

1 **Gut Microbiome Signatures Linked to HIV-1 Reservoir Size and Viremia Control**

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37 **Abstract**

38

39 **Background**

40 The potential role of the gut microbiome as a predictor of immune-mediated HIV-1 control in the absence of
41 antiretroviral therapy (ART) is still unknown. In the BCN02 clinical trial, which combined the
42 MVA.HIVconsv immunogen with the latency-reversing agent romidepsin in early-ART treated HIV-1
43 infected individuals, 23% (3/13) of participants showed sustained low-levels of plasma viremia during 32
44 weeks of a monitored ART pause (MAP). Here, we present a multi-omics analysis to identify compositional
45 and functional gut microbiome patterns associated with HIV-1 control in the BCN02 trial.

46

47 **Results**

48 Viremic controllers during the MAP (controllers) exhibited higher *Bacteroidales/Clostridiales* ratio and lower
49 microbial gene richness before vaccination and throughout the study intervention when compared to non-
50 controllers. Longitudinal assessment indicated that the gut microbiome of controllers was enriched in pro-
51 inflammatory bacteria and depleted in butyrate-producing bacteria and methanogenic archaea. Functional
52 profiling also showed that metabolic pathways, including methanogenesis and carbohydrate biosynthesis,
53 were significantly decreased in controllers. Fecal metaproteome analyses confirmed that baseline functional
54 differences were mainly driven by *Clostridiales*. Participants with high baseline *Bacteroidales/Clostridiales*
55 ratio had increased pre-existing immune activation-related transcripts. The *Bacteroidales/Clostridiales* ratio as
56 well as host immune-activation signatures inversely correlated with HIV-1 reservoir size.

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58 **Conclusions**

59 This proof-of-concept study suggests the *Bacteroidales/Clostridiales* ratio as a novel gut microbiome
60 signature associated with HIV-1 reservoir size and immune-mediated viral control after ART interruption.

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74 **Background**

75 A major obstacle to HIV-1 cure is the persistence of viral reservoirs. This mainly refers to latently-infected
76 cells carrying transcriptionally-silent, replication-competent viruses which evade antiretroviral therapy (ART)
77 as well as immune-mediated clearance[1–3]. The immune system is generally unable to contain HIV-1
78 replication in the absence of ART[4]. However, up to 10-20% of subjects that initiate ART within first weeks
79 after HIV-1 acquisition may temporarily achieve HIV-1 viremia suppression after ART interruption (ATI)[5].
80 Understanding the mechanisms behind immune-mediated viremia control after ATI is key to progress towards
81 a functional HIV cure. Broader and higher-magnitude CTL (cytotoxic T-lymphocyte) responses against less
82 diverse HIV-1 epitopes[6,7] in the context of favorable HLA class I genotypes[8] and smaller HIV-1 reservoir
83 size[9] have all been related to such post-treatment HIV-1 control.

84 There is indirect evidence that the gut microbiome might also contribute to immune-mediated control of HIV-
85 1 replication[10,11]. Vaccine-induced gut microbiome alterations, consisting in lower bacterial diversity and
86 negative correlation between richness and CD14⁺DR⁻ monocytes in colorectal intraepithelial lymphocytes,
87 have been recently associated with HIV/SIV (SHIV) protection in a non-human primate challenge study after
88 mucosal vaccination with HIV/SIV peptides, modified vaccinia Ankara–SIV and HIV-gp120–CD4 fusion
89 protein plus adjuvants through the oral route[12]. In the HVTN 096 trial[13] , where the impact of the gut
90 microbiota on HIV-specific immune response to a DNA-prime, poxvirus-boost strategy in human adults was
91 assessed, baseline and vaccine-induced gp41-reactive IgG titers were associated with different microbiota
92 community structures, in terms of richness and composition[14]. In particular, co-occurring bacterial groups,
93 such as *Ruminococcaceae*, *Peptoniphilaceae*, and *Bacteroidaceae*, were associated with vaccine-induced IgG
94 response and inversely correlated with pre-existing gp41 binding IgG antibodies, suggesting that the
95 microbiome may influence the immune response and vaccine immunogenicity[15]. Further evidence emerged
96 from other studies in typhoid Ty21[16], rotavirus[17] and oral polio virus, tetanus-toxoid, bacillus Calmette-
97 Guérin and hepatitis B immunization strategies[18], in which specific gut microbiome signatures
98 (*Bifidobacterium*, *Streptococcus bovis* and *Clostridiales*, respectively) positively correlated with vaccine-
99 induced immune response. In the absence of immune correlates of viral control, HIV cure trials usually
100 incorporate an ART interruption phase to address the efficacy of a therapeutic intervention[19]. Data on the
101 role of gut microbiome composition in the responsiveness to a curative strategy and the relationship with viral
102 control after ART interruption are lacking. The BCN02 study[20] was a single-arm, proof of concept “kick &
103 kill” clinical trial evaluating the safety and the *in vivo* effects of the histone deacetylase inhibitor romidepsin
104 given as a latency reversing agent[21] in combination with a therapeutic HIV vaccine (MVA.HIVcons) in a
105 group of early-ART treated HIV-1-infected individuals[22,23]. During a monitored ART interruption (MAP),
106 23% of individuals showed sustained viremia control up to 32 weeks of follow-up.

107 Here, we aimed to identify salient compositional and functional gut microbiome patterns associated with
108 control of HIV-1 viremia after ART interruption in the “kick & kill” strategy used in the BCN02 study.

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111 **Materials and Methods**

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113 **Study design**

114 This was a sub-study derived from the BCN02 clinical trial (NCT02616874). The BCN02 was a multicenter,
115 open-label, single-arm, phase I, proof of concept clinical trial in which 15 HIV-1-infected individuals with
116 sustained viral suppression who started ART within the first six months after HIV transmission were enrolled
117 to evaluate the safety, tolerability, immunogenicity and effect on the viral reservoir of a kick&kill strategy
118 consisting of the combination of HIVconsv vaccines with romidepsin[20] (Additional Figures: Figure S1a).
119 Fifteen individuals enrolled in the BCN02 trial (procedures for recruitment and eligibility criteria are detailed
120 elsewhere[20]) were immunized with a first dose of MVA.HIVconsv (MVA1, 2×10^8 pfu intramuscularly),
121 followed by three weekly-doses of romidepsin (RMD₁₋₂₋₃, 5 mg/m² BSA intravenously) and a second boost of
122 MVA.HIVconsv (MVA2, 2×10^8 pfu intramuscularly) four weeks after the last RMD₃ infusion. To assess the
123 ability for viral control after ART interruption, participants underwent a monitored antiviral pause (MAP), 8
124 weeks after the second vaccination (MVA2), for a maximum of 32 weeks or until any ART resumption
125 criteria were met (plasma viral load $> 2,000$ copies/ml, CD4⁺ cell counts < 500 cells/mm³ and/or
126 development of clinical symptoms related to an acute retroviral syndrome[20]). The study was conducted
127 between February 2016 and October 2017 at two HIV-1 units from university hospitals in Barcelona (Hospital
128 Germans Trias i Pujol and Hospital Clínic) and a community center (BCN-Checkpoint, Barcelona). The
129 microbiome sub-study concept, design and patient information were reviewed and approved by the
130 institutional ethical review board of the participating institutions (Reference Nr AC-15-108-R) and by the
131 Spanish Regulatory Authorities (EudraCT 2015-002300-84). Written informed consent was provided by all
132 study participants in accordance to the principles expressed in the Declaration of Helsinki and local personal
133 data protection law (LOPD 15/1999).

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135 **Sample disposition and data analysis**

136 Fourteen participants from the BCN02 trial consented to participate in the BCN02-microbiome study, one was
137 excluded due to a protocol violation during MAP and thirteen were included for multi-omics analyses. Twelve
138 from the thirteen participants that finalized the “kick & kill” intervention completed the MAP phase (n=3
139 controllers and n=9 non-controllers) and one subject (B07) did not enter the MAP period due to immune
140 futility pre-defined criteria and absence of protective HLA class I protective alleles associated with natural
141 HIV-1 control (Additional Figures: Figure S1b). Based on the gut microbiome similarity with non-controllers
142 at study entry and over the “kick & kill” intervention, the participant B07 was included in the non-controller
143 arm to increase the statistical power in this microbiome sub-study.

144 Fecal specimens were longitudinally collected at BCN02 during the intervention period at study entry (pre-
145 Vax), 1 week after 1st vaccination (MVA1), 1 week after RMD₃ (RMD) and 4 weeks after 2nd vaccination
146 (MVA2). Samples were also collected over the MAP period (from 4 to 34 weeks after ART interruption) and

147 24 weeks after ART resumption (Additional Figures: Figure S1a). All samples were processed for shotgun
148 metagenomics analysis. Taxonomical classification, microbial gene content and functional profiling were
149 inferred using Metaphlan2[24], IGC reference catalog[25] and HUMAnN2[26], respectively. Sequencing
150 analysis and quality control of metagenomics data are provided in the Additional Results section (Additional
151 Text). To facilitate the interpretation, longitudinal time points were schematically grouped into three phases
152 (Additional Figures: Figure S2a). Fecal material, peripheral blood mononuclear cells (PBMC) and plasma
153 samples were also sampled at baseline to assess fecal metaproteome, host transcriptome profiles and soluble
154 inflammation biomarkers, respectively (Additional Figures: Figure S2b). Microbial proteins from fecal
155 samples were measured by mass spectrometry and protein identification performed using Mascot search
156 engine (v2.4, Matrix Science) and Scaffold Q+ software (v4.9.0, Proteome Software)[27]. PBMC
157 transcriptomes were evaluated using RNA-sequencing and sequence reads aligned to the human reference
158 genome by STAR v2.5.3a[28]. Read counts estimation was inferred using RSEM v1.3.0[29] and differential
159 expression analysis performed by DESeq2[30]. Plasma proteins were estimated using the Proximity Extension
160 Assay based on the Olink Inflammation Panel[31]. Correlations between 'omic' datasets were computed using
161 Spearman's correlation coefficients and integrative multi-omics analysis was assessed based on the mixOmics
162 R package[32]. A detailed description of wet-lab procedures, bioinformatic methods and statistical analysis of
163 metagenome, metaproteome, transcriptome, soluble plasma markers and multi-omics data is available in the
164 Additional Methods section (Additional Text).

165

166 **Results**

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168 **Patient characteristics**

169 In this microbiome sub-study, we evaluated 13 participants of the BCN02 study. Three had sustained plasma
170 HIV-1 viremia (<2,000 copies/ml) during 32 weeks of MAP (viremic controllers), whereas 9 developed HIV-
171 1 RNA rebound (>2,000 copies/ml) during MAP (non-controllers). One additional subject (B07) did not
172 qualify for MAP due to pre-specified immune futility criteria and absence of protective HLA alleles, and
173 therefore, was also considered a non-controller in this microbiome study. (Additional Figures: Figure S1b).
174 Study participants were predominantly MSM (92%) of Caucasian ethnicity (92%), with median age of 42
175 years and median body mass index of 22.9 kg/m² (Table 1). Median baseline CD4⁺ T-cell counts were 728
176 (416-1408) cells/ mm³ and median CD4/CD8 T-cell ratio was 1.4 (0.97-1.9). All subjects had been on
177 integrase strand-transfer inhibitor-based triple ART for >3 years, begun during the first 3 months after HIV-1
178 infection. Median baseline HIV-1 proviral DNA was 140 copies/10⁶ CD4⁺ T-cells, being numerically lower in
179 controllers than in non-controllers (65 vs 165 copies/10⁶ CD4⁺ T-cells, *p*=0.29).

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181 **Baseline gut-associated *Bacteroidales* / *Clostridiales* ratio discriminates between viremic controllers and** 182 **non-controllers**

183 Viremic controllers had significantly higher *Bacteroidales* levels than non-controllers at study entry (pre-Vax)
184 ($p = 0.007$) and during all the intervention phase (after the first MVA dose: MVA1 $p = 0.049$, after the three
185 romidepsin doses: RMD $p = 0.049$ and after the second MVA dose: MVA2, $p = 0.014$) (Fig. 1a, Additional
186 Figures: Figures S3-S4) as well as lower *Clostridiales* ($p = 0.014$) levels before vaccination (Fig. 1b,
187 Additional Figures: Figures S3-S4). The *Bacteroidales/Clostridiales* ratio remained significantly higher in
188 controllers throughout the intervention (pre-Vax, $p = 0.007$ and MVA2, $p = 0.028$) (Fig. 1c). In addition, non-
189 controllers were enriched in *Erysipelotrichales* and *Coriobacteriales* (Additional Figures: Figure S4a) and
190 showed significantly higher *Methanobacteriales* levels (Additional Figures: Figure S4b). More detailed
191 analyses at lower taxonomic levels using the LEfSe algorithm (Additional Figures: Figure S5) showed that
192 controllers were mainly enriched in *Prevotella copri*, as well as in *Haemophilus pittmaniae* and *Streptococcus*
193 *parasanguinis*. In comparison, non-controllers were enriched in the *Clostridiales* species *Eubacterium rectale*
194 and *siraeum*, *Subdoligranulum spp.* *Coprococcus*, and *Dorea longicatena*, as well as in *Collinsella*
195 *aerofaciens* and *Methanobrevibacter spp.* A longitudinal analysis using the ‘feature-volatility’ function from
196 qiime2 (Additional Figures: Figure S6) showed that such differences were sustained over the whole
197 intervention period. All 9 non-controllers analyzed here resumed ART by week 4 after the MAP initiation,
198 whereas the 3 controllers remained off ART for at least 28 weeks and up to 32 weeks. During the MAP,
199 *Bacteroidales* showed an initial increase up to week 4 followed by a reduction by weeks 8-12 in controllers
200 (Fig. 1a). Inversely, *Clostridiales* levels increased by weeks 8-12 and remained stable thereafter (Fig. 1b) (no
201 statistical support provided during MAP). No significant differences in bacterial composition were found
202 following ART resumption; however, there was a limited sample availability at ART resumption phase (Figs.
203 1a-c).

204

205 **Viremic controllers display lower gut microbial diversity and richness over the intervention**

206 Controllers had lower microbial gene counts than non-controllers at the study entry and throughout the study
207 intervention, although such differences lost statistical significance in the RMD and MVA2 assessments (Fig.
208 2a). Intra-individual diversity (Shannon index) also remained numerically lower in controllers, but differences
209 were not statistically significant (Fig. 2b). During the MAP, gut microbial diversity increased around weeks 8-
210 10 in controllers, and remained stable thereafter (Figs. 2a-b). No statistically significant differences were
211 found in microbial diversity following ART re-initiation (Figs. 2a-b). Using the Bray-Curtis index, controllers
212 exhibited lower beta-diversity and higher similarity, particularly already at the study entry (Additional
213 Figures: Figure S7), and showed less intra-host longitudinal evolution (Fig. 2c) than non-controllers
214 (PERMANOVA, $r^2 = 0.591$, $p = 0.001$). Whereas the gut microbiome composition of controllers was
215 significantly different from that of non-controllers (PERMANOVA, $r^2 = 0.112$, $p = 0.001$), no significant
216 longitudinal differences were observed within each group (PERMANOVA, $r^2 = 0.043$, $p = 0.815$), suggesting
217 that the combined intervention with MVA.HIVconsv vaccines and three weekly low-dose infusion of
218 romidepsin did not significantly alter the gut-microbiome composition (Fig. 2c). Of note, results did not
219 change after removing B07 from the non-controller arm (Additional Figures: Figure S8).

220

221 **Longitudinal microbial metabolic pathways differ between viremic controllers and non-controllers**

222 Functional profiling based on HUMAnN2[26] identified 28 differential metabolic pathways between
223 controllers and non-controllers at study entry (unadjusted $p < 0.05$, Wilcoxon test) (Additional Figures: Figure
224 S9 and S10a). Twelve out of the 28 pathways identified at study entry were differentially abundant throughout
225 the intervention (Additional Figures: Figure S10b). Controllers were enriched in ‘fatty acid and lipid
226 biosynthesis’ and ‘amino acid biosynthesis’ pathways. Conversely, metabolic pathways overrepresented in
227 non-controllers included ‘methanogenesis from H₂ and CO₂’, ‘carbohydrate biosynthesis’ and ‘generation of
228 precursor metabolite and energy’. Longitudinal variations of such metabolic pathways during MAP and after
229 ART re-initiation are shown (Additional Figures: Figure S11), although low numbers did not allow for
230 statistical testing. The ‘methanogenesis from H₂ and CO₂’ pathway was the most discriminant feature between
231 the two groups (fold-change=11.5, $p=0.04$). Consistently, methanogenic archaea (*Methanobrevibacter smithii*
232 and *Methanospaera stadtmanae*) were detected in most non-controllers and were rare or absent in controllers
233 (Additional Figures: Figure S12). Taken together, these data show that differences between controllers and
234 non-controllers emerged from resident microbial communities, before any intervention was started in BCN02
235 study. Thus, subsequent analyses were focused on characterizing further potentially discriminant signatures at
236 study entry.

237

238 **Increased *Bacteroidales/Clostridiales* ratio in viremic controllers negatively correlated with longitudinal**
239 **HIV-1 viral reservoir size**

240 The *Bacteroidales/Clostridiales* ratio inversely and significantly correlated with longitudinal total CD4⁺ T
241 cell-associated HIV-1 DNA measured at study entry ($\rho = -0.6, p = 0.03$) and over the “kick & kill”
242 intervention, whereas an opposite trend was observed for gene richness ($\rho = 0.65, p = 0.01$ at study entry)
243 (Fig. 3a). Alpha-diversity (Shannon index) exhibited weak positive correlation with the viral reservoir, being
244 the correlation not significant. In line with the gut microbiota patterns found in controllers, the ratio
245 *Bacteroidales/Clostridiales* showed a strong negative correlation with gene richness ($rho = -0.87, p = 0.0001$) (Fig. 3a). In the longitudinal comparison, controllers tended to display lower viral
246 reservoir size, although differences were not statistically significant (Fig. 3b). A similar trend was observed
247 for cell-associated (CA) HIV-1 RNA (Figs. 3c-d), although stronger correlations were found at RMD and
248 MVA2 timepoints with both *Bacteroidales/Clostridiales* ratio (RMD; $\rho = -0.76, p = 0.002$ and MVA2; $\rho = -0.74, p = 0.003$) and gene richness (RMD; $\rho = 0.72, p = 0.005$ and MVA2; $\rho = 0.71, p = 0.006$) (Fig. 3c).
249 Moreover, in the longitudinal comparison, controllers displayed significantly lower CA HIV-1 RNA at RMD
250 and MVA2 ($p = 0.03$) (Fig. 3d). A set of clinical and vaccine-response variables was screened for association
251 with gut microbial signatures. Absolute CD4⁺ T-cell count before ART initiation was the only factor
252 significantly associated with the *Bacteroidales/Clostridiales* ratio ($\rho = 0.65, p = 0.01$) and gene richness
253 ($rho = -0.62, p = 0.02$), whereas a strong and inverse correlation was found between the Shannon index
254 and CD4/CD8 ratio at BCN02 study entry ($\rho = 0.9, p = 2.83e-05$) (Additional Figures: Figure S13).

257

258 **Distinct bacterial protein signatures associated with viremia control**

259 Baseline metaproteome analysis identified 15,214 bacterial proteins, annotated to 24 unique orders and 69
260 genera across samples. The abundance of total *Clostridiales* or *Bacteroidales* was not different between
261 groups (Figs. 4a-c). However, several *Clostridiales* genera were decreased in controllers, i.e.: *Eubacterium* (-
262 3.71%; $p=0.03$), *Pseudoflavonifractor* (-0.49%; $p=0.049$), *Oscillibacter* (-0.14%; $p=0.07$), whereas *Blautia*
263 was increased (+5.02%; $p=0.03$) (Fig. 4a). At the genus level, the relative abundance of *Erysipelotrichales* (-
264 0.28%; $p=0.07$), and *Coprobacillus* (-0.22%; $p=0.07$) showed a decreasing trend in controllers, although
265 differences were not statistically significant (Fig. 4b). Unbiased hierarchical clustering showed protein
266 differences ($p<0.025$) between groups (Fig. 4d). Viremic controllers were enriched in bacterial proteins from
267 *Blautia* and *Ruminococcus*, and depleted in proteins derived from other *Clostridiales* such as *Clostridium*,
268 *Eubacterium*, *Coprococcus*, *Faecalibacterium*, *Oscillibacter*, and *Pseudoflavonifactor*. Pathways associated
269 with *Blautia* included galactose, starch/sucrose and glyoxylate/dicarboxylate metabolism as well as ribosome
270 activity. (Fig. 4e). Butyrate and other short-chain fatty acid metabolism pathways were similar in both groups
271 (Figs. 4d-e).

272 **Increased baseline immune activation and inflammatory response gene expression in viremic
273 controllers**

274 Full-PBMC gene expression analysis detected a total of 27,426 transcripts at baseline, after filtering for low-
275 expressed genes. Using DESeq2[30], a total of 31 differentially expressed genes (DEGs) were identified (\log_2
276 FoldChange = 0 and BH-adjusted p -value < 0.1), of which 15 and 16 were upregulated in controllers and non-
277 controllers, respectively (Fig. 5a). The proportion of protein-coding genes, pseudogenes, non-coding
278 transcripts, TEC (genes to be experimentally confirmed), and long-intergenic noncoding genes within DEGs
279 was 61.29%, 12.90%, 12.90%, 9.68 and 3.23%, respectively (Additional Table 1). We found 10 and 3 DEGs
280 showing \log_2 FC above 2 and 4, respectively; moreover 8 out 10 genes with \log_2 FC >2 at a Benjamini-
281 Hochberg-adjusted p -value significance < 0.05 (Fig. 5a and Additional Table 1). Hierarchical clustering based
282 on transcriptional DEG profiles showed that controllers grouped together, while non-controllers separated into
283 two distinct expression groups (Additional Fig. 13a). Upregulated genes in non-controllers included 11
284 transcripts with unknown function (Additional Table 2), which were excluded from downstream analyses. The
285 most statistically differentially-expressed gene was myeloperoxidase (*MPO*, adjusted p -value = 1.38e-06),
286 followed by a member of the folate receptor family (*FOLR3*, adjusted p -value = 2.68e-05) (Additional Figs.
287 13b-c and Additional Table 2). Interestingly, both neutrophil-related transcripts *MPO* and *FOLR3* were
288 upregulated in controllers (Fig. 5b) and have been implicated in immune response signaling and regulation of
289 inflammatory processes[33,34]. Other transcripts with known associations to host defense and neutrophil-
290 mediated immunity and higher expression in controllers included defensin alpha 1 and 4 (*DEFA1*, *DEFA4*),
291 bactericidal permeability-increasing protein (*BPI*), cathepsin G (*CTSG*) and neutrophil elastase (*ELANE*) (Fig.
292 5b and Additional Table 2). Gene Ontology (GO) enrichment analysis identified a number of biological
293 processes associated with genes highly expressed in controllers (Additional Table 3). To reduce redundancy,

294 the complete list of GO terms was collapsed into representative subclasses using REVIGO. Many of these
295 functions were associated with immune response, such as neutrophil-mediated immunity, leukocyte
296 degranulation and antimicrobial humoral response (Fig. 5c).

297

298 **Increased baseline inflammation-related plasma proteins in viremic controllers**

299 Soluble factors in plasma from the 92 inflammation-related protein panel were assessed using the Proximity
300 Extension Assay (see Methods in Additional Text). Plasma protein levels did not independently separate
301 controllers from non-controllers using unbiased hierarchical clustering or principal component analysis
302 (Additional Figs. 14a-b). Of the 92 plasma proteins characterized, only 7 were differentially expressed
303 (Wilcoxon, uncorrected $p < 0.05$) and were increased in controllers (Additional Fig. 14c): adenosine deaminase
304 ADA ($p = 0.012$), decoy receptor osteoprotegerin OPG ($p = 0.024$), self-ligand receptor of the signaling
305 lymphocytic activation molecule family SLAMF-1 ($p = 0.048$), chemokines CCL23, CCL28, MCP-2 ($p =$
306 0.048) and the neurotrophic factor NT-3 ($p = 0.048$) (Additional Fig. 14d).

307

308 **Integration analysis between *Bacteroidales/Clostridiales* ratio, host immune activation-related
309 transcripts, bacterial proteins and HIV-1 reservoir size**

310 The *Bacteroidales / Clostridiales* ratio was positively correlated (Spearman rho=0.55 and q -value < 0.05) with
311 DEGs involved in inflammatory response and immune system activation, including *DEFA1*, *DEFA4*,
312 *TOP1MT*, *CTSG*, *MPO*, *AZU1*, *ELANE* (Fig. 6a), Spearman rho and q value are given in Additional Dataset 1.
313 Functional enrichment analysis including all transcripts significantly correlated with the ratio
314 *Bacteroidales/Clostridiales* (n. transcripts=453, q -value < 0.05 , see Additional Dataset 1) identified four main
315 functional clusters related to neutrophil activation, disruption of cells of other organism, antimicrobial
316 humoral response and regulation of immune response to tumor cell (Additional Fig. 15a and Additional Table
317 4). Of these, the *Bacteroidales / Clostridiales* ratio strongly correlated ($\rho > 0.8$) with transcripts within such
318 functional clusters, which were highly expressed in controllers (Fig. 6b).

319 Moreover, 61 and 70 out 453 transcripts (Additional Dataset 2) correlated ($\rho = 0.5$) with the baseline CA
320 HIV-1 RNA and HIV-1 DNA, respectively, and were enriched in immune-mediated response functions
321 (Additional Figs. 15b-c and Additional Table 5). In the integrated analysis of metagenomic, transcriptomic
322 and metaproteomic data to identify signatures discriminating between controllers and non-controllers,
323 *Bacteroidales* and *Clostridiales* were clearly separated through the component (Additional Fig. 16a). While
324 *Bacteroidales* clustered with immune activation-related transcripts (*MPO*, *AZU1*, *ELANE*, *TCN1*, *DEFA1*,
325 *BPI*, *DEF4*) and proteins from *Ruminococcus*, *Blautia*, *Prevotella* and *Faecalibacterium* genera, *Clostridiales*
326 inversely correlated with these features (Additional Figs. 16a-b). Such associations were assessed at a lower
327 taxonomic scale and confirmed that *Bacteroidales* species (*B. dorei* and *B. eggerthii*) inversely correlated with
328 HIV-1 DNA levels, whereas members of *Clostridiales* (*S. unclassified*, *D. formicigenerans* and *E. siraeum*)
329 positively correlated with HIV-1 DNA and CA HIV-1 RNA levels. The viral reservoir (HIV-1 DNA and CA
330 HIV-1 RNA) negatively correlated with genes involved in ‘neutrophil mediated immunity’, ‘antimicrobial

331 humoral response' and 'cell killing'. Weak correlations were observed with bacterial proteins involved in the
332 regulation of metabolism (Fig. 6c and Additional Dataset 2). Overall, these findings further supported positive
333 associations between *Bacteroidales* abundance and transcripts related to immune response in controllers,
334 which in turn negatively correlated with the viral reservoir size.

335

336 Discussion

337 In this proof-of-concept study, a longitudinal multi-'omics' analysis identified the *Bacteroidales/Clostridiales*
338 ratio as a novel gut microbiome signature associated with HIV-1 reservoir size and viremic control during a
339 monitored ART pause. Individuals with high *Bacteroidales/Clostridiales* ratio showed gene expression
340 signatures related to immune activation, particularly neutrophil-mediated immunity and antimicrobial humoral
341 response, which negatively correlated with the viral reservoir size. Our findings largely arise from
342 unsupervised analyses where many other signatures could have emerged, especially given the relatively low
343 number of individuals analyzed. However, they are internally coherent and consistent with a theoretical
344 framework where increased inflammation might contribute to immune-mediated HIV-1 control. They also
345 suggest a putative biomarker for safer ART interruptions in HIV cure studies.

346 The baseline gut microbiome of controllers was enriched in pro-inflammatory species, such as *P. copri*[35],
347 and depleted in bacteria, traditionally associated with the maintenance of gut homeostasis through production
348 of SCFAs[36], including *R. intestinalis* and *Subdoligranulum spp.* Lower microbial diversity and gene
349 richness in controllers were consistent with a previous work from our group in people living with HIV[37], as
350 well as other studies[38], in which higher gene richness associated with increased levels of butyrate-producing
351 bacteria and methanogenic archaea. Microbial functional enrichment in 'lipid and fatty acid biosynthesis' in
352 controllers might be reflective of mechanisms of lipopolysaccharide biosynthesis and production of
353 inflammatory mediators[39] mediated by members of *Bacteroidales*[40]. No discernible longitudinal
354 variations were observed in the gut microbiome of BCN02 participants, in line with a previous evidence in
355 oral typhoid immunization strategy[16]. Of note, the gut microbiome in healthy population has been described
356 generally resilient to perturbations[41]. Taken together, these observations would suggest a trend toward the
357 maintenance of a relative stability in the gut microbiome composition, with resident microbial communities
358 potentially associated with viral control during ART interruption. Baseline metaproteome analysis confirmed
359 that functional differences between controllers and non-controllers were mainly driven by *Clostridiales*,
360 which were actively producing microbial proteins in both groups albeit in distinct functional contexts. Further
361 discriminating baseline signatures linked to increased immune system activation and inflammatory response
362 in controllers emerged from PBMC transcriptome and inflammation-related plasma proteins profiling. It also
363 emerged that the ratio *Bacteroidales/Clostridiales* inversely correlated with the viral reservoir size in terms of
364 HIV-1 DNA and CA HIV-1 RNA. Although controllers did not display significantly lower viral reservoir size
365 compared to non-controllers, these associations are consistent with previous studies suggesting a role of low
366 viral reservoir on ART interruption outcomes[9].

367 Taken together, these findings suggest that baseline immune activation potentially associated with a microbial
368 shift toward pro-inflammatory bacteria and lower viral reservoir may contribute to sustained post ART
369 interruption HIV-1 control. While there is evidence suggesting a strong impact of the gut microbiota
370 composition on host immune system and inflammatory status[42], the mechanistic basis of how microbial
371 communities may interact with the viral reservoir and, in turn, exert immunomodulatory effects on HIV-1
372 control during ART interruption remains to be delineated. We speculate that a pre-existing, altered balance of
373 ‘beneficial’ gut microbial groups, such as *Clostridiales*, and concomitant overabundance of pro-inflammatory
374 bacteria would boost host immune system activation, thus triggering a prompt control of rebounding virus, as
375 observed in controllers. In support of this hypothesis, increased abundance of members from *Clostridiales*
376 were previously associated with neutrophilia and lower poliovirus and tetanus-hepatitis B vaccine
377 response[18]. Moreover, baseline transcriptional pro-inflammatory and immune activation signatures were
378 suggested as potential predictors of increased influenza[43], systemic lupus erythematosus[44] and hepatitis
379 B[45] vaccine-induced immune response, with weaker responses in elderly[43,45]. It is thus reasonable to
380 postulate that immune activation prior to vaccination together with microbiome-associated factors may affect
381 vaccine outcomes.

382 This study has several limitations. Due to eligibility criteria in the parental BCN02 study, there was a limited
383 sample size, and we were unable to include a control arm without the intervention. Therefore, our
384 considerations were narrowed to three individuals that showed viremic control during ART interruption.
385 Bearing these limitations in mind, our results should be interpreted with caution, emphasizing the need of
386 independent validation in randomized and placebo-controlled trials to assess potentially unmeasured
387 confounders and provide further perspectives on factors that might induce gut microbial shifts. Upcoming
388 analyses in larger longitudinal trials, including the recently reported AELIX-002 trial[46], where fecal
389 samples have been stored longitudinally, are expected to validate our findings. These preliminary findings
390 might have important implications in the design of HIV-1 cure intervention trials that include ART
391 interruption. As proposed for other therapeutic areas[47], microbiome-associated predictive patterns could
392 help to optimize patient stratification, thus resulting in more targeted studies and higher efficacy of HIV-1
393 interventions. In addition, if a given resident microbial community is to be defined that is indeed predictive of
394 viral control during ART interruption, then modulating participants’ gut microbiota before immunization
395 might potentially modulate vaccine responsiveness and ultimately, clinical outcomes. While host-genetics and
396 other vaccine-associated factors as baseline predictors are less amendable, the gut microbiome is potentially
397 modifiable and even transferrable to another host. Strategies manipulating the gut microbiota composition and
398 relative by-products via prebiotics and/or probiotics administration[48] or microbiota engraftment following
399 fecal microbiota transplantation[49] are under intense evaluation[50], albeit with several limitations.

400

401 **Conclusions**

402 In conclusion, in this exploratory study, we identified pre-existing gut microbial and immune activation
403 signatures as potential predictors of sustained HIV-1 control in the absence of ART, providing a potential
404 target for future treatment strategies and opening up new avenues for a functional HIV cure.

405

406 **Availability of data and materials**

407 Datasets supporting the conclusions of this study are available as Additional information (Additional
408 Datasets). Metagenome and RNA-seq data have been deposited in the European Nucleotide Archive
409 (ENA) and are accessible through ENA accession numbers PRJEB42384 and PRJEB43195. The code and
410 databases used for data analysis are available as Additional information (Additional Code) and at
411 10.5281/zenodo.4876340.

412

413 **Abbreviations**

414 **ART:** antiretroviral therapy

415 **ATI:** antiretroviral therapy interruption

416 **HUMAnN2:** HMP unified metabolic analysis network v.2

417 **MAP:** monitored antiretroviral therapy pause

418 **MVA:** MVA.HIVcons

419 **RMD:** romidepsin

420 **SCFA:** short chain fatty acids

421

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541
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574

575 **Author Contributions**

576 R.P, B.M, J.M, B.C, J.M.M and C.B, conceived and designed the study. B.M, R.P, C.M, J.M.M and C.B
577 recruited the study participants and performed their clinical evaluations. M.P and M.C performed fecal DNA
578 extraction, library preparation and sequencing, under the supervision of M.N.J, Y.G and R.P. B.O and C.D
579 performed PBMC transcriptomics and soluble factors determinations, under the supervision of M.R.R and
580 C.B. M.C.P performed the viral reservoir size determinations, under the supervision of J.M.P. L.N.R, M.D.L,
581 S.K and K.B performed fecal metaproteomics experiments and data analysis, under the supervision of A.Bu.
582 A.Bo performed bioinformatics and statistical analyses of metagenome, transcriptome, soluble factors, clinical
583 and integration data, under the supervision of M.N.J and R.P. L.N.R performed the bioinformatics and
584 statistical analyses of fecal metaproteome data, under the supervision of A.Bu. F.C.M, M.N.J, A.Bo, B.O and
585 Y.G contributed to data management. A.Bo and R.P wrote the paper, which was reviewed, edited and
586 approved by all authors.

587

588 **Competing interests**

589 The authors declare no competing interests.

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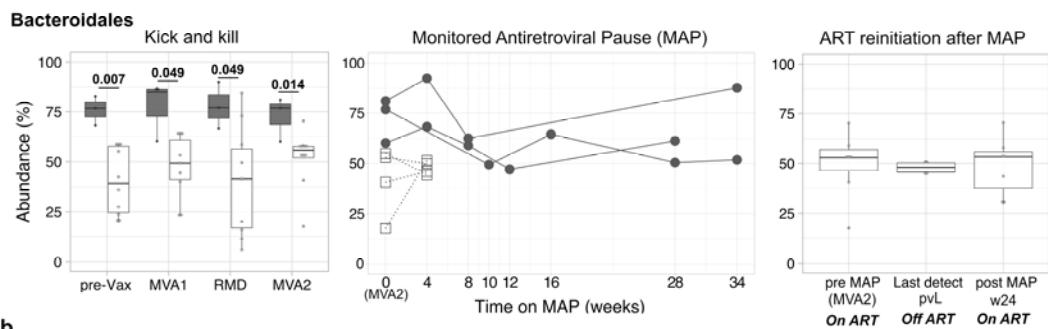
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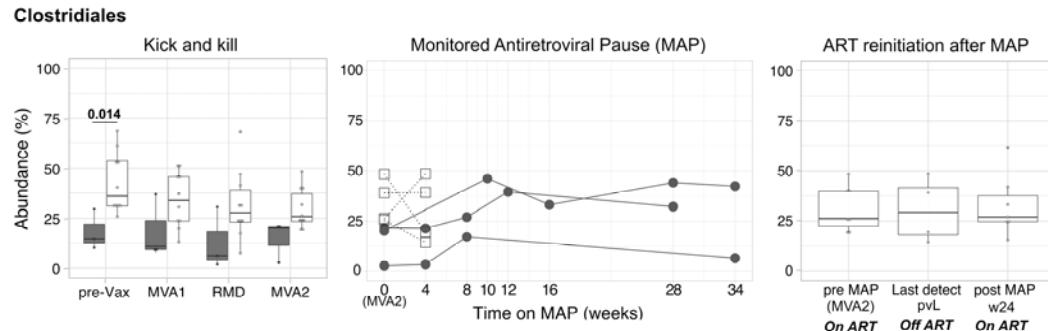
594 **Figures**

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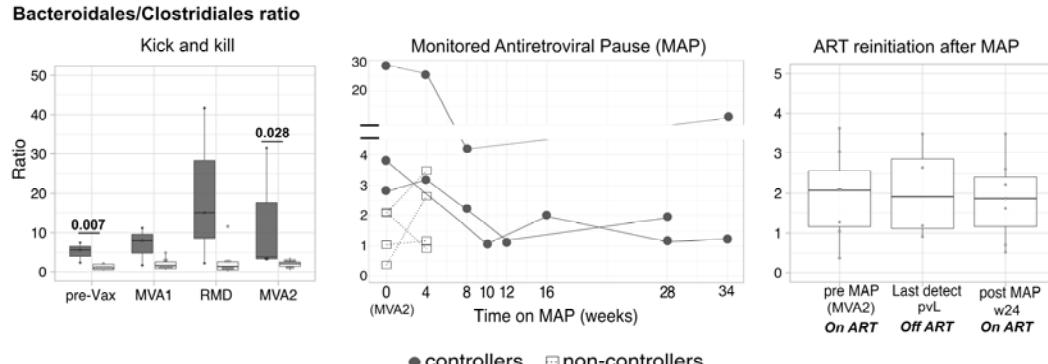
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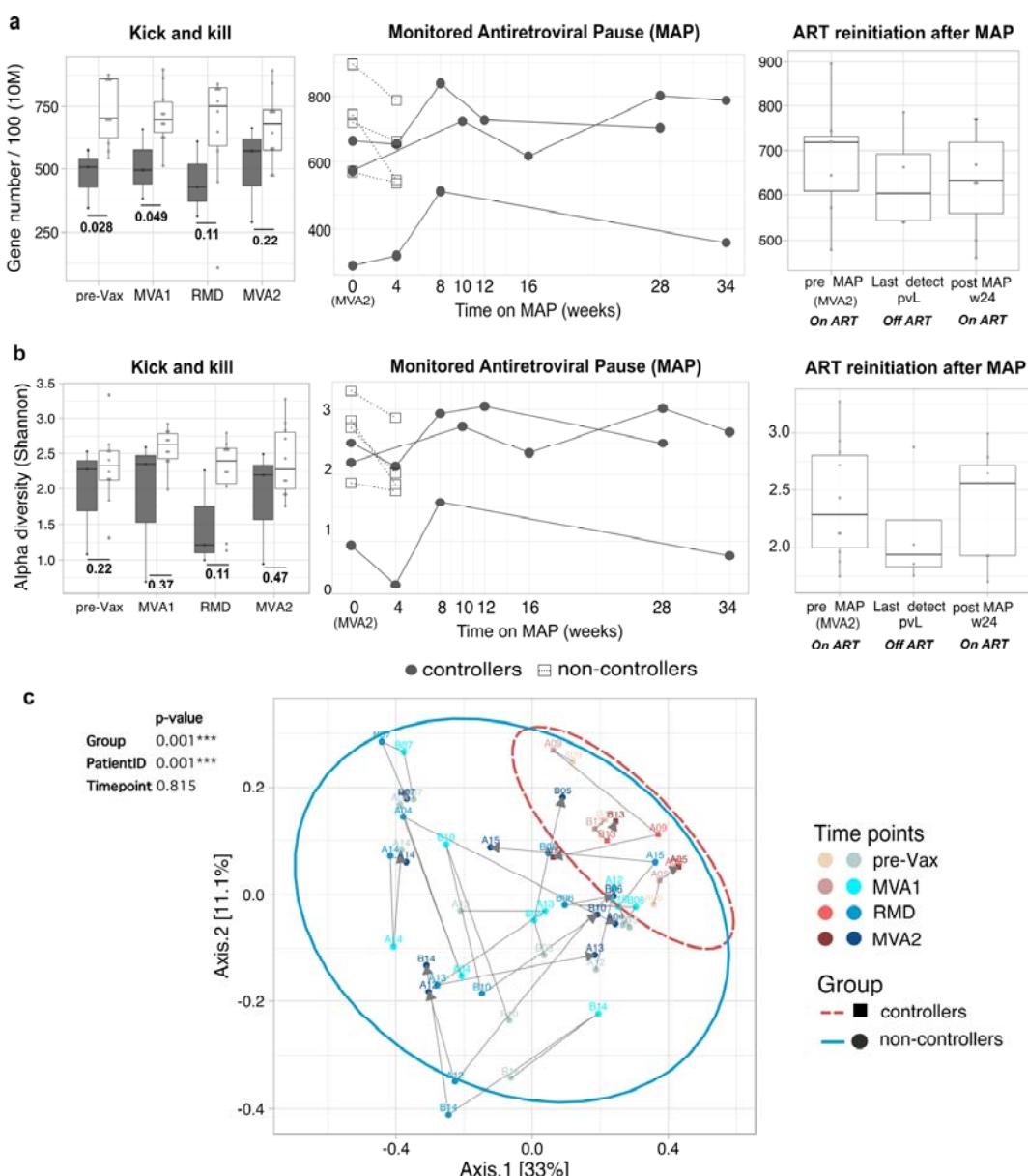


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598 **Fig 1. Higher longitudinal Bacteroidales/Clostridiales ratio in viremic controllers.** Relative abundance
599 expressed as percentage of (a), *Bacteroidales*, (b) *Clostridiales* and (c) their ratio in controllers (gray) and
600 non-controllers (white) are represented by boxplots (left and right vertical panels) and line plots (middle
601 vertical panels). In line plots, values for each subject are illustrated by white squares (non-controllers) and
602 grey dots (viremic controllers). Boxplots show the median (horizontal black line) and interquartile range
603 between the first and third quartiles (25th and 75th, respectively). Third vertical panels show non-controllers
604 before ART interruption (pre-MAP, n=7), last timepoint on MAP before ART resumption (Last detect pVL,
605 n=4) and 24 weeks after ART resumption (post MAP w4, n=7). Abbreviations: MAP, monitored antiretroviral
606 pause; pre-Vax, baseline (1 day before first MVA vaccination); MVA1, 1 week after first MVA vaccination;
607 RMD, 1 week after third romidepsin infusion; MVA2, 4 weeks after second MVA vaccination.



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610 **Fig 2. Lower microbial diversity and richness in controllers.** Longitudinal (a) microbial gene richness at
 611 10 million (10M filtered reads) down-sampling size and (b) alpha diversity based on Shannon index in
 612 viremic controllers (gray) and non-controllers (white). c, Principal coordinates analysis (PCoA) of microbial
 613 diversity based on Bray-Curtis distances at pre-vaccination and during 'kick and kill' intervention. Proportion
 614 of variance explained by each principal coordinate axis is reported in the corresponding axis label. Subjects
 615 per each group are represented by squares (controllers) and circles (non-controllers). Each point stands for one
 616 subject, color coded by group and time point. The increase in purple (controllers) and blue (non-controllers)
 617 colors reflects sequential time points from baseline (pre-Vax) to the second vaccine administration (MVA2).
 618 Ellipses delineate the distribution of points per each group. Gray arrows link directional changes in bacterial
 619 abundance throughout the kick and kill intervention from baseline (pre-Vax). PERMANOVA statistical

620 analysis of samples grouped by Group, PatientID (patient internal identifier) and time point is shown on the
621 top of the panel. Abbreviations: MAP, monitored antiretroviral pause; pre-Vax, baseline (1 day before first
622 MVA vaccination); MVA1, 1 week after first MVA vaccination; RMD, 1 week after third romidepsin
623 infusion; MVA2, 4 weeks after second MVA vaccination.

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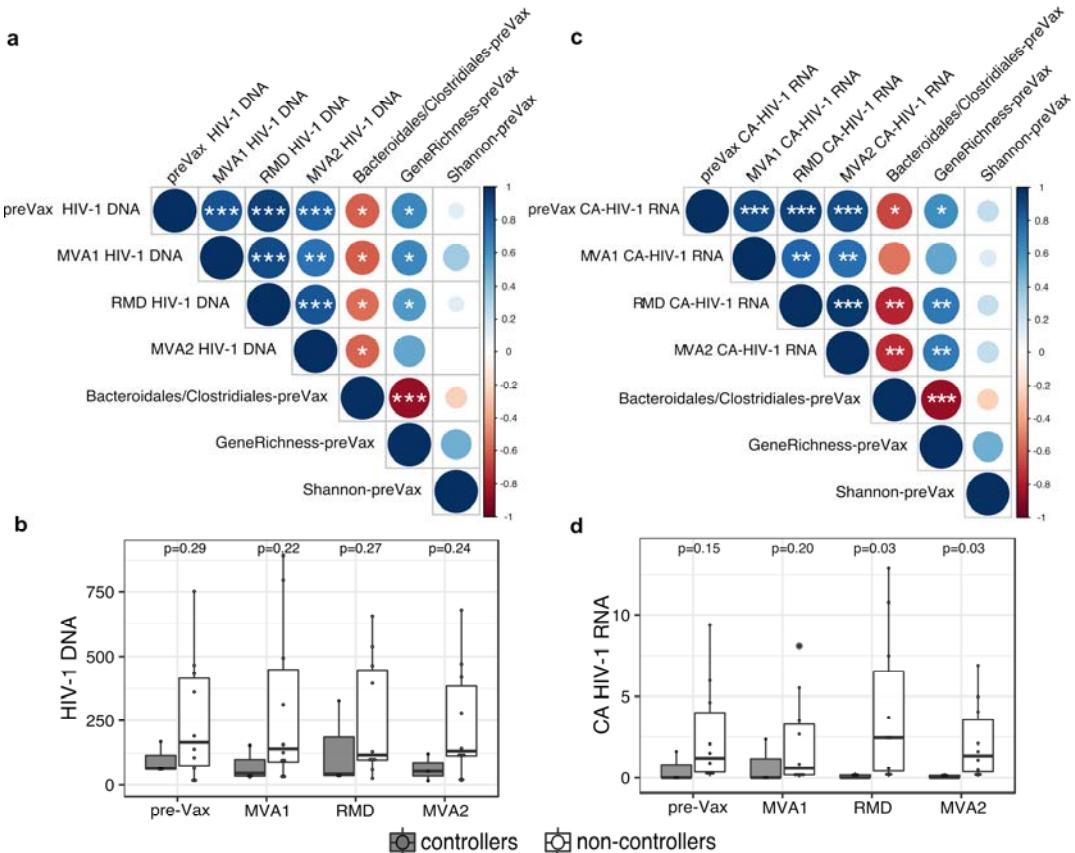
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641 **Fig 3. Associations between HIV-1 reservoir size and gut microbial signatures.** Spearman's correlations
642 between gut microbial signatures (ratio *Bacteroidales/Clostridiales*, gene richness and alpha-diversity
643 Shannon index) and longitudinal (a) HIV-1 DNA (HIV-1 DNA copies/10⁶ CD4⁺ T-cells) and (c) cell-
644 associated (CA) HIV-1 RNA (HIV-1/TBP relative expression). Positive correlations are indicated in blue and
645 negative correlations, in red. Color and size of the circles indicate the magnitude of the correlation. White
646 asterisks indicate significant correlations (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, Benjamini–Hochberg
647 adjustment for multiple comparisons). Boxplots showing longitudinal comparison of (b) HIV-1 DNA and (d)
648 cell-associated (CA) HIV-1 RNA between controllers and non-controllers. Abbreviations: MAP, monitored
649 antiretroviral pause; pre-Vax, baseline (1 day before first MVA vaccination); MVA1, 1 week after first MVA
650 vaccination; RMD, 1 week after third romidepsin infusion; MVA2, 4 weeks after second MVA vaccination.

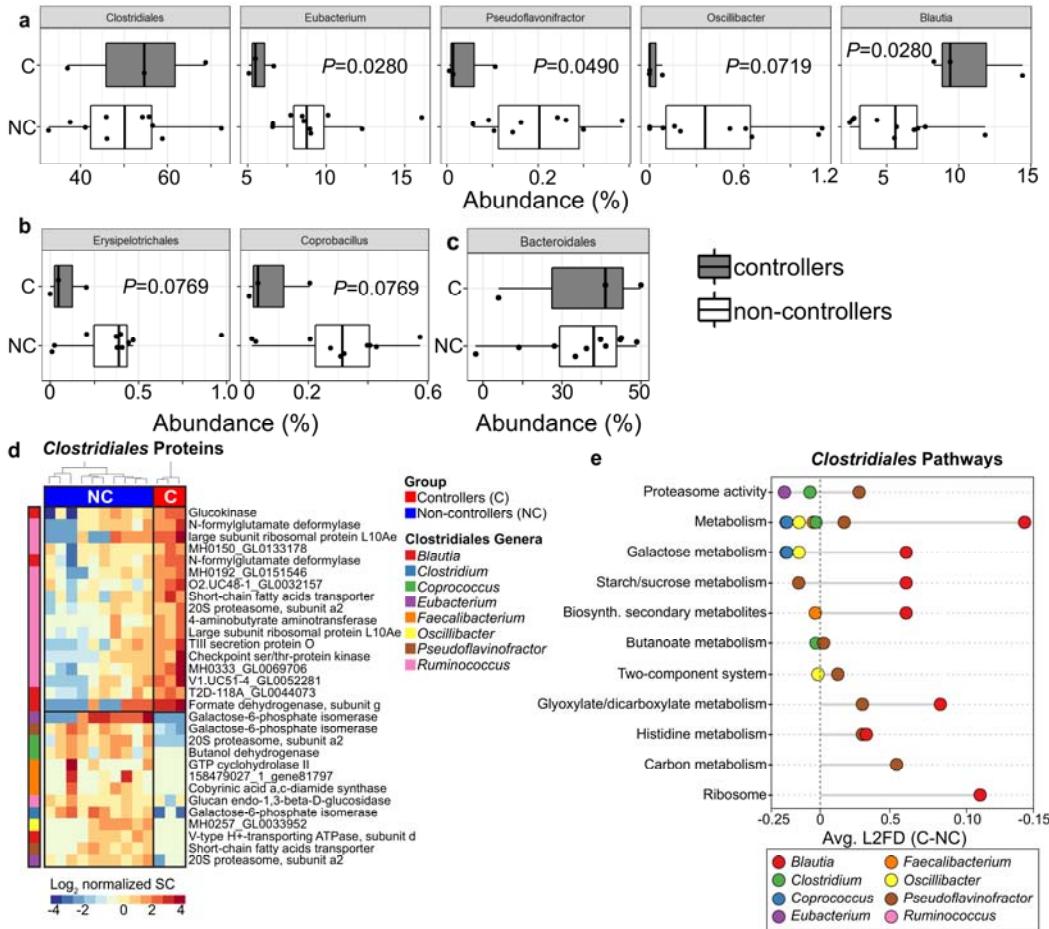
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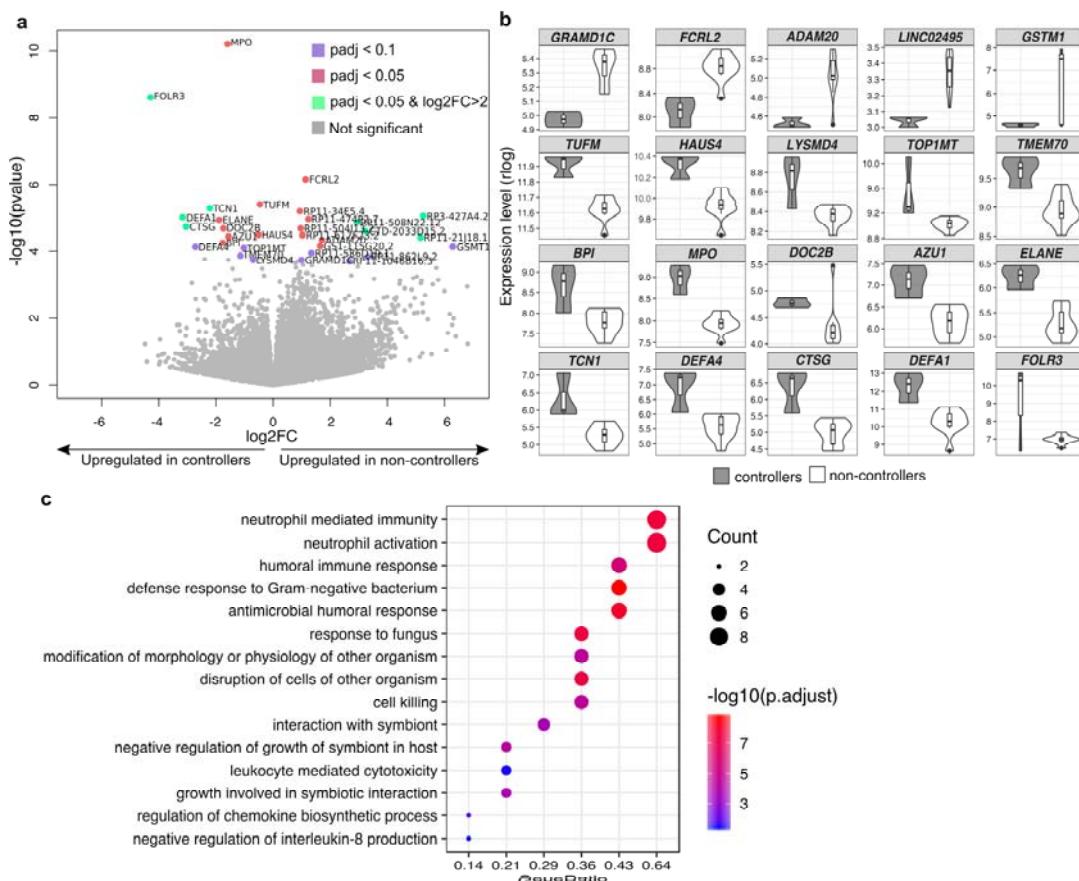


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658 **Fig 4. Baseline metaproteomic signatures associated with HIV control after ART interruption. a-c,**
659 Metaproteomic analysis of gut microbiome in BNC02 participants. Proteins from *Clostridiales* genera and
660 *Erysipelotrichales* were relatively underabundant in controllers compared to non-controllers at baseline pre-
661 vaccination. No differences in *Clostridiales* or *Bacteroidales* proteome at the order-level were observed. **d,**
662 Baseline levels of *Clostridiales* proteins (96 proteins) distinguished controllers from non-controllers.
663 Overabundant proteins belonged to the *Blautia* and *Ruminococcus* genera, while under-abundant proteins
664 belonged to the *Clostridium*, *Coprococcus*, *Eubacterium*, *Faecalibacterium*, *Oscillibacter* and
665 *Pseudoflavinifactor* genera. **e,** Functional annotation of *Clostridiales* bacterial proteins using KEGG gene
666 ontology identified differences in cellular metabolism pathways at baseline between groups. Abbreviations:
667 C=controllers, NC=non-controllers, SC=spectral count.

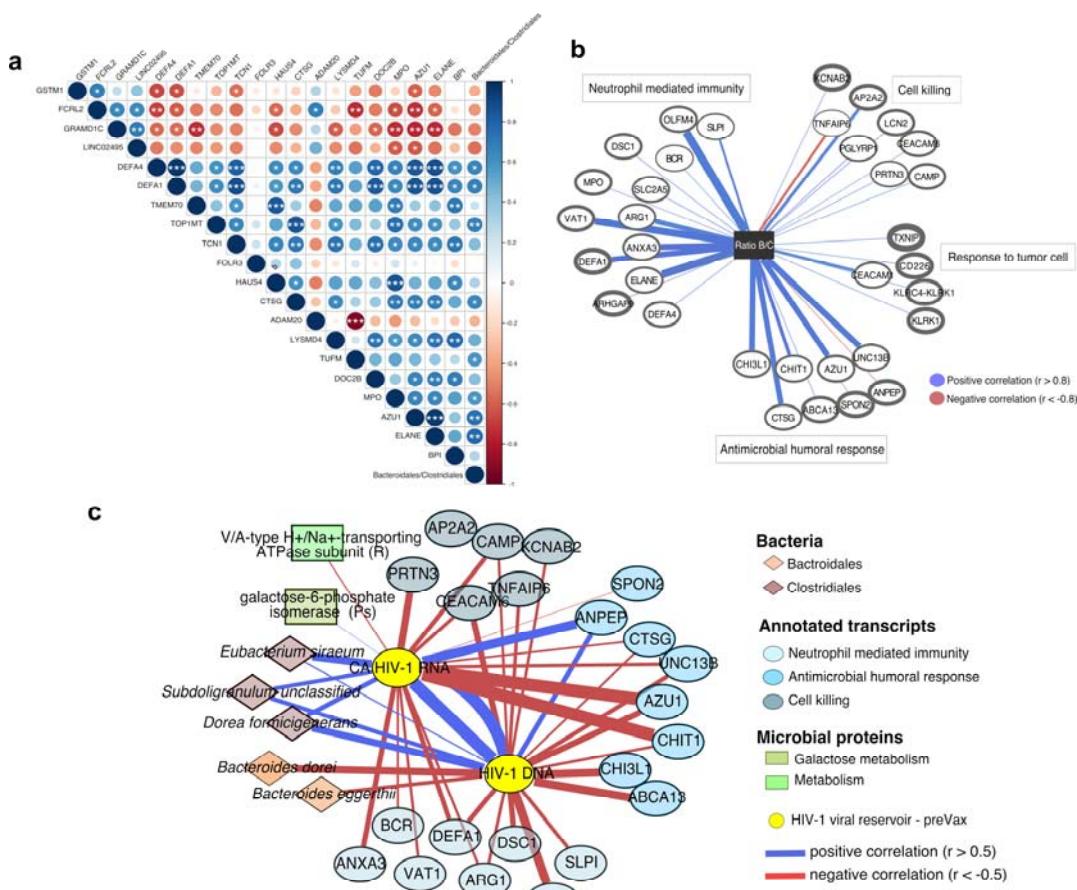
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671 **Fig 5. Baseline functional enrichment in levels of immune activation and inflammatory**
672 **response in viremic controllers. a**, Volcano plot of differentially expressed genes between
673 controllers and non-controllers at baseline (pre-Vax) with adjusted p -value < 0.1 (violet dots),
674 adjusted p -value < 0.05 (red dots) and \log_2 (FoldChange) > 2 and adjusted p -value < 0.05
675 (green dots). Gray-colored dots represent genes not displaying statistical significance (adjusted
676 p -value > 0.1). The \log_2 Fold Change on the x-axis indicates the magnitude of change, while
677 the $-\log_{10}$ (p-value) on the y-axis indicates the significance of each gene. A total of 31 genes
678 showed significant differential expression (adjusted p -value < 0.1): 15 and 16 upregulated in
679 controllers and non-controllers, respectively. **b**, Violin plots showing relative expression levels
680 (rlog, regularized log transformation) of differentially expressed genes with functional
681 annotation. **c**, Gene ontology (GO) enrichment analysis of upregulated genes in Controllers. In
682 the y-axis, only representative enriched GO terms (biological process) are reported (terms
683 obtained after redundancy reduction by REVIGO). X-axis reports the percentage of genes in a
684 given GO terms, expressed as ‘Gene ratio’. The color key from blue to red indicates low to high
685 Bonferroni-adjusted $\log_{10} p$ -value. Dot sizes are based on the “count” (genes) associated to
686 each GO term. Significantly enriched GO terms, number of genes associated to each GO term
687 and adjusted p -values are provided in Additional Table 3.



690 **Fig 6. Integrative analysis of baseline gut microbial signatures, immune activation-related**
691 **transcripts, bacterial proteins and HIV-1 reservoir. a,** Spearman's correlations between the
692 ratio *Bacteroidales/Clostridiales* and DEGs (annotated transcripts). Positive correlations are
693 indicated in blue and negative correlations, in red. Color and size of the circles indicate the
694 magnitude of the correlation. White asterisks indicate significant correlations (* $p < 0.05$; ** $p <$
695 0.01; *** $p < 0.001$, Benjamini–Hochberg adjustment for multiple comparisons). **b,** Network
696 visualizing significant Spearman's correlations between the ratio *Bacteroidales/Clostridiales*
697 and transcripts involved in the enrichment analysis described in Additional Table 4. Transcripts
698 are represented as vertices and border width is proportional to transcript expression (\log_2
699 $|cpmTMM_w0 + 1|$) in controllers. Edge width indicates the magnitude of correlation. Red and
700 blue edges represent positive and negative correlation, respectively. **c,** Network showing
701 Spearman's correlation between viral reservoir (CA HIV-1 RNA and HIV-1 DNA), bacterial
702 species within *Bacteroidales* and *Clostridiales*, human transcripts correlated with the ratio
703 *Bacteroidales / Clostridiales* and differentially abundant bacterial proteins ($p \leq 0.025$). Features
704 are showed as vertices and colored by 'omic' dataset. Positive and negative correlations are
705 presented as blue and red edges, respectively. Edge width indicates the magnitude of correlation

706 coefficient. Protein-associated bacterial genera are reported in parentheses. Abbreviations:
707 DEGs, differentially expressed genes between controllers and non-controllers; R,
708 *Ruminococcus*; Ps, *Pseudoflavonifactor* and pre-Vax, baseline timepoint (1 day before first
709 MVA vaccination).

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744 **Tables**

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Table 1. Study participant demographics and clinical characteristics.

Variable	All participants (n=13)	non-controllers (n=10)	controllers (n=3)
Demographics			
Sex (M/F), <i>n</i>	12/1	9/1	3/0
Risk group (MSM/HTS), <i>n</i>	12/1	9/1	3/0
Ethnic group (Caucasian/Latin), <i>n</i>	12/1	9/1	3/0
Age (years)	42 (39-47)	43 (39-47)	34 (33-38)
BMI (Kg/m ²)	22.9 (20.9-24)	22.3 (21.1-23.4)	24.3 (22.2-25)
Treatment and clinical characteristics			
ART regimen, <i>n</i> (TDF_FTC_RAL/ABC_3TC_RAL/ABC_3TC_DTG)	2/9/2	2/6/2	0/3/0
Viral reservoir (HIV-1 DNA cp/10 ⁶ CD4 ⁺ T-cells)	140 (65-361)	165 (76.2-415.7)	65 (62.5-116.5)
CD4 ⁺ T-cell (cells/mm ³)	728 (648-1182)	839 (581.8-1293.8)	657 (652.5-814)
CD4 ⁺ T-cell (%)	42.9 (42.2-49.3)	43.4 (42.3-48.1)	42.2 (38.4-48.1)
CD4/CD8 T-cell counts ratio	1.4 (1.2-1.6)	1.4 (1.2-1.5)	1.3 (1.1-1.6)

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747 Continuous data are presented using median, 25% and 75% interquartile range, unless otherwise

748 described.

749 M, male; F, female; MSM, men who have sex with men; HTS, heterosexual; BMI, body mass

750 index; ART, antiretroviral therapy; cp, copies; TDF, Tenofovir Disoproxil Fumarate; FTC,

751 Emtricitabine; RAL, Raltegravir; ABC, Abacavir; 3TC, Lamivudine; DTG, Dolutegravir. No

752 statistically significant differences were observed (*p* ≤ 0.05; Wilcoxon rank-sum test

