be subjective and could overinterpret the significance of our findings in the absence of an established benchmark for MSI-derived tissue coverage. We have therefore removed this qualitative descriptor and now report the measured tissue coverage objectively, without interpretation. In the Discussion section, we contextualize these findings by comparing them with data obtained from NHP studies using 4-drug antiretroviral regimens.

2. The authors conclude that the magnitudes of reservoir decay by week 48 are comparable between dual and triple therapy (Lines 105 and 185) and that fold change estimates support the comparable reductions in vDNA and vRNA (Line 195). This reviewer isn’t convinced. At baseline, both vDNA+ and RNA+ were numerically higher in the 3DR group (Table 1). The week 48 fold change ratios (week 48/baseline) for both vDNA+ and RNA+ (Table 1) were lower for the 3DR group (Table 1). These data would indicate the 3DR group had a greater reduction quantitatively in both vDNA+ and vRNA+ cells. My question, acknowledging the very small sample size of this study, is could these data suggest the 3DR regimen is more potent/suppressive than the 2DR regimen?

**Response:** We thank the reviewer for this thoughtful observation. We agree that baseline lymph node vDNA- and vRNA-positive cell counts were numerically higher in the 3DR group, which consequently yielded lower week-48-to-baseline fold-change estimates. However, this exploratory imaging substudy included only four independent participants per treatment arm at each time point, with each participant contributing a single lymph node biopsy. As such, the study was not powered to assess treatment superiority, and no formal statistical comparisons between regimens were prespecified. We therefore believe that these numerical differences should be interpreted cautiously and do provide sufficient evidence to support conclusions regarding greater antiviral potency of one regimen over the other. To address this concern more explicitly, we have revised the Discussion section to emphasize that the observed differences may reflect baseline variability arising from the limited sample size rather than true treatment-related effects. Accordingly, the findings should be interpreted as showing broadly similar patterns of reservoir decline in lymph nodes, rather than demonstrating equivalence or superiority of either regimen.

Importantly, this imaging substudy was nested within a randomized clinical trial in which both treatment strategies achieved comparable virological efficacy in peripheral blood.

The primary objective of the substudy was to characterize viral dynamics and antiretroviral distribution within lymph node tissue, rather than to compare clinical efficacy of the two regimens.

3. TFV concentrations (in the 3DR regimen) were not measurable by MSI (Lines 136-141), and therefore the authors only reported the results of the spatial analysis for participants in the 2DR group (DTG and 3TC). An objective of the study, however, (Lines 78-79) was to investigate whether the 2DR regimen (DTG/3TC) achieves sufficient tissue penetration compared with triple therapy. This objective is not presently met. I recommend the authors include the spatial analysis for participants in the 3DR group based on DTG and FTC. This would allow some comparison between the 2DR and 3DR groups and may (or may not) provide some insight into the question above of whether the 3DR regimen could be more potent than the 2DR regimen? It would also double the sample size for DTG in LN tissue by MSI, which is useful for comparison and rigor and reproducibility.

**Response:** We deliberately restricted the spatial analyses to the DTG/3TC arm because our objective was to characterize the spatial distribution of the complete antiretroviral regimen. The only substantial pharmacological difference between the two treatment arms was the inclusion of TAF in the triple-drug regimen. However, tenofovir concentrations were below the lower limit of quantification of the MSI assay, preventing reconstruction of the full spatial distribution of the triple-drug regimen. Consequently, a comparison between DTG/3TC and DTG/FTC, excluding the component that distinguishes the triple regimen, would not constitute a true comparison between the two-drug treatment regimens. We therefore considered such an analysis potentially misleading and chose not to perform it. This rationale has now been clarified in the revised manuscript.

4. In Line 140, the authors state because the MSI LLOQ exceeded the relevant in vitro IC<sub>90</sub> for DTG and 3TC that the locations where DTG and 3TC were measured had efficacious drug concentrations. In Table 2, the 3TC-TP reference value is 564 fmol/10<sup>6</sup> while the measured median 3TC-TP concentration in the LN was 527 fmol/10<sup>6</sup> cells with a range down to 5, indicating approximately 50% of concentrations were less than the reference value. This is also the case for FTC-TP in the LN. The authors need to explain this discrepancy. The authors should also provide a conversion for DTG in the LN (in Table 2) so that DTG values can be compared with the reference value of 64 ng/mL provided.

Finally, there is the question of whether just exceeding the in vitro IC<sub>90</sub> in the LN enough, when, for example, the usual goal in plasma (for integrase inhibitors) is to exceed by 4-fold or greater? A little commentary on the “how much is enough” problem and how to assess regimen (vs. individual drug) activity would be appropriate.

**Response:** We have clarified two key points in the discussion that are important to consider when comparing IR-MALDESI MSI and LC-MS/MS findings. First, MSI analysis only measured parent drug (eg DTG, 3TC) due to rapid enzymatic degradation of phosphorylated metabolites following sectioning (as cited in the methods section). These measurements showed that drug exposure was not uniformly distributed within sample LNs and there were portions of each tissue section where no drug was detectable by MSI. Because MSI LLOQs were higher than DTG and 3TC IC<sub>90</sub> values, we know that anywhere drug was measured by MSI had concentrations above these values. Second, LC-MS/MS concentrations of parent drugs and metabolites (eg 3TC-TP, FTC-TP) in

Table 2 are derived from bulk tissue homogenates and reflect average drug exposure within the homogenized tissue. Even in instances where a bulk homogenate tissue metabolite concentration is below a reference IC90 value, our MSI findings suggest that there are likely gradients in metabolite exposure within the tissue that may have similar proximity to vDNA+ and vRNA+ cells as FTC and 3TC. Hence, these findings are complimentary rather than discrepant. We have underscored in the text that the study was not designed to determine the contribution of individual drugs or establish a causal relationship between local drug distribution and reservoir decay. As the first study to longitudinally integrate spatial antiretroviral imaging with tissue HIV reservoir measurements in human LN during the first year of ART, our findings reflect a reference point for further analysis of causality that would be needed to elucidate what safety factor in tissue concentrations relative to in vitro IC90 (4-fold or otherwise) is needed in LN tissue.

#### Other

1. Line 51. Should modify the DTG/3TC recommendation to “certain people” or include exceptions (from the DHHS guidelines) of for persons with (1) HIV RNA > 500,000 copies/mL, (2) HBV coinfection or (3) before the results of HIV genotype resistance testing for RT or HBV testing are available, should be added.

**Response:** We have revised the Introduction to better align with the terminology adopted in current clinical guidelines.

2. Line 170, clarify that there was no change in collagen in both treatment groups.

**Response:** We thank the reviewer for this valuable suggestion. We have clarified the text to explicitly state that collagen I did not show a consistent pattern of temporal change following ART initiation in either treatment group.

3. Line 329, clarify that the tissue homogenate for LC-MS/MS analysis was prepared from the flash-frozen sample, and whether before or after tissue sectioning (line 284)? If after sectioning, how many sections and how was the final concentration for the homogenate determined?

**Response:** We have added text in the methods section to indicate that LC-MS/MS analysis was performed on snap-frozen LN tissue that was homogenized following cryosectioning. The number of tissue sections collected for IHC/ISH and MSI analysis prior to tissue homogenization has been added to the text describing tissue sectioning. Procedures for determination of homogenate concentrations by LC-MS/MS are well documented in the citations for the validated assays.

4. Table 2. Add citations for the reference values (eg, IC90, IC50, etc).

**Response:** We have added the corresponding references supporting the pharmacodynamic reference values (IC50, IC90, and EC50) reported in Table 2.

Reviewer #2 (Remarks to the Author):

This is a huge international collaborative effort on an important topic relating to oral antiviral drug combinations (ARVs or cART), anchored by integrase inhibitor dolutegravir on residual virus in the cells presented as viral DNA and RNA located in the lymph nodes and blood circulation. There are some key information relating to the timing of initiating the cART for the first time in people who are verified HIV positive. The degree and extend of viral integration to host DNA within the lymph nodes and periphery may be related to the timing of HIV infection as it related to the timing of initiating treatment for the first time. While the attempt to probe drug distribution, in the node tissue, using Infrared Matrix-Assisted Laser Desorption Electrospray Ionization (IR-MALDESI) Mass Spectrometry Imaging (MSI) is laudable, the spatial resolution [voxel (volumetric pixel) ~100x100µm], may be insufficient to support some of the statement regarding drug-coregistration in the cells linking to reduction on HIV reservoir within the lymph nodes and related cellular viral load in the cells of periphery.

**Response:**

We appreciate the reviewer's perspective on the limitations of spatial resolution offered by our MSI approach. We have been careful to emphasize the spatial scale of our colocalization assessment and have focused this analysis on characterizing proximity of exposure to vDNA+ and vRNA+ cells. The nonlinear relationship between MSI probed volume and spatial resolution renders cellular-level analyte sensitivity for ARVs to be extremely challenging, though a continued future goal for our work. Nevertheless, it is physiologically unlikely that an adjacent cell will hold a very different drug concentration from another within the dimensions of one voxel and the present spatial resolution can capture a level of heterogeneity that may arise from tissue microvessel penetration, drug transporter expression, collagen disposition, and other factors to assist with pharmacologic interpretation. As noted above, we have further clarified that that the study was not designed to determine the contribution of individual drugs or establish a causal relationship between local drug distribution and reservoir decay.

Providing key critical information on estimated initial HIV positive confirmation before initiating treatment in this cohort will be helpful.

**Response:** We thank the reviewer for this valuable suggestion. We agree that the duration of untreated HIV infection may influence both the size and composition of the HIV reservoir. In the DUALITY trial, randomization was stratified according to two factors known to influence reservoir size: (i) an estimated time since HIV acquisition of less than versus greater than 3 months, and (ii) a screening plasma HIV-1 RNA level above versus below 100,000 copies/mL. The estimated time since HIV acquisition was determined based on the interval since the last documented HIV-negative test and/or the presence of symptoms consistent with acute retroviral syndrome, rather than an exact date of infection. We have clarified this aspect of the study design in the revised manuscript.

While there appeared to be reduction in viral load within the cells, whether and mechanistic proposal of which specific drug within found within the cells may linked to the observed viral load reduction in cells is not fully supported by the data.

**Response:** We agree that our data do not establish a mechanistic relationship between the localization of individual antiretroviral drugs and the observed reduction in HIV reservoir markers. The imaging analyses demonstrate spatial proximity between antiretroviral drugs and HIV-infected cells; however, they cannot determine the relative contribution of individual agents nor establish a causal relationship. We have revised the

Discussion to clarify this point and to avoid mechanistic interpretations that are not directly supported by the data.

With respect to the main point of 2 drugs (DTG/3TC) vs 3 drugs (DTG/FTC/TAF), the two cohort has different degree of median viral load at treatment initiation; and by the end of 48 weeks, the outcomes of viral load- vDNA or vRNA in the nodes and cells are reduced but again highly variable for a small number of participant in the n=4 per group. Thus it may be challenging to make a broad statement; but could suggest a comparable outcomes, with 3D potentially be more favorable when individuals are presented with higher viral load at the time of treatment initiation in ARV naive individuals. However a time-frame within which one would expect significant vDNA reduction can be expected in the nodes and cells could be helpful.

**Response:** We agree that the numerical differences in baseline viral burden, together with the limited sample size, warrant cautious interpretation of these findings. As also noted in our response to Reviewer #1, we have revised the Discussion to explicitly acknowledge these limitations and to clarify that this exploratory substudy was neither designed nor powered to establish the superiority or equivalence of the treatment regimens. Accordingly, we do not believe that the present data support conclusions regarding the greater efficacy of one regimen over the other. We believe that the revised Discussion more appropriately reflects the exploratory nature of these comparisons.

**Reviewer #3 (Remarks to the Author):**

Over the past decades there have been incredible advances in drug development for HIV-1 antiretroviral therapies. From the co-formulation of combination antiretroviral therapy into single-tablet regimens HIV treatment regimens have become easily administered, tolerable, minimal adverse events, strong efficacy, and simple to take longer term for patient use. An evolving regimen is 2-drugs that contain antiretroviral drugs with a strong therapeutic index. This includes, but is not limited to treatments for people living with HIV (PLWH) that include the integrase inhibitor dolutegravir (DTG) and the nucleoside reverse transcriptase inhibitor (NRTI) lamivudine (3TC). The preferable pharmacokinetic and pharmacodynamics profile, and clinical trial efficacy and safety of DTG/3TC affirm the importance as current treatment ARV options. Overall similar efficacy and tolerability were achieved with DTG within two-or three drug regimens in clinical trials. The effectiveness in people living with HIV-1 (PLHIV) switching to DTG with lamivudine (3TC) or rilpivirine (RPV) is now well established. Indeed, DTG and 3TC or DTG/RPV demonstrates low rate of virological failure and a high rate of viral suppression in virologically suppressed infected person. notably DTG/3TC is an effective treatment option for people with HIV with a viral load <50 copies/mL independent of age, sex, or race. In the present study peripheral HIV-1 reservoir markers declined similarly in patients treated with DTG/3TC or triple therapy. The present work evaluated 39 person undergoing inguinal LN excision at baseline or during the first year of treatment with DTG/3TC for HIV-1 DNA and RNA by DNA/RNAscope and antiretroviral spatial distribution by mass spectrometry imaging. HIV-1–infected cells declined after treatment with comparable reductions between each of the treatment groups. Antiretrovirals were detected in LN containing HIV-positive cells. The results provide spatial evidence that DTG/3TC achieves effective LN penetration and supports effective antiviral exposure within key anatomical HIV reservoirs. The investigators show that HIV-infected cells

within LN decline rapidly after ART initiation and that the magnitude and kinetics of reservoir decay are comparable between dual and triple therapy with DTG/3TC and triple regimens. The findings extend the prior observations made by this group in peripheral blood and provide direct tissue-level evidence of antiretroviral responses in anatomical reservoirs. The data is well documented and affirmed. The impact, however, is more limited based on the fact that this report is supportive of a range of prior reports and provides only affirmation of what is already known clinically.

**Response:** We thank the reviewer for the positive assessment of our work and for recognizing the technical rigor of the study. We agree that previous clinical trials, including our own DUALITY study, have demonstrated comparable virological efficacy between dual- and triple-drug therapy in peripheral blood. The primary objective of the present study was not to reassess the clinical efficacy of DTG/3TC, but rather to provide direct tissue-level evidence addressing a longstanding biological question: whether dual therapy achieves adequate drug penetration and antiviral activity within lymph node reservoirs. To our knowledge, this is the first study to combine longitudinal lymph node sampling during the first year of ART with multimodal spatial imaging of both HIV persistence and antiretroviral distribution in humans. We have further emphasized this rationale in the Discussion to better highlight the biological and mechanistic insights provided by the study.