A randomized comparison of antiretroviral therapy alone versus antiretroviral therapy with a 'kick-and-kill' approach, on measures of the HIV reservoir amongst participants with recent HIV infection: the RIVER trial

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Summary

Background: Antiretroviral therapy (ART) cannot cure HIV infection because of a persistent reservoir of latently infected cells. Approaches that force HIV transcription from these cells, making them susceptible to killing - termed ‘kick and kill’ - have been explored as a strategy towards an HIV cure. RIVER is the first randomized trial to determine the impact of ART alone versus ART plus ‘kick-and-kill’ on markers of the HIV reservoir.

Methods: RIVER (Trial registration: NCT02336074) was an open-label, multicenter, 1:1 randomized controlled trial of ART-only (control) versus ART plus the histone deacetylase inhibitor vorinostat (the ‘kick’) and replication-deficient viral vector vaccines encoding conserved HIV sequences ChAdV63.HIVconsv-prime, MVA.HIVconsv-boost T-cell vaccination (the ‘kill’) (ART+V+V; intervention) in HIV-positive adults treated in recent HIV-infection. The primary endpoint was total HIV DNA in peripheral blood CD4+ T-cells at weeks 16 and 18 post-randomization. Secondary endpoints included safety, alternative measures of the HIV reservoir including quantitative viral outgrowth, HIV-specific T-cell frequencies, and CD8+ T-cell mediated viral inhibition.

Findings: Between December 2015 and November 2017, 60 HIV-positive male participants were randomized (computer-based and stratified by time since diagnosis; 30 participants in each trial arm) and completed the study interventions, with no loss-to-follow-up. There were no intervention-related serious adverse events. Mean total HIV DNA at weeks 16 and 18 was 3.02 log_{10} copies HIV DNA/10^6 CD4+ T-cells in the control and 3.06 log_{10} copies HIV DNA/10^6 CD4+ T-cells in the intervention arm, with no statistically significant difference (mean difference of 0.04 (95%CI -0.03, 0.11) log_{10} total HIV DNA copies/10^6 CD4+ T-cells (p=0.26)).

Interpretation: This ‘kick-and-kill’ approach conferred no significant benefit compared to ART alone on measures of the HIV reservoir. Although this does not disprove the ‘kick and
kill’ strategy, for future trials significant enhancement of both ‘kick’ and ‘kill’ agents will be required.

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### Research in context panel

#### Evidence before this study
This randomised clinical trial was designed to test the concept of ‘kick and kill’ as a strategy to achieve a cure for HIV infection. Prior to this study, there was evidence from *in vitro* and single arm clinical studies that the histone deacetylase inhibitor (HDACi) class of drugs could induce viral transcription from latently infected cells, potentially creating a target for the immune system. In conjunction with this ‘kick’ to the latent HIV reservoir there was evidence that T cell immunity – which determines HIV disease progression - could be enhanced through vaccination-induced responses, providing the ‘kill’. Although the strategy of ‘kick and kill’ looked promising, there had been no powered RCTs to test it.

#### Added value of the study
RIVER tested ‘kick and kill’ using the HDACi vorinostat as the ‘kick’ combined with a vaccine strategy targeting conserved regions of the HIV genome. The vaccine aimed to produce T cells to kill latently-infected cells in which viral transcription had been induced by the HDACi. RIVER showed that the intervention was safe, with outstanding adherence to the complex trial protocol by the participants. However, even though there was evidence for both increased histone acetylation and potent vaccine-induced T-cell responses, the intervention did not confer any additional benefit on any measures of the HIV reservoir compared with antiretroviral therapy alone.

#### Implications of all the available evidence.
RIVER was the first RCT in treated recent HIV infection, and was not able to show any impact of ‘kick and kill’ on the primary outcome measure, or any marker of the HIV reservoir size. This is consistent with other studies which had tested HDACi alone. We did not, however, stop antiretroviral therapy in the RIVER trial participants, and future studies may include a treatment interruption as a further measure of impact. Whilst the RIVER trial suggest that this specific ‘kick and kill’ approach may not be an effective approach towards achieving HIV cure, the overall principle cannot yet be dismissed, as more potent future interventions may have a greater impact.
Introduction

Antiretroviral therapy (ART) has significantly improved survival for people living with HIV (PLWH), but alone is unable to cure HIV due to a reservoir of latently infected cells harbouring transcriptionally silent, but replication competent, integrated HIV DNA. These latently infected cells are not visible to the immune system as they do not express viral antigens. The HIV reservoir persists in CD4+ cells, is established soon after viral acquisition and, despite suppressive ART, is the main source of viral recrudescence if ART is interrupted. The size of the HIV reservoir, which can be limited by starting ART in recent or primary HIV infection (PHI), predicts clinical progression and can impact time to viral rebound upon ART cessation. Any approaches towards curing HIV or achieving viral control off ART must therefore target eliminating or controlling the HIV reservoir.

In addition to ART, which blocks ongoing viral replication, one strategy to deplete the HIV reservoir involves forcing HIV transcription using latency reversing agents to induce viral antigen expression in latently infected cells. When synergised with interventions that enhance HIV-specific cytolytic T cell activity, immune recognition may confer elimination of these HIV antigen expressing cells; this approach has been termed ‘kick-and-kill’. Several single-arm studies have shown that inhibitors of histone deacetylation (HDACi) reverse HIV latency by inducing viral gene transcription, although none of these studies to date have shown a significant impact on the measured HIV reservoir. This observation may be explained by the fact that HDACi agents alone, whilst forcing viral transcription, maybe insufficient to induce cell death, and hence it is argued that an HIV-specific ‘kill’ may be necessary. T-cell inducing vaccines that employ potent viral vectors to deliver conserved HIV immunogens have been postulated to fulfil this role. We have developed a heterologous viral vector vaccine strategy employing replication-deficient chimpanzee adenovirus (ChAd) and modified
vaccinia Ankara (MVA) vectors that each encode an HIV conserved region immunogen, HIVconsv, in a prime-boost regimen (ChAdV63.HIVconsv/ MVA.HIVconsv). The HIVconsv immunogen, was specifically designed to focus T cell responses towards highly conserved regions of the viral proteome and thus provide ‘universal coverage’ of diverse HIV subtypes, while avoiding variable regions that may act as a decoy for the immune system. In addition, targeting responses to functionally constrained epitopes, in which mutation carries a fitness cost, should mitigate against viral escape. This prime-boost vaccination approach was initially tested in individuals with primary HIV infection receiving ART at the time of HIV diagnosis in the BCN01 study. In the subsequent rollover study BCN02, a further MVA.HIVconsv vaccine boost was administered together with the HDACi romidepsin, followed by an ATI. In a preliminary analysis, a period of post-ART viral control was demonstrated in a subset of individuals, although the absence of ART-only controls makes interpretation difficult.

The RIVER trial is the first randomized controlled study to compare ART combined with a ‘kick and kill’ approach using two previously tried interventions, the latency-reversing HDACi vorinostat and prime-boost vaccinations with ChAdV63.HIVconsv and MVA.HIVconsv, with ART-alone on measures of the HIV reservoir amongst participants starting ART in primary HIV infection. The benefit of recruiting during treated PHI is that the HIV reservoir is smaller and the immune system better preserved than in chronic infection. However all studies using HDACi to date have enrolled during chronic infection, where there may be a lower barrier to stimulating viral transcription. Vorinostat was selected as the ‘kick’ agent, as when RIVER was designed vorinostat was the HDACi with the best evidence of both induced viral transcription in human studies and acceptable safety data. Although the licensed HDACi’s
differ in their potency against HDAC inhibition in vitro, the level of target engagement (quantified by histone acetylation) and induction of HIV CA-RNA are similar\textsuperscript{11-13}.

\textbf{Methods}

\textit{(See also Supplementary materials S1-S10; Figure S1a, b and c).}

\textbf{Study design and participants}

RIVER was a randomized, controlled, phase II trial performed in six UK clinical sites between December 2015 and November 2017. Ethics committee approval was obtained from all participating centres (14/SC/1372) in accordance with the principles of the Declaration of Helsinki.

Participants with confirmed recently acquired HIV infection were enrolled according to fulfilling at least one of the following criteria:

a) Positive HIV-1 serology within < 12 weeks of negative HIV-1 serology or point-of-care test

b) Positive p24 antigen and a negative HIV antibody test,

c) Negative antibody test with either detectable HIV RNA or proviral DNA,

d) Public Health England recency HIV antibody avidity assay (RITA)\textsuperscript{19} reported “Incident” (within the preceding 16 weeks),

e) Weakly reactive or equivocal 4th generation HIV antibody antigen test,

f) Equivocal or reactive antibody test with <4 bands on western blot.

All participants were aged 18-60 years, and initiated ART within 4 weeks of confirmed primary HIV infection (PHI). Eligible consenting participants included two strata, the second introduced after enrolment began in order to optimise recruitment. The majority of participants
(52/60) were recruited to stratum 1, where ART was initiated at enrolment and randomization occurred after 24 weeks provided HIV RNA was <50 copies/ml. The eight participants recruited via stratum 2 had been initiated on ART up to 4 weeks after a previous diagnosis of PHI defined using the same criteria as for stratum 1, and were randomized at enrolment provided they had received ART for >24 weeks but <2 years since PHI, and HIV RNA <50 copies/ml. Based on data demonstrating a biphasic decay in total HIV DNA following ART initiation (with a plateau from 6 months to approximately 2 years after acute infection\textsuperscript{20}), we modelled that individuals initiating ART in PHI and remaining on suppressive therapy for up to 2 years had comparable levels of HIV reservoirs at that point to those randomized after 6 months ART. Furthermore, since the primary endpoint of the RIVER study design compares total HIV DNA between the two arms from randomization to post-randomization weeks 16 & 18, stratum 1 and 2 can be considered comparable. All inclusion/exclusion criteria are described in the Supplementary Material (S4).

Randomization

Eligible individuals were randomly assigned 1:1 to either ART-only or ART plus vaccination with ChAdV63.HIVconsv prime and MVA.HIVconsv boost followed by vorinostat taken orally in 10 doses of 400mg every 3 days for 28 days (ART+V+V). Dosing was based on optimised use of vorinostat as previously described\textsuperscript{21}. The vaccine design and manufacture are described elsewhere\textsuperscript{16}. The randomization list was computer-generated and preprepared by the trial statistician using permuted blocks of randomly varying sizes, stratified by time since diagnosis (stratum 1 versus stratum 2). Sites faxed screening forms to the trial coordinating centre, where trial staff confirmed eligibility and did the randomisation (they could access the next number on the list, but not the whole list). Treatment allocation was open label.
Study procedures

Participants attended study centres at screening, randomization, <1 week post-randomization (PR) (ChAdV63.HIVconsv vaccine in ART+V+V arm), PR week 8 day 1 (MVA.HIVconsv boost vaccine in ART+V+V), following previously described dosing intervals PR week 8 day 3 (first dose of vorinostat in ART+V+V) and then at PR weeks 9, 10, 11, 12, 16 and 18 in accordance with previously described schedules. Visits included assessment of adverse events and blood collection (and are detailed in Table S20). In stratum 1, four additional pre-randomization visits were attended at enrolment, weeks 4, 12, and 22 (Figures S1a & S1b).

ART was chosen according to British HIV Association guidelines. Participants were recommended to use a 3 or 4 ART drug regimen which included the integrase inhibitor, raltegravir.

Outcomes

Safety outcomes included all clinical and laboratory adverse events (AEs) of all grades using the Division of AIDS toxicity grading scale, including Serious AEs (S7a).

Primary outcome: total HIV DNA in CD4\(^+\) T-cells isolated from peripheral blood averaged across post-randomization weeks 16 and 18. Total HIV DNA measurement was undertaken according to methods previously described in CD4\(^+\) T-cells isolated from cryopreserved peripheral blood mononuclear cells (PBMC)\(^7,24\) (S7b).

Secondary outcomes are described in full in the Supplementary Material (S7c-l). In brief, they include: integrated HIV DNA (S7c) measured using nested qPCR\(^24,25\); cell-associated unspliced RNA (CA-RNA) (S7d)\(^26\); quantitative viral outgrowth assay (qVOA) (S7e)\(^27\), and single copy HIV RNA assay (SCA) (S7f). CD4\(^+\) and CD8\(^+\) HIV-specific T-cell responses: Antigen-specific phenotype and cytokine secretion profiles of CD4\(^+\) and CD8\(^+\) T-cells were assessed by intracellular cytokine staining (S7g) to peptides corresponding the
HIVconsv vaccine insert, as described previously\textsuperscript{28}; \textbf{CD8\textsuperscript{+} T cell antiviral suppressive activity} (S7h), calculated as described previously\textsuperscript{29}.

Other exploratory post-hoc outcomes: \textbf{cell-associated HIV gag p24 protein (CA-p24)} (S7i)\textsuperscript{30}; \textbf{cell viability} (S7j); \textbf{histone H4 acetylation} (S7k) using a H4K5/8/12/16 immunoassay with thawed PBMC derived cell lysates added to an ELISA using anti-H4 monoclonal antibody; and \textbf{in vitro HDACi stimulation} (S7l)\textsuperscript{30}. Viability and yield exclusion criteria were established before the trial to determine which samples would be usable for the primary and secondary outcomes.

\textbf{Statistical analysis}

The sample size calculation was based on assumptions; a) the combination intervention conferred a 50\% reduction in the primary endpoint compared with ART-alone (i.e. difference of log\textsubscript{10}), assumed to be the same in both strata; b) standard deviation is 0.4 for a single measurement in both arms; c) treatment arms are compared in terms of absolute total HIV-DNA level at PR 16\&18 adjusted for baseline level; d) correlation coefficient is 0.5 between a baseline measurement and a PR 16\&18 measurement, and 0.7 for measurements at PR 16\&18; e) two-sided $\alpha = 0.05$ for the null hypothesis. Under these assumptions, a sample size of 52 individuals provided 94\% power to detect a 50\% reduction in total HIV DNA (86\% power for a 45\% reduction). Allowing for loss to follow-up, 60 participants were enrolled.

The primary endpoint was analysed on a log\textsubscript{10}-scale, comparing arms in terms of absolute total HIV-DNA levels at PR16\&18 adjusted for the baseline level and by stratum using analysis of covariance. When either the PR week 16 or the PR week 18 result was missing but not both, the primary endpoint consisted of the single available result. For the primary analysis, two missing baseline total HIV DNA results were imputed using a multiple imputation method.
based on stratum and total HIV DNA at PR16&18. The two treatment groups were compared
as randomized, according to the intention-to-treat principle (ITT). Pre-specified sensitivity
analyses for the primary endpoint were an analysis excluding participants with incomplete
intervention (temporary/complete stop of ART, vaccination, or vorinostat), and a “complete
case analysis” excluding participants with missing total HIV DNA.

ITT analyses of secondary endpoints were performed unadjusted for multiple comparisons.
Binary outcomes were compared between the arms using chi\(^2\)/exact test and logistic regression.
Continuous outcomes were compared using linear regression, interval regression, generalised
estimating equations or rank tests and median regression. Data were analysed with Stata
(StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC).

**Role of the funders:**
The funder (MRC-DPFS) was not involved in the data collection, analysis interpretation nor
writing of the manuscript. The Partner funders Merck contribute data on HDACi activity and
p24 Simoa assay and for that aspect of the analysis they were partners. They also reviewed
the manuscript, contributed to relevant text around their assay and approved submission. The
funders GSK who part-owned the vaccine did not have any input into data collection,
analysis or manuscript preparation or submission and are not named co-authors.

**Results**

**Participants**
82 participants were screened to randomize 60 (52 stratum 1; 8 stratum 2) (Figure 1). Median
time between confirmed PHI and ART start was 2 weeks (IQR 1, 3), with no significant
difference between randomization characteristics by study arm (Tables 1, and S1). PHI
diagnoses were by recency-testing algorithm\(^19\) (RITA) in 40 (67\%) participants, (Table S2). At
randomization HIV Viral Load (VL) was <50 copies/ml in 30 (100%) ART+V+V and 29 (97%) ART-only; median CD4+ T-cell count in the ART+V+V and ART-only arms was 710 (IQR 579, 759) and 694 (IQR 561, 844) cells/mm³ respectively.

Follow-up

No participant withdrew or was lost after randomization. Overall attendance of visits was good (Table S3), with all 60 participants attending both primary endpoint visits (PR16&18). All 60 participants randomized stayed on ART throughout the trial. Integrase inhibitor containing ART was prescribed for 58 (97%). Self-reported ART non-adherence was low and similar across the study arms (Figure S2). All 30 participants in the ART+V+V arm received the two vaccines as scheduled (Figure S3); 27 (90%) participants received all 10 doses of vorinostat as scheduled (Table S4).

Primary endpoint

Mean total HIV DNA was 3.84 (SD 0.58) log₁₀ copies/10⁶ CD4⁺ T-cells at enrolment (stratum 1), decreasing to 3.14 (SD 0.49) at randomization (Table S5). Total HIV DNA (Table S5b) then decreased further post randomization, similarly in the two arms (Figure 2), with estimated mean total HIV DNA at PR16&18 of 3.02 log₁₀ copies HIV DNA/10⁶ CD4⁺ T-cells in ART only and 3.06 log₁₀ copies HIV DNA/10⁶ CD4⁺ T-cells in ART+V+V. The difference in the primary endpoint (ART+V+V minus ART only in mean log₁₀ HIV DNA copies/10⁶ CD4⁺ T-cells) was 0.04 (95% CI: -0.03 to 0.11), p=0.26, i.e. approximately 9% higher in ART+V+V. There also was no evidence of a treatment difference in the study primary endpoint with sensitivity analyses excluding participants not completing all doses of vorinostat or excluding participants with missing total HIV-DNA at randomization (Table S6), and excluding stratum 2 participants.
Other measures of the HIV reservoir

Integrated HIV DNA

Results were available for 52 participants for both randomization and either PR16 or PR18. Mean integrated HIV DNA was 2.83 (SD 0.51) and 2.81 (SD 0.48) at randomization and 2.79 (SD 0.51) and 2.83 (SD 0.45) log_{10} copies/10^6 CD4+ T-cells at PR16&18 for ART-only, and ART+V+V respectively (Figure 3a). There was no significant difference between integrated HIV DNA values for the arms at PR16&18 (ART+V+V minus ART-only: 0.05 log_{10} copies/10^6 cells (95% CI -0.15 to 0.25; p=0.60)). There was a strong correlation between total and integrated HIV DNA \( r=0.72 \) (p<0.001) (Supplementary Figure S4).

qVOA

Results were available for 51 participants at randomization, of which viral outgrowth was undetectable in 20 (39%). At week 16, viral outgrowth was undetectable in 12/29 (41%) ART-only, and 6/27 (22%) in ART+V+V arm (adjusted Odds Ratio 0.41 (95% CI 0.13-1.35); p=0.14). There was no significant difference between the arms in viral outgrowth at week PR16, with an adjusted difference in median (ART+V+V minus ART-only) of 0.05 IUPM (95% CI -0.28 to 0.39; p=0.76) in participants with detectable results, and of 0.13 log_{10} IUPM (95% CI -0.38 to 0.65; p=0.61) in participants including those with undetectable results (Figure 3b; for the raw data see Table S19).

CA unspliced RNA

Results were available in 50 participants for randomization and PR16 & 18. Overall median baseline CA-RNA was 0.10 (IQR 0.01 - 0.66) copies/ng, with no difference by arm at PR16 &
18, with adjusted difference in median (ART+V+V minus ART-only) of 0.02 copies/ng (95% CI -0.19 to 0.24; p=0.83) (Figure 3c).

Single Copy Assay HIV RNA
HIV RNA was available in 59 participants between randomization and week PR18. Median HIV RNA was 14 copies/ml in both arms at randomization, and between 4-9 copies/ml in ART-only, and between 5-7 copies/ml in ART+V+V, post-randomization visits with no difference between the arms (Figure 3d; Table S7).

Impact of Vaccination on T-cell responses and function

CD4+ and CD8+ HIV-specific T-cell responses
Immunogenicity of the vaccination regimen was assessed by determining the frequency of HIVconsv-specific CD4+ and CD8+ T cells in intracellular cytokine assays measured at randomization, PR-9 (9 weeks post randomization and 1 week after final vaccine dose) and PR-12 (12 weeks post randomization and after completion of vorinostat course). Participants in the ART+V+V arm showed significantly higher percentages of HIV-specific CD4+ T-cells positive for IFN-γ, IL-2, TNF-α, and CD154 than ART-only at PR9 and PR12 (Table S8). Vaccinated participants also had significantly higher proportions of CD8+ T-cells positive for IFNγ and TNFα at both time-points, and for IL-2 at PR9 (Table S9). In post-hoc analyses of polyfunctionality, vaccinated participants showed a higher proportion of CD154+ INF-γ+ CD4+ cells and CD107a+ INFγ+ CD8+ cells (Figures 4a&b).

CD8+ T-cell antiviral inhibitory activity
The capacity of vaccine-induced or vaccine-boosted CD8+ T cells to target HIV-infected cells was assessed in viral inhibition assays. Mean viral inhibition at randomization was 54% (SD
40% overall. Post randomization, it significantly decreased in the ART-only arm but did not change in the ART+V+V arm: adjusted change from randomization to PR9 was -7% and 7% in ART-only and ART+V+V arm, respectively (p=0.10 for difference between the arms), and -18% and 2% to PR12 (p=0.026) (Figure 4c).

CD4+ T-cell count and CD4/CD8 ratio

Median CD4+ T-cell count remained unchanged between randomization (708; IQR 568 - 788), weeks 16 (742; IQR 538 - 849) & 18 (706; IQR 562 - 880 cells/mm3), with no significant difference between study arms across any timepoint. Median CD4/CD8 ratio was 0.67 at enrolment, and increased to 1.08 at randomization. CD4/CD8 ratio increased further post-randomization without significant difference by study group (Figure 4d).

Impact of vorinostat on viral transcription

Histone H4 Acetylation

Twenty-two participants in the ART+V+V arm provided a total of 41 pre/post vorinostat results. Averaged across all time-points, acetylation two hours post vorinostat increased by a factor of 3.2 (95%CI 2.4-4.2; p<0.001) compared to pre-vorinostat (Table S10, Figure S7).

CA-RNA

For 17/30 participants in the ART+V+V arm with pre and 2 hours post vorinostat data, there was no significant difference between pre and post vorinostat CA-RNA levels at the three timepoints tested (Table S11).
Exc-vivo vorinostat stimulation led to a statistically significant 3.86-fold increase in mean HIV CA-RNA compared to untreated controls. (Supplementary Figure S6; p=0.009), showing that HIV gene transcription could be stimulated by vorinostat under optimised conditions.

Cell-associated p24

Most of the results fell in the range 0.01 to 0.02 AEB (‘average amount of enzyme per bead’), regarded as ‘undetectable’ (Table S12). P24 was above the limit of quantification (>0.02 AEB) in only 4 samples overall, with no difference between the randomization groups: 1/57 at randomization, 1/35 at PR11 (both ART only), and 1/30 in each randomization group at PR16.

HIV RNA Single Copy Assay

For 28 participants in the ART+V+V arm, pre and 2 hours post vorinostat SCA results were available (n=22, 20, and 21 at PR visits week 8, 9, 12). At none of the 3 time-points, was there a significant difference in HIV RNA pre and post-vorinostat, and medians pre and post were 5.5 (IQR 2-20) and 4.5 (IQR 2-15) copies/mL at PR8 (p=0.67), 5.5 (IQR 1-20.5) and 7 (IQR 3.5-11) copies/mL at PR9 (p=0.76), and 5 (IQR 1-7) and 4 (IQR 2-14) copies/mL at PR12 (p=0.11), (Figure S5).

Cell viability post vorinostat dosing viability

Samples collected two hours post vorinostat treatment had significantly lower viability when compared to all other samples (p<0.001, medians 65.5% vs 87%, respectively) as well as lower % expected yields (p=0.016, medians 54% vs 64%, respectively).

Safety
There were no deaths or notable events. One SAE of vasovagal syncope likely secondary to blood draw-related venepuncture (ART+V+V arm) was reported post-randomization (Table S13). Clinical adverse events were reported for 29 (97%) participants in the ART+V+V arm and 22 (73%) in the control arm (p=0.026) (Table 2 and Tables S14-16, S22, S23). Most laboratory AE were mild-moderate and similar by arm (Table S17). There was no significant difference in QTc change from PR week 8 between the two arms (Table S18). Solicited general symptoms and injection site reactions in the ART+V+V arm are shown in Figure S8.

Discussion

The RIVER trial provides the first randomized controlled assessment of the effect of a ‘kick and kill’ intervention on measures of the HIV reservoir in study participants with treated recent HIV infection. Despite significantly enhanced vaccine-induced HIV-specific CD4⁺ and CD8⁺ T-cell responses and evidence for in vivo vorinostat-induced HDAC inhibition, there was no difference in any measures of the HIV reservoir between the ART-only arm and the ART+V+V arm. That we were able to demonstrate evidence for an impact of both the ‘kick’ (increased histone acetylation following vorinostat) and ‘kill’ (enhanced functional T cell responses induced by vaccination) but no change in measures of the HIV reservoir raises challenging questions.

In the vaccine-only BCN01 study¹⁷, there was no impact on measures of the HIV reservoir despite potent HIV-specific immune responses in a similar cohort treated in primary infection, suggesting that enhancing the ‘kill’ alone is not adequate for HIV cure. Our use of the same vaccination approach demonstrated similarly enhanced HIV-specific T-cell immunity. In studies that have solely employed a ‘kick’, using HDAC inhibition, several have failed to show a convincing impact on HIV reservoir size¹¹-¹³,³¹. However, some
studies demonstrated induction of viral transcription following single\textsuperscript{11} and multiple daily
dosing of vorinostat\textsuperscript{12} (using identical vorinostat dosing strategies to RIVER\textsuperscript{21}), supporting
the premise that inhibition of histone deacetylation could potentially enhance viral protein
presentation to effector T cells. While the magnitude of HDAC inhibition in RIVER was in
keeping with previous studies, we did not observe the same increases in viral transcription
reported by some groups\textsuperscript{9,11-13}, although not all\textsuperscript{31}. It should be noted that our choice of dosing
regime for vorinostat was based on limited data, and further optimization, such as a longer
treatment course might have enhanced the impact.

Three possible explanations are considered to explain this discrepancy: 1) Differential
susceptibility to HDAC inhibition. Some previous studies pre-selected participants based on
susceptibility to HDAC inhibition due to concerns that sensitivity to viral reactivation varied
between individuals\textsuperscript{9,11,21}. We chose not to do this as others had demonstrated HDACi
induced viral transcription in un-selected participants\textsuperscript{12,13}. Of note, \textit{ex vivo} stimulation of
CD4$^+$ T-cells from RIVER participants identified responsiveness to vorinostat measured by
increased HIV RNA transcript production, making this explanation less likely. 2) The
reservoir in primary HIV-infected participants is somehow different from those with treated
chronic infection. Previous studies with HDACi have recruited participants with chronic HIV
infection\textsuperscript{9,11-13,21,31,32}. Compared to PHI, the reservoir in chronic infection is larger,
containing greater viral diversity, possible higher levels of basal transcription, more defective
proviral sequences, and with variable susceptibility to latency reversal\textsuperscript{33}. Although it remains
unproven, the heterogeneity in the character of the reservoir may have impacted susceptibility
to our intervention. 3) The HDAC inhibitor vorinostat impacted cell viability. We observed a
reduction in viability for samples taken two hours after vorinostat dosing, in keeping with
other groups\textsuperscript{34}, which may have contributed to our findings. It is also possible that the 2-hour
timepoint chosen to measure HDAC-inhibition and viral transcription (coinciding with peak plasma drug concentration) was too soon to detect significant viral transcription and subsequent data suggest that 8 hours post-dosing might be preferable. Of previous studies using T-cell vaccination with HDACi, the BCN 02 study which enrolled participants with treated PHI is most comparable to RIVER, using identical vaccines\textsuperscript{18} with the HDACi, romidepsin\textsuperscript{35}. The BCN 02 study investigators more recently demonstrated both romidepsin-induced acetylation and viral transcription, as well as enhanced vaccine-induced T-cell responses\textsuperscript{36}. Subsequent ART interruption led to post-treatment sustained viral control among three out of 13 (23%) study participants, although in the absence of a control arm, one cannot comment on whether this was driven by the interventions.

The REDUC study\textsuperscript{32} evaluated the combined effects of an HIV Gag peptide vaccine, recombinant human granulocyte macrophage colony-stimulating factor and romidepsin on the HIV reservoir. There was no significant impact of the intervention on measures of the reservoir in evaluable patients. There are no other completed RCT testing alternative HIV ‘kick and kill’ approaches.

Although the existence of an HIV reservoir has been acknowledged for over 20 years\textsuperscript{2,5} there remains no consensus on how best to quantify it or determine whether it has been successfully targeted by an intervention, and no biomarkers definitively predict viral rebound after stopping ART\textsuperscript{37,38}. Accordingly, clinical trials exploring HIV cure strategies often include a variety of HIV reservoir measurements\textsuperscript{39}, as we have here. Ultimately the only outcome measure of clinical value may be analytical treatment interruption (ATI). Although at the time of writing there is a growing consensus that ATIs can be conducted safely and there is some progress to
unifying protocols to do this\textsuperscript{40}, when RIVER was designed ATIs were still considered high risk due to concerns over harm associated with viral rebound, the risk of onward HIV transmission, uncertainty of individual level clinical and immunological harm and the requirement for frequent viral load monitoring.

Accordingly, the use of HIV DNA quantitation as a primary endpoint for the RIVER trial may be criticized, with conflicting evidence on its value as a predictor of post-treatment control\textsuperscript{7,8,37}. It might be argued that an ATI\textsuperscript{38} would have provided an alternative more clinically relevant measure of impact of the intervention, even given the caveats above. We also felt that in the absence of any change in any parameter relating to reservoir size or activation, an ATI would have been unlikely to provide additional information. Although in hindsight this may be a weakness of the design, we maintain that an ATI is unlikely to have demonstrated a different overall result to the one we report in regards to the efficacy of this ‘kick and kill’ approach.

More detailed analysis of “responder” and “non-responder” subgroups is planned which could inform underlying mechanisms and variability.

The relative timing of the ‘kick and kill’ intervention tested in this trial may be a further explanation for lack of efficacy. The vaccine dosing schedule, comprising prime and boost separated by an 8-week interval was previously shown to be safe and immunogenic in the BNC01 study\textsuperscript{17}. The subsequent timing of vorinostat, with respect to vaccination was based on the estimated kinetic of CD8\textsuperscript{+} T-cell responses, with the peak coinciding with vorinostat-induced viral transcription. However whether the viral antigens expressed on latently infected cells as a result of HDACi-induced viral transcription are the same as those epitopes selected by a candidate T-cell vaccine remains unknown. However, this may be over-simplifying a complex issue. Even if it is speculated that vorinostat did induce adequate protein expression
in HIV-infected CD4+ T cells, they might still not be killed by vaccine-induced CD8+ T cells because of Nef-mediated HLA class I downregulation, insufficient peptide presentation, presentation of epitopes other than those recognised by vaccine-induced responses, archived mutations in the epitopes targeted by CD8+ T cells, or CD8+ T cell dysfunction or exhaustion.

RIVER is the first randomized trial in the HIV cure field testing the ‘kick and kill’ approach in treated PHI. That no difference compared with ART-alone was reported raises the key question of whether the concept is fundamentally flawed or if a significant impact on the reservoir could be induced with different agents. Whilst the findings represent a set-back in some ways, RIVER helps set the standard for how future trials in this research arena might be conducted as new insights have been gained with regard to trial design, the inclusion of community representation and the need for better interventions, and the necessity for clarity regarding the most relevant measures of the reservoir, and probably an ATI. Finally, the outstanding participant and community engagement with RIVER reflects the need for, and deep involvement of, those living with HIV in clinical research aimed at ending the epidemic.

Contributors

SF and JF were co-PIs. SF was clinical chief investigator. JF was laboratory lead. AB and WS were the trial statisticians. MP was responsible for developing the trial primary endpoint analysis. LD, JK and HY led the HIV-immunology laboratory endpoints assays. AL, AF, MW and MB were responsible for running the secondary endpoint viral outgrowth assays. SP was the trial physician at the MRC CTU and a site clinical PI. SKdL provided clinical and research oversight for all vaccination visits at the Royal Free Hospital. JFo, JT, AC, MN, MJ were clinical PIs responsible for recruitment. MK and SK were responsible for undertaking the single copy HIV RNA secondary endpoint assay. DK was the community representative
for people living with HIV. TH designed the vaccines used in the trial and provided the GMP IMPs with related documentation, and contributed to the trial design. RB was the trial manager. BH, GX and RB from Merck provided the laboratory expertise for pharmacological guidance on HDACi. All authors contributed to the writing and review of the manuscript. The members of the RIVER trial study group are listed in the supplementary materials.

**Declaration of Interests**

The authors have no interests to declare.
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