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

4

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79 HIV, vorinostat, T-cell vaccine, kick-and-kill, RCT, ART, HIV reservoir.

80 **Summary**

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

86 **Methods:** RIVER (Trial registration: NCT02336074) was an open-label, multicenter, 1:1
87 randomized controlled trial of ART-only (control) versus ART plus the histone deacetylase
88 inhibitor vorinostat (the 'kick') and replication-deficient viral vector vaccines encoding
89 conserved HIV sequences ChAdV63.HIVconsv-prime, MVA.HIVconsv-boost T-cell
90 vaccination (the 'kill') (ART+V+V; intervention) in HIV-positive adults treated in recent HIV-
91 infection. The primary endpoint was total HIV DNA in peripheral blood $CD4^+$ T-cells at weeks
92 16 and 18 post-randomization. Secondary endpoints included safety, alternative measures of
93 the HIV reservoir including quantitative viral outgrowth, HIV-specific T-cell frequencies, and
94 $CD8^+$ T-cell mediated viral inhibition.

95 **Findings:** Between December 2015 and November 2017, 60 HIV-positive male participants
96 were randomized (computer-based and stratified by time since diagnosis; 30 participants in
97 each trial arm) and completed the study interventions, with no loss-to-follow-up. There were
98 no intervention-related serious adverse events. Mean total HIV DNA at weeks 16 and 18 was
99 3.02 $\log_{10}$ copies HIV DNA/10^6 $CD4^+$ T-cells in the control and 3.06 $\log_{10}$ copies HIV
100 DNA/10^6 $CD4^+$ T-cells in the intervention arm, with no statistically significant difference
101 (mean difference of 0.04 (95%CI -0.03, 0.11) $\log_{10}$ total HIV DNA copies/10^6 $CD4^+$ T-cells
102 (p=0.26)).

103 **Interpretation:** This 'kick-and-kill' approach conferred no significant benefit compared to
104 ART alone on measures of the HIV reservoir. Although this does not disprove the 'kick and

105 kill' strategy, for future trials significant enhancement of both 'kick' and 'kill' agents will be

106 required.

107

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110

111

112 **Research in context panel**

113

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 suggests that this specific 'kick and kill' approach may not be an effective approach towards achieving HIV cure, the overall principle can not yet be dismissed, as more potent future interventions may have a greater impact.

114

115 **Introduction**

116 Antiretroviral therapy (ART) has significantly improved survival for people living with HIV
117 (PLWH)[1], but alone is unable to cure HIV due to a reservoir of latently infected cells
118 harbouring transcriptionally silent, but replication competent, integrated HIV DNA[2]. These
119 latently infected cells are not visible to the immune system as they do not express viral antigens.
120 The HIV reservoir persists in $CD4^+$ cells[3], is established soon after viral acquisition[4] and,
121 despite suppressive ART, is the main source of viral recrudescence if ART is interrupted[5]. The
122 size of the HIV reservoir, which can be limited by starting ART in recent or primary HIV
123 infection (PHI)[6], predicts clinical progression[7] and can impact time to viral rebound upon ART
124 cessation[7,8]. Any approaches towards curing HIV or achieving viral control off ART must
125 therefore target eliminating or controlling the HIV reservoir.

126

127 In addition to ART, which blocks ongoing viral replication, one strategy to deplete the HIV
128 reservoir involves forcing HIV transcription using latency reversing agents to induce viral
129 antigen expression in latently infected cells[9]. When synergised with interventions that enhance
130 HIV-specific cytolytic T cell activity, immune recognition may confer elimination of these
131 HIV antigen expressing cells; this approach has been termed 'kick-and-kill'[10]. Several single-
132 arm studies have shown that inhibitors of histone deacetylation (HDACi) reverse HIV latency
133 by inducing viral gene transcription[11-13], although none of these studies to date have shown a
134 significant impact on the measured HIV reservoir. This observation may be explained by the
135 fact that HDACi agents alone, whilst forcing viral transcription, maybe insufficient to induce
136 cell death[14], and hence it is argued that an HIV-specific 'kill' may be necessary[15]. T-cell
137 inducing vaccines that employ potent viral vectors to deliver conserved HIV immunogens have
138 been postulated to fulfil this role[15-17]. We have developed a heterologous viral vector vaccine
139 strategy employing replication-deficient chimpanzee adenovirus (ChAd) and modified

140 vaccinia Ankara (MVA) vectors that each encode an HIV conserved region immunogen,
141 HIVconsv, in a prime-boost regimen (ChAdV63.HIVconsv/ MVA.HIVconsv). The HIVconsv
142 immunogen, was specifically designed to focus T cell responses towards highly conserved
143 regions of the viral proteome and thus provide 'universal coverage' of diverse HIV subtypes,
144 while avoiding variable regions that may act as a decoy for the immune system. In addition,
145 targeting responses to functionally constrained epitopes, in which mutation carries a fitness
146 cost, should mitigate against viral escape[16-17]. This prime-boost vaccination approach was
147 initially tested in individuals with primary HIV infection receiving ART at the time of HIV
148 diagnosis in the BCN01 study[17]. In the subsequent rollover study BCN02, a further
149 MVA.HIVconsv vaccine boost was administered together with the HDACi romidepsin,
150 followed by an ATI. In a preliminary analysis, a period of post-ART viral control was
151 demonstrated in a subset of individuals[18], although the absence of ART-only controls makes
152 interpretation difficult.
153
154 The RIVER trial is the first randomized controlled study to compare ART combined with a
155 'kick and kill' approach using two previously tried interventions, the latency-reversing HDACi
156 vorinostat and prime-boost vaccinations with ChAdV63.HIVconsv and MVA.HIVconsv, with
157 ART-alone on measures of the HIV reservoir amongst participants starting ART in primary
158 HIV infection. The benefit of recruiting during treated PHI is that the HIV reservoir is smaller
159 and the immune system better preserved than in chronic infection. However all studies using
160 HDACi to date have enrolled during chronic infection, where there may be a lower barrier to
161 stimulating viral transcription. Vorinostat was selected as the 'kick' agent, as when RIVER
162 was designed vorinostat was the HDACi with the best evidence of both induced viral
163 transcription in human studies and acceptable safety data[11,12]. 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[11-13].

Methods

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

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)[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[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[21]. The vaccine design and manufacture are described elsewhere[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.

214 **Study procedures**

215 Participants attended study centres at screening, randomization, <1 week post-randomization
216 (PR) (ChAdV63.HIVconsv vaccine in ART+V+V arm), PR week 8 day 1 (MVA.HIVconsv
217 boost vaccine in ART+V+V), following previously described dosing intervals[21] PR week 8 day
218 3 (first dose of vorinostat in ART+V+V) and then at PR weeks 9, 10, 11, 12, 16 and 18 in
219 accordance with previously described schedules. Visits included assessment of adverse events
220 and blood collection (and are detailed in Table S20). In stratum 1, four additional pre-
221 randomization visits were attended at enrolment, weeks 4, 12, and 22 (Figures S1a & S1b).
222 ART was chosen according to British HIV Association guidelines[22]. Participants were
223 recommended to use a 3 or 4 ART drug regimen which included the integrase inhibitor,
224 raltegravir.

225

226 **Outcomes**

227 **Safety outcomes** included all clinical and laboratory adverse events (AEs) of all grades using
228 the Division of AIDS toxicity grading scale[23], including Serious AEs (S7a).

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

233 **Secondary outcomes** are described in full in the Supplementary Material (S7c-l). In brief,
234 they include: **integrated HIV DNA** (S7c) measured using nested qPCR[24,25]; **cell-associated**
235 **unspliced RNA (CA-RNA)** (S7d)[26]; **quantitative viral outgrowth assay (qVOA)** (S7e)[27],
236 and **single copy HIV RNA assay (SCA)** (S7f). **$CD4^+$and $CD8^+$ HIV-specific T-cell**
237 **responses:** Antigen-specific phenotype and cytokine secretion profiles of $CD4^+$ and $CD8^+$ T-
238 cells were assessed by intracellular cytokine staining (S7g) to peptides corresponding the

239 HIVconsv vaccine insert, as described previously[28]; **CD8$^+$ T cell antiviral suppressive**

240 **activity** (S7h), calculated as described previously[29].

241

242 Other exploratory post-hoc outcomes: **cell-associated HIV *gag* p24 protein** (**CA-p24**) (S7i)

243 [30]; **cell viability** (S7j); **histone H4 acetylation** (S7k) using a H4K5/8/12/16 immunoassay with

244 thawed PBMC derived cell lysates added to an ELISA using anti-H4 monoclonal antibody;

245 and ***in vitro HDACi stimulation*** (S7l)[30]. Viability and yield exclusion criteria were established

246 before the trial to determine which samples would be usable for the primary and secondary

247 outcomes.

248

249 **Statistical analysis**

250 The sample size calculation was based on assumptions; a) the combination intervention

251 conferred a 50% reduction in the primary endpoint compared with ART-alone (i.e. difference

252 of $\log_{10}$), assumed to be the same in both strata; b) standard deviation is 0.4 for a single

253 measurement in both arms; c) treatment arms are compared in terms of absolute total HIV-

254 DNA level at PR 16&18 adjusted for baseline level; d) correlation coefficient is 0.5 between a

255 baseline measurement and a PR 16&18 measurement, and 0.7 for measurements at PR 16&18;

256 e) two-sided $\alpha = 0.05$ for the null hypothesis. Under these assumptions, a sample size of 52

257 individuals provided 94% power to detect a 50% reduction in total HIV DNA (86% power for

258 a 45% reduction). Allowing for loss to follow-up, 60 participants were enrolled.

259 The primary endpoint was analysed on a $\log_{10}$-scale, comparing arms in terms of absolute total

260 HIV-DNA levels at PR16&18 adjusted for the baseline level and by stratum using analysis of

261 covariance. When either the PR week 16 or the PR week 18 result was missing but not both,

262 the primary endpoint consisted of the single available result. For the primary analysis, two

263 missing baseline total HIV DNA results were imputed using a multiple imputation method

264 based on stratum and total HIV DNA at PR16&18. The two treatment groups were compared
265 as randomized, according to the intention-to-treat principle (ITT). Pre-specified sensitivity
266 analyses for the primary endpoint were an analysis excluding participants with incomplete
267 intervention (temporary/complete stop of ART, vaccination, or vorinostat), and a "complete
268 case analysis" excluding participants with missing total HIV DNA.

269 ITT analyses of secondary endpoints were performed unadjusted for multiple comparisons.
270 Binary outcomes were compared between the arms using chi^2/exact test and logistic regression.
271 Continuous outcomes were compared using linear regression, interval regression, generalised
272 estimating equations or rank tests and median regression. Data were analysed with Stata
273 (StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC).
274

275 **Role of the funders:**

276 The funder (MRC-DPFS) was not involved in the data collection, analysis interpretation nor
277 writing of the manuscript. The Partner funders Merck contribute data on HDACi activity and
278 p24 Simoa assay and for that aspect of the analysis they were partners. They also reviewed
279 the manuscript, contributed to relevant text around their assay and approved submission. The
280 funders GSK who part-owned the vaccine did not have any input into data collection,
281 analysis or manuscript preparation or submission and are not named co-authors.
282

283 **Results**

284 ***Participants***

285 82 participants were screened to randomize 60 (52 stratum 1; 8 stratum 2) (Figure 1). Median
286 time between confirmed PHI and ART start was 2 weeks (IQR 1, 3), with no significant
287 difference between randomization characteristics by study arm (Tables 1, and S1). PHI
288 diagnoses were by recency-testing algorithm[19] (RITA) in 40 (67%) participants, (Table S2). At

289 randomization HIV Viral Load (VL) was <50 copies/ml in 30 (100%) ART+V+V and 29

290 (97%) ART-only; median CD4+ T-cell count in the ART+V+V and ART-only arms was 710

291 (IQR 579, 759) and 694 (IQR 561, 844) cells/mm^3 respectively.

292

293 ***Follow-up***

294 No participant withdrew or was lost after randomization. Overall attendance of visits was good

295 (Table S3), with all 60 participants attending both primary endpoint visits (PR16&18). All 60

296 participants randomized stayed on ART throughout the trial. Integrase inhibitor containing

297 ART was prescribed for 58 (97%). Self-reported ART non-adherence was low and similar

298 across the study arms (Figure S2). All 30 participants in the ART+V+V arm received the two

299 vaccines as scheduled (Figure S3); 27 (90%) participants received all 10 doses of vorinostat as

300 scheduled (Table S4).

301

302 ***Primary endpoint***

303 Mean total HIV DNA was 3.84 (SD 0.58) $\log_{10}$ copies/10^6 $CD4^+$ T-cells at enrolment (stratum

304 1), decreasing to 3.14 (SD 0.49) at randomization (Table S5). Total HIV DNA (Table S5b)

305 then decreased further post randomization, similarly in the two arms (Figure 2), with estimated

306 mean total HIV DNA at PR16&18 of 3.02 $\log_{10}$ copies HIV DNA/10^6 $CD4^+$ T-cells in ART

307 only and 3.06 $\log_{10}$ copies HIV DNA/10^6 $CD4^+$ T-cells in ART+V+V. The difference in the

308 primary endpoint (ART+V+V minus ART only in mean $\log_{10}$ HIV DNA copies/10^6 $CD4^+$ T-

309 cells) was 0.04 (95% CI: -0.03 to 0.11), p=0.26, i.e. approximately 9% higher in ART+V+V.

310 There also was no evidence of a treatment difference in the study primary endpoint with

311 sensitivity analyses excluding participants not completing all doses of vorinostat or excluding

312 participants with missing total HIV-DNA at randomization (Table S6), and excluding stratum

313 2 participants.

314

315 ***Other measures of the HIV reservoir***

316 *Integrated HIV DNA*

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

324

325 *qVOA*

326 Results were available for 51 participants at randomization, of which viral outgrowth was
327 undetectable in 20 (39%). At week 16, viral outgrowth was undetectable in 12/29 (41%) ART-
328 only, and 6/27 (22%) in ART+V+V arm (adjusted Odds Ratio 0.41 (95% CI 0.13-1.35);
329 p=0.14). There was no significant difference between the arms in viral outgrowth at week
330 PR16, with an adjusted difference in median (ART+V+V minus ART-only) of 0.05 IUPM
331 (95% CI -0.28 to 0.39; p=0.76) in participants with detectable results, and of 0.13 $\log_{10}$ IUPM
332 (95% CI -0.38 to 0.65; p=0.61) in participants including those with undetectable results (Figure
333 3b; for the raw data see Table S19).

334

335 *CA unspliced RNA*

336 Results were available in 50 participants for randomization and PR16 & 18. Overall median
337 baseline CA-RNA was 0.10 (IQR 0.01 - 0.66) copies/ng, with no difference by arm at PR16 &

338 18, with adjusted difference in median (ART+V+V minus ART-only) of 0.02 copies/ng (95%
339 CI -0.19 to 0.24; p=0.83)(Figure 3c).
340
341 *Single Copy Assay HIV RNA*
342 HIV RNA was available in 59 participants between randomization and week PR18. Median
343 HIV RNA was 14 copies/ml in both arms at randomization, and between 4-9 copies/ml in ART-
344 only, and between 5-7 copies/ml in ART+V+V, post-randomization visits with no difference
345 between the arms (Figure 3d; Table S7).
346
347 **Impact of Vaccination on T-cell responses and function**
348 *$CD4^+$and CD8+ HIV-specific T-cell responses*
349 Immunogenicity of the vaccination regimen was assessed by determining the frequency of
350 HIVconsv-specific $CD4^+$ and $CD8^+$ T cells in intracellular cytokine assays measured at
351 randomization, PR-9 (9 weeks post randomization and 1 week after final vaccine dose) and
352 PR-12 (12 weeks post randomization and after completion of vorinostat course). Participants
353 in the ART+V+V arm showed significantly higher percentages of HIV-specific $CD4^+$ T-cells
354 positive for IFN-γ, IL-2, TNF-α, and CD154 than ART-only at PR9 and PR12 (Table S8).
355 Vaccinated participants also had significantly higher proportions of $CD8^+$ T-cells positive for
356 IFNγ and TNFα at both time-points, and for IL-2 at PR9 (Table S9). In *post-hoc* analyses of
357 polyfunctionality, vaccinated participants showed a higher proportion of $CD154^+$ $INF\text{-}\gamma^+$ $CD4^+$
358 cells and $CD107a^+$ $INF\gamma^+$ $CD8^+$ cells (Figures 4a&b).
359
360 *$CD8^+$ T-cell antiviral inhibitory activity*
361 The capacity of vaccine-induced or vaccine-boosted $CD8^+$ T cells to target HIV-infected cells
362 was assessed in viral inhibition assays. Mean viral inhibition at randomization was 54% (SD

363 40%) overall. Post randomization, it significantly decreased in the ART-only arm but did not
364 change in the ART+V+V arm: adjusted change from randomization to PR9 was -7% and 7%
365 in ART-only and ART+V+V arm, respectively (p=0.10 for difference between the arms), and
366 -18% and 2% to PR12 (p=0.026) (Figure 4c).
367
368 *$CD4^+$ T-cell count and CD4/CD8 ratio*
369 Median $CD4^+$ T-cell count remained unchanged between randomization (708; IQR 568 - 788),
370 weeks 16 (742; IQR 538 - 849) & 18 (706; IQR 562 - 880 cells/mm3), with no significant
371 difference between study arms across any timepoint. Median CD4/CD8 ratio was 0.67 at
372 enrolment, and increased to 1.08 at randomization. CD4/CD8 ratio increased further post-
373 randomization without significant difference by study group (Figure 4d).
374
375 ***Impact of vorinostat on viral transcription***
376 *Histone H4 Acetylation*
377 Twenty-two participants in the ART+V+V arm provided a total of 41 pre/post vorinostat
378 results. Averaged across all time-points, acetylation two hours post vorinostat increased by a
379 factor of 3.2 (95%CI 2.4-4.2; p<0.001) compared to pre-vorinostat (Table S10, Figure S7).
380
381 *CA-RNA*
382 For 17/30 participants in the ART+V+V arm with pre and 2 hours post vorinostat data, there
383 was no significant difference between pre and post vorinostat CA-RNA levels at the three time-
384 points tested (Table S11).
385

386 *Ex-vivo* vorinostat stimulation led to a statistically significant 3.86-fold increase in mean HIV
387 CA-RNA compared to untreated controls. (Supplementary Figure S6; p=0.009), showing that
388 HIV gene transcription could be stimulated by vorinostat under optimised conditions.
389
390 *Cell-associated p24*
391 Most of the results fell in the range 0.01 to 0.02 AEB ('average amount of enzyme per
392 bead'), regarded as 'undetectable' (Table S12). P24 was above the limit of quantification
393 (>0.02 AEB) in only 4 samples overall, with no difference between the randomization
394 groups: 1/57 at randomization, 1/35 at PR11 (both ART only), and 1/30 in each
395 randomization group at PR16.
396
397 *HIV RNA Single Copy Assay*
398 For 28 participants in the ART+V+V arm, pre and 2 hours post vorinostat SCA results were
399 available (n=22, 20, and 21 at PR visits week 8, 9, 12). At none of the 3 time-points, was there
400 a significant difference in HIV RNA pre and post-vorinostat, and medians pre and post were
401 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
402 3.5-11) copies/mL at PR9 (p=0.76), and 5 (IQR 1-7) and 4 (IQR 2-14) copies/mL at PR12
403 (p=0.11), (Figure S5).
404
405 *Cell viability post vorinostat dosing viability*
406 Samples collected two hours post vorinostat treatment had significantly lower viability when
407 compared to all other samples (p<0.001, medians 65.5% vs 87%, respectively) as well as
408 lower % expected yields (p=0.016, medians 54% vs 64%, respectively).
409
410 ***Safety***

411 There were no deaths or notable events. One SAE of vasovagal syncope likely secondary to
412 blood draw-related venepuncture (ART+V+V arm) was reported post-randomization (Table
413 S13). Clinical adverse events were reported for 29 (97%) participants in the ART+V+V arm
414 and 22 (73%) in the control arm (p=0.026) (Table 2 and Tables S14-16, S22, S23). Most
415 laboratory AE were mild-moderate and similar by arm (Table S17). There was no significant
416 difference in QTc change from PR week 8 between the two arms (Table S18). Solicited general
417 symptoms and injection site reactions in the ART+V+V arm are shown in Figure S8.
418
419 **Discussion**
420 The RIVER trial provides the first randomized controlled assessment of the effect of a 'kick
421 and kill' intervention on measures of the HIV reservoir in study participants with treated recent
422 HIV infection. Despite significantly enhanced vaccine-induced HIV-specific $CD4^+$ and $CD8^+$
423 T-cell responses and evidence for *in vivo* vorinostat-induced HDAC inhibition, there was no
424 difference in any measures of the HIV reservoir between the ART-only arm and the ART+V+V
425 arm. That we were able to demonstrate evidence for an impact of both the 'kick' (increased
426 histone acetylation following vorinostat) and 'kill' (enhanced functional T cell responses
427 induced by vaccination) but no change in measures of the HIV reservoir raises challenging
428 questions.
429
430 In the vaccine-only BCN01 study[17], there was no impact on measures of the HIV reservoir
431 despite potent HIV-specific immune responses in a similar cohort treated in primary
432 infection, suggesting that enhancing the 'kill' alone is not adequate for HIV cure. Our use of
433 the same vaccination approach demonstrated similarly enhanced HIV-specific T-cell
434 immunity. In studies that have solely employed a 'kick', using HDAC inhibition, several
435 have failed to show a convincing impact on HIV reservoir size [11-13, 31]. However, some

436 studies demonstrated induction of viral transcription following single[11,] and multiple daily
437 dosing of vorinostat[12,] (using identical vorinostat dosing strategies to RIVER[21]), supporting
438 the premise that inhibition of histone deacetylation could potentially enhance viral protein
439 presentation to effector T cells. While the magnitude of HDAC inhibition in RIVER was in
440 keeping with previous studies, we did not observe the same increases in viral transcription
441 reported by some groups[9,11-13], although not all[31]. It should be noted that our choice of dosing
442 regime for vorinostat was based on limited data, and further optimization, such as a longer
443 treatment course might have enhanced the impact.
444
445 Three possible explanations are considered to explain this discrepancy: 1) Differential
446 susceptibility to HDAC inhibition. Some previous studies pre-selected participants based on
447 susceptibility to HDAC inhibition due to concerns that sensitivity to viral reactivation varied
448 between individuals[9,11,21]. We chose not to do this as others had demonstrated HDACi
449 induced viral transcription in un-selected participants[12,13]. Of note, *ex vivo* stimulation of
450 $CD4^+$ T-cells from RIVER participants identified responsiveness to vorinostat measured by
451 increased HIV RNA transcript production, making this explanation less likely. 2) The
452 reservoir in primary HIV-infected participants is somehow different from those with treated
453 chronic infection. Previous studies with HDACi have recruited participants with chronic HIV
454 infection[9,11-13,21,31,32] . Compared to PHI, the reservoir in chronic infection is larger,
455 containing greater viral diversity, possible higher levels of basal transcription, more defective
456 proviral sequences, and with variable susceptibility to latency reversal[33]. Although it remains
457 unproven, the heterogeneity in the character of the reservoir may have impacted susceptibility
458 to our intervention. 3) The HDAC inhibitor vorinostat impacted cell viability. We observed a
459 reduction in viability for samples taken two hours after vorinostat dosing, in keeping with
460 other groups[34], which may have contributed to our findings. It is also possible that the 2-hour

461 timepoint chosen to measure HDAC-inhibition and viral transcription (coinciding with peak
462 plasma drug concentration) was too soon to detect significant viral transcription and
463 subsequent data suggest that 8 hours post-dosing might be preferable [12,13].
464
465 Of previous studies using T-cell vaccination with HDACi, the BCN 02 study which enrolled
466 participants with treated PHI is most comparable to RIVER, using identical vaccines[18] with
467 the HDACi, romidepsin[35]. The BCN 02 study investigators more recently demonstated both
468 romidepsin-induced acetylation and viral transcription, as well as enhanced vaccine-induced
469 T-cell responses[36]. Subsequent ART interruption led to post-treatment sustained viral control
470 among three out of 13 (23%) study participants, although in the absence of a control arm, one
471 cannot comment on whether this was driven by the interventions.
472
473 The REDUC study[32] evaluated the combined effects of an HIV *Gag* peptide vaccine,
474 recombinant human granulocyte macrophage colony-stimulating factor and romidepsin on the
475 HIV reservoir. There was no significant impact of the intervention on measures of the reservoir
476 in evaluable patients. There are no other completed RCT testing alternative HIV 'kick and kill'
477 approaches.
478
479 Although the existence of an HIV reservoir has been acknowledged for over 20 years[2,5] there
480 remains no consensus on how best to quantify it or determine whether it has been succesfully
481 targeted by an intervention, and no biomarkers definitively predict viral rebound after stopping
482 ART[37,38]. Accordingly, clinical trials exploring HIV cure strategies often include a variety of
483 HIV reservoir measurements[39], as we have here. Ultimately the only outcome measure of
484 clinical value may be analytical treatment interruption (ATI). Although at the time of writing
485 there is a growing consensus that ATIs can be conducted safely and there is some progress to

486 unifying protocols to do this[40], when RIVER was designed ATIs were still considered high risk
487 due to concerns over harm associated with viral rebound, the risk of onward HIV transmission,
488 uncertainty of individual level clinical and immunological harm and the requirement for
489 frequent viral load monitoring.
490
491 Accordingly, the use of HIV DNA quantitation as a primary endpoint for the RIVER trial may
492 be criticized, with conflicting evidence on its value as a predictor of post-treatment control
493 [7,8,37]. It might be argued that an ATI[38] would have provided an alternative more clinically
494 relevant measure of impact of the intervention, even given the caveats above. We also felt that
495 in the absence of any change in any parameter relating to reservoir size or activation, an ATI
496 would have been unlikely to provide additional information. Although in hindsight this may be
497 a weakness of the design, we maintain that an ATI is unlikely to have demonstrated a different
498 overall result to the one we report in regards to the efficacy of this 'kick and kill' approach.
499 More detailed analysis of "responder" and "non-responder" subgroups is planned which could
500 inform underlying mechanisms and variability.
501
502 The relative timing of the 'kick and kill' intervention tested in this trial may be a further
503 explanation for lack of efficacy. The vaccine dosing schedule, comprising prime and boost
504 separated by an 8-week interval was previously shown to be safe and immunogenic in the
505 BNC01 study[17]. The subsequent timing of vorinostat, with respect to vaccination was based on
506 the estimated kinetic of $CD8^+$ T-cell responses, with the peak coinciding with vorinostat-
507 induced viral transcription. However whether the viral antigens expressed on latently infected
508 cells as a result of HDACi-induced viral transcription are the same as those epitopes selected
509 by a candidate T-cell vaccine remains unknown. However, this may be over-simplifying a
510 complex issue. Even if it is speculated that vorinostat did induce adequate protein expression

511 in HIV-infected $CD4^+$ T cells, they might still not be killed by vaccine-induced $CD8^+$ T cells
512 because of Nef-mediated HLA class I downregulation, insufficient peptide presentation,
513 presentation of epitopes other than those recognised by vaccine-induced responses, archived
514 mutations in the epitopes targeted by $CD8^+$ T cells, or $CD8^+$ T cell dysfunction or exhaustion.
515

516 RIVER is the first randomized trial in the HIV cure field testing the 'kick and kill' approach in
517 treated PHI. That no difference compared with ART-alone was reported raises the key question
518 of whether the concept is fundamentally flawed or if a significant impact on the reservoir could
519 be induced with different agents. Whilst the findings represent a set-back in some ways, RIVER
520 helps set the standard for how future trials in this research arena might be conducted as new
521 insights have been gained with regard to trial design, the inclusion of community representation
522 and the need for better interventions, and the necessity for clarity regarding the most relevant
523 measures of the reservoir, and probably an ATI. Finally, the outstanding participant and
524 community engagement with RIVER reflects the need for, and deep involvement of, those
525 living with HIV in clinical research aimed at ending the epidemic.
526

527 **Contributors**

528 SF and JF were co-PIs. SF was clinical chief investigator. JF was laboratory lead. AB and
529 WS were the trial statisticians. MP was responsible for developing the trial primary endpoint
530 analysis. LD, JK and HY led the HIV-immunology laboratory endpoints assays. AL, AF,
531 MW and MB were responsible for running the secondary endpoint viral outgrowth assays. SP
532 was the trial physician at the MRC CTU and a site clinical PI. SKdL provided clinical and
533 research oversight for all vaccination visits at the Royal Free Hospital. JFo, JT, AC, MN, MJ
534 were clinical PIs responsible for recruitment. MK and SK were responsible for undertaking
535 the single copy HIV RNA secondary endpoint assay. DK was the community representative

536 for people living with HIV. TH designed the vaccines used in the trial and provided the GMP

537 IMPs with related documentation, and contributed to the trial design. RB was the trial

538 manager. BH, GX and RB from Merck provided the laboratory expertise for pharmacological

539 guidance on HDACi. All authors contributed to the writing and review of the manuscript.

540 The members of the RIVER trial study group are listed in the supplementary materials.

541

542 **Declaration of Interests**

543 The authors have no interests to declare

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