

1 **More than *Mycobacterium tuberculosis*: specific site-of-disease microbial**
2 **communities, functional capacities, and their distinct clinical profiles in tuberculous**
3 **lymphadenitis**

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29 **Keywords:** Microbiome, Extrapulmonary Tuberculosis, Tuberculous Lymphadenitis, HIV

30 **Word count:** 2995

31 **ABSTRACT**

32 **Background:** Lymphadenitis is the most common extrapulmonary tuberculosis (EPTB)
33 manifestation and a major cause of death. The microbiome is important to human health but
34 uninvestigated in EPTB. We profiled the site-of-disease lymph node microbiome in
35 tuberculosis lymphadenitis (TBL).

36 **Methods:** Fine needle aspiration biopsies (FNABs) were collected from 159 pre-treatment
37 presumptive TBL patients in Cape Town, South Africa. 16S Illumina MiSeq rRNA gene
38 sequencing was done.

39 **Results:** We analysed 89 definite TBLs (dTBLs) and 61 non-TBLs (nTBLs), which had
40 similar α - but different β -diversities ($p=0.001$). Clustering identified five lymphotypes prior to
41 TB status stratification: *Mycobacterium*-, *Prevotella*- and *Streptococcus*-dominant
42 lymphotypes were more frequent in dTBLs whereas a *Corynebacterium*-dominant
43 lymphotype and a fifth lymphotype (no dominant taxon) were more frequent in nTBLs. When
44 restricted to dTBLs, clustering identified a *Mycobacterium*-dominant lymphotype with low α -
45 diversity and other non-*Mycobacterium*-dominated lymphotypes (termed *Prevotella*-
46 *Corynebacterium* and *Prevotella-Streptococcus*). The *Mycobacterium* dTBL lymphotype was
47 associated with HIV-positivity and clinical features characteristic of severe lymphadenitis
48 (e.g., node size). dTBL microbial communities were enriched with potentially
49 proinflammatory microbial short chain fatty acid metabolic pathways (propanoate, butanoate)
50 vs. those in nTBLs. 11% (7/61) of nTBLs had *Mycobacterium* reads.

51 **Conclusions:** TBL at the site-of-disease is not microbially homogenous and distinct
52 microbial community clusters exist that are associated with different immunomodulatory
53 potentials and clinical characteristics. Non-*Mycobacterium*-dominated dTBL lymphotypes,
54 which contain taxa potentially targeted by TB treatment, represent less severe potentially
55 earlier stage disease. These investigations lay foundations for studying the microbiome's
56 role in lymphatic TB and the long-term clinical significance of lymphotypes requires
57 prospective evaluation.

58 250/250

59 INTRODUCTION

60 Tuberculosis (TB), which kills 1.5 million people globally each year (including 214 000
61 people with HIV), causes extrapulmonary tuberculosis (EPTB) ¹. EPTB accounts for ~15% of
62 all TB, and as much as half of all TB in in people living with HIV (PLHIV) in some settings ².
63 EPTB is difficult to diagnose ³ and has high mortality.

64
65 TB lymphadenitis (TBL) is the most common EPTB manifestation, accounting for 70% of
66 EPTB and most frequently affects peripheral and cervical lymph nodes ⁴. TBL occurs after
67 *Mtb* enters the airways, is taken up by phagocytic cells, and transported to a thoracic lymph
68 node where granulomas may form. These steps are also necessary for priming T-cells to
69 generate adaptive immune responses for microbial killing mediated by cytokines and other
70 effector mechanisms ^{5 6}.

71
72 Lymph nodes have an important role in TB pathogenesis: enlargement has been
73 documented following exposure, even if only a fraction of patients with enlarged nodes
74 develop active disease ⁷. Furthermore, *Mtb* DNA is often found in the lymph nodes of
75 exposed yet healthy people. Lymph nodes are therefore hypothesised to serve as a *Mtb*
76 growth and persistence niche ⁷ that can spread to bodily sites ⁸ (in animals lymph node
77 infection almost always accompanies infection in the lungs ^{9 10}); suggesting that TB may
78 primarily be a lymphatic rather than pulmonary disease ¹¹. For example, prior to
79 development of active TB, lymph nodes demonstrate enhanced metabolic activity on PET-
80 CT scans ¹². Together these studies show the lymph nodes have an important role in TB
81 pathogenesis, however, the determinants of why *Mtb* sometimes successfully establishes
82 itself in the lymph nodes and subsequently proliferates, including the potential role of other
83 microbes, is understudied. Key to understanding this is characterizing the local site-of-
84 disease.

85

86 The human microbiome influences immune function ¹³. Two studies assessed lymph node
87 microbial content ^{14 15}, both in mesenteric lymph nodes in Crohn's disease where reduced
88 diversity was observed. The site-of-disease microbiome in TB is underexamined ¹⁶: in
89 bronchoalveolar lavage fluid (BALF), active pulmonary TB was associated with
90 *Mycobacterium* enrichment and *Streptococcus* depletion ^{17 18}.

91

92 The site-of-disease microbiome in TBL (including in HIV-endemic settings where TB is
93 common) remains uncharacterised. Therefore, given the apparent role of the lymph nodes in
94 TB pathogenesis, and the importance of the microbiome as a modulator of immunity, we
95 characterised the site-of-disease lymph microbiome in presumptive TBL patients from a high
96 HIV burden setting before the potentially confounding effects of antibiotic-based TB
97 treatment.

98 **METHODS**

99 **Patient recruitment and follow-up**

100 Presumptive TBL participants (≥ 18 years) were recruited from Tygerberg Academic Hospital
101 in Cape Town, SA (25 January 2017-11 December 2018). Participants were
102 programmatically referred for a routine fine needle aspiration biopsy (FNAB) for the
103 investigation of lymphadenopathy as described ¹⁹. Eligible participants were not on TB
104 treatment within six months. Clinical and demographic data were collected by interview and
105 medical record review. Patients with TBL were programmatically diagnosed with TB, initiated
106 on treatment, and study staff assessed treatment response by telephonic follow-up ≥ 12
107 weeks. The study had no role in patient management.

108

109 **Ethics**

110 Patients provided written informed consent. The study was approved by the Health
111 Research and Ethical Committee of Stellenbosch University (N16/04/050), Tygerberg
112 Hospital (Project ID:4134), and the Western Cape Department of Health
113 (WC_2016RP15_762).

114

115 **Specimen collection and processing**

116 For each patient, two background DNA sampling controls were collected in microcentrifuge
117 tubes prior to lymph node aspiration: a skin swab (collected into saline; Ysterplaat Medical
118 Supplies, Cape Town, South Africa) of the site to be punctured, followed by a saline flush of
119 the syringe to be used for aspiration. Aspiration and microbiological procedures are in the
120 Supplement. Aspirated material from the third pass was collected into 500 μ L sterile saline
121 and stored at -80 °C until batched DNA extraction.

122

123 **Routine specimen testing**

124 Patients were categorised based on lymphatic or non-lymphatic mycobacteriological
125 evidence, provided by the government programmatic laboratory (National Health Laboratory

126 Service [NHLS]), and/or clinical decision to start treatment (**Figure 1**) by responsible clinician
127 thereafter. Case definitions are described in the Supplement (**Table S1**).

128

129 **Microbial DNA extraction and sequencing**

130 DNA was extracted from specimens and controls using the PureLink Microbiome DNA
131 Purification Kit (Invitrogen, Carlsbad, USA). The 16S rRNA gene V4 hypervariable region
132 (150 bp read length) was amplified and sequenced (paired-ends) on the Illumina MiSeq
133 platform. Lymph, skin swab, and 1 in 5 saline flushes were extracted and sequenced.

134

135 **Microbiome data analysis**

136 16S rRNA gene sequences ([Sequence Read Archive](#) PRJNA738676) were processed,
137 denoised and analysed in Quantitative Insights Into Microbial Ecology (QIIME 2, v2020.8) ²⁰
138 and DADA2 ²¹ using closed-reference picking by assigning taxonomy at a 97% similarity
139 against representative sequences in Greengenes (v13.8) ²². QIIME2 outputs (phylogenetic
140 tree, feature table, taxonomy) and metadata imported into R (v3.5.2) and analyses done
141 using *phyloseq* ²³. Shannon's index was calculated using *vegan* ²⁴ to measure α -diversity
142 (within-sample diversity). Bray-Curtis distances were used to measure β -diversity (between-
143 sample diversity) and construct Principal Coordinate Analysis (PCoA) plots. Dirichlet-
144 Multinomial Mixtures (DMM) modelling was done to estimate the optimal number of clusters
145 (microbial community states known as lymphotypes) based on compositional similarity ²⁵.

146

147 **Inferred metagenome**

148 Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
149 (PICRUSt) v.2.1.3-b ²⁶ was used to predict gene family abundance with PICRUSt2 default
150 options (`picrust2_pipeline.py`). The resulting gene table was mapped against the Kyoto
151 Encyclopaedia of Genes and Genomes (KEGG) database, and pathway abundances were
152 inferred from predicted KEGG ORTHOLOGY (KO) abundances.

153

154 **Differential abundance analysis**

155 Differentially abundant taxa and pathways were identified using *DESeq2* (v1.22.2) ²⁷ with
156 Benjamini-Hochberg multiple testing correction. Feature tables were pruned to taxa with
157 $\geq 5\%$ relative abundance in 0.5% of samples ²⁸. Adjusted p-values < 0.2 and < 0.05 was
158 considered significant for taxa and pathways, respectively ([DESeq2 tables](#)). PICRUSt
159 *DeSeq2* outputs were used to identify common pathways in L4 vs. each lymphotype (overall
160 patients), and L3 vs. each other lymphotype (dTBLs).

161

162 **Statistical analyses**

163 Statistical analysis was done in GraphPad Prism Version 7.00 (GraphPad Software, USA)
164 and R. For α -diversity comparisons, the non-parametric Mann-Whitney test or the Kruskal-
165 Wallis ANOVA with Dunn's multiple comparisons was used. For paired comparisons, the
166 Wilcoxon signed rank test was used. Permutational multivariate analysis of variance
167 (PERMANOVA) was computed with 999 permutations to test β -diversity differences and R^2
168 used to measure the proportion variation explained by a variable. For correlation analysis,
169 the non-parametric Spearman ranking and parametric Pearson ranking tests were used. A p-
170 value < 0.05 was significant for all comparisons.

171

172 RESULTS

173 Cohort characteristics

174 We had 89 dTBLs, 61 nTBLs (**Figure 1**) and 9 pTBLs (latter subsequently excluded due to
175 small numbers). dTBLs were more likely to have supraclavicular or head lymph node
176 involvement than nTBLs, if HIV-positive were more likely to have a lower CD4 count (**Table**
177 **1**) and were more likely to have a FNAB that appeared bloody rather than chylous.

178 **Table 1:** Demographic and clinical characteristics of patients with presumptive TBL. dTBLs
179 were more likely to have HIV and a lower CD4 count if HIV-positive, supraclavicular lymph
180 node involvement, and a bloody FNAB. Data are n/N (%) or median (IQR).

	Patients with presumptive TB (n=158)			p-value
	Total (n=158)	dTBL (n=89)	nTBL (n=61)	
Age, years	36 (21-44)	35 (29-40)	38 (30-49)	0.053
Female	85/159 (53)	48/89 (54)	35/61 (57)	0.677
HIV	77/156 (49)	49/89 (55)	23/59 (39)	0.055
CD4+	166 (90-308)	155 (76-251)	250 (139-458)	0.027
CD4+ <200 cells/ μ l	47/77 (61)	32/49 (65)	11/23 (48)	0.159
On ART	38/76 (50)	21/49 (43)	14/22 (64)	0.105
Previous TB	36/156 (23)	24/88 (27)	9/60 (15)	0.078
Tobacco smoking	44/157 (28)	21/89 (24)	22/60 (37)	0.084
Antibiotic use within 1 year of recruitment	41/155 (26)	22/87 (25)	16/60 (27)	0.851
At recruitment	24/41 (59)	10/22 (45)	11/16 (69)	0.154
Lymph node characteristics: sites				
Neck	138/158 (87)	78/89 (88)	55/61 (90)	0.632
Deep anterior cervical	65/138 (47)	36/78 (46)	24/55 (47)	0.774
Deep lateral cervical	25/138 (18)	15/78 (19)	10/55 (18)	0.879
Superficial	15/138 (11)	6/78 (8)	9/55 (16)	0.120
Supraclavicular	19/138 (14)	16/78 (21)	3/55 (5)	0.015
Head	13/138 (10)	4/78 (5)	9/55 (16)	0.032
Thorax	20/158 (13)	11/89 (12)	6/61 (10)	0.632
Axillary (vs. breast)	16/21 (81)	9/11 (82)	3/5 (60)	0.350
Lymph node characteristics: size, cm ²	4 (2-9)	4 (2-9)	4 (4-9)	0.150
Specimen appearance				
Bloody (vs. chylous)	130/158 (82)	66/89 (72)	57/61 (93)	0.003

181

182 Bolded items indicate that p values are significant at p<0.05

183 *8 Probable TBLs (pTBLs) excluded from table

184 Abbreviations: dTBLs: definite tuberculous lymphadenitis; nTBLs: non-tuberculous lymphadenitis; pTBLs: probable-tuberculous;

185 ART: Antiretroviral therapy.

186 †Missing data: HIV (n=2); Specimen appearance (n=3)

187

188 **Lymph microbiome is distinct from background sampling controls.**

189 Lymph fluid had similar α -diversity to background controls (skin, saline) but different β -
190 diversity resulting from an enrichment of *Mycobacterium* (**Figure S1A-D**), suggesting
191 environmental contamination unlikely.

192

193 **Mycobacterium enrichment in dTBLs drives differences with nTBLs**

194 α -Diversity was similar in dTBLs and nTBLs (**Figure 2A**) and, in β -diversity analyses,
195 *Mycobacterium* was the most discriminatory taxon (**Figure 2B-C**) appearing at several fold
196 higher frequencies than in nTBLs (**Figure 2D**). Bray distances within nTBLs were greater
197 than within dTBLs (**Figure 2E**), thus dTBLs were more like each other than nTBLs to each
198 other (likely reflecting the mixture of different disease pathologies in the nTBLs and relative
199 homogeneity of dTBLs). *Mycobacterium* reads were present in 64% (57/89) of dTBLs and
200 11% (7/61; $p<0.0001$) of nTBLs (**Figure S2**) and, when sequences underwent BLAST, all
201 reads matched with *Mtb*.

202

203 **Correlation between 16S rRNA gene sequencing and TB diagnostic test results**

204 As expected, there was a higher relative abundance of *Mycobacterium* reads in dTBLs
205 [median 0.034 (IQR 0.001-0.460) vs. 0.001 (0.001-0.001), $p<0.0001$; **Figure 2D**].
206 *Mycobacterium* relative abundance in dTBLs showed a positive correlation with bacillary
207 load (based on Xpert and Ultra cycle threshold values; $r_s=-0.774$, 95% CI [-0.777, -0.514],
208 $p<0.0001$; **Figure 2F**), and culture days-to-positivity; $r_p=-0.642$, [-0.833, -0.31.], $p=0.001$;
209 **Figure 2G**). Furthermore, a non-significant trend towards a positive correlation between
210 lymph node size and mycobacterial load (relative abundance, Xpert/Ultra C_{Tmin} values) was
211 observed (**Figure S3A-B**).

212

213 **Differences by HIV status**

214 Overall: α -diversity did not differ by HIV status (**Figure 3A**) and although β -diversity
215 did (**Figure 3B**) no differentially enriched taxa were found (not shown), however, the relative
216 abundance of *Mycobacterium* was higher in PLHIV (**Figure S4A**).

217 Comparisons within dTBLs or nTBLs by HIV status: There were 55% (49/89) and
218 39% (23/59) HIV-positive dTBLs and nTBLs, respectively. Within dTBLs or nTBLs, α -
219 diversities did not differ by HIV status (**Figure 3A**) and β -diversity differed within dTBLs by
220 HIV status ($p=0.017$, **Figure 3C**) but not within nTBLs. HIV-positive dTBLs had higher
221 *Mycobacterium* relative abundance than HIV-negative dTBLs (**Figure S4A**).

222 Comparisons within HIV-positives or -negatives by TB status: In people with the
223 same HIV status, α -diversity did not differ by TB status (**Figure 3A**) and β -diversity only
224 differed between dTBLs vs. nTBLs in HIV-positives ($p=0.009$, **Figure 3E**) where dTBLs were
225 enriched in *Mycobacterium* (**Figure S4B**). In HIV-negatives, there were no differences
226 between dTBLs and nTBLs (**Figure 3F**).

227

228 **Lymphotype identification and their associations with clinical characteristics**

229 Overall: Five lymphotypes with differing α - and β -diversities were identified (**Figure**
230 **4A-C, Table S3**). L1 had no dominant taxa (**Figure 4D**), whilst L4 was *Mycobacterium*-
231 dominated and had the least α -diversity, and L2, L3 and L5 were *Corynebacterium*-,
232 *Prevotella*- and *Streptococcus*-dominated, respectively. While no taxa were differentially
233 abundant in L1 vs. other lymphotypes (**Figure S5A-C**), L2, L3, and L5 were enriched relative
234 to L4 in *Corynebacterium*, *Prevotella*, and *Streptococcus*, respectively (**Figure 4E-G**). The
235 proportions of dTBLs in L1, L2, L3, L4, and L5 were 35% (17/48), 63% (28/44), 57% (12/21),
236 100% (21/21), and 69% (11/16), respectively. The patients in these lymphotypes are
237 associated with distinct clinical characteristics. The majority of nTBLs occurred in highly
238 diverse lymphotypes with a heterogenous mixtures of taxa; likely reflecting the spectrum of
239 pathologies in people with TBL ruled out. L1 was associated with characteristics indicative of
240 less severe lymphadenitis. Compared separately to L2, L4, and L5, L1s were less likely to
241 have dTBL. Furthermore, L1s were less likely to be HIV-positive vs. L4s but, L1 PLHIVs had

242 lower CD4 counts vs. L2 and L3 PLHIVs. In contrast, L4 was associated with characteristics
243 resembling more severe lymphadenitis. L4 was more likely to contain dTBL patients than
244 each other lymphotype. Furthermore, compared to L2s, L4s were more likely to have a
245 bigger lymph node, chylous FNABs and, of PLHIV, a smaller proportion on ART. Compared
246 to L3s, L4s were more likely to have previous TB and HIV, and those with HIV were more
247 likely to have lower CD4 counts. Compared to L5s, L3s with HIV had lower CD4 counts.
248 Therefore, in summary, L1 appears to be associated with less severe forms of
249 lymphadenitis, whereas L4 was associated more severe forms (**Table S4**).

250 Within patients of the same TB status: Within dTBLs, three lymphotypes with differing
251 β -diversities were identified (**Figure 5A-B**). L1 was abundant in *Prevotella* and
252 *Corynebacterium*, L2 in *Prevotella* and *Streptococcus*, and L3 in *Mycobacterium* (**Figure 5C**)
253 and these taxa were differentially abundant (**Figure 5D-F**). These lymphotypes were termed
254 *Prevotella-Corynebacterium*, *Prevotella-Streptococcus* and *Mycobacterium*, respectively.
255 L3s were more likely to be HIV-positive, with larger lymph nodes, compared to L1s. In
256 addition, L3s were more likely to have larger lymph nodes than L2s. Lastly, L2s are more
257 likely to be female than L1s (**Table S5**). Together, these differences suggest L3 is
258 associated with more severe TBL than other lymphotypes. Within nTBLs, no lymphotypes
259 were identified (**Figure S6**).

260

261 **Predictive metagenome profiling shows increased short chain fatty acid**

262 **metabolism**

263 dTBLs vs. nTBLs: 139 inferred microbial metabolic pathways were differentially
264 enriched (75 in dTBLs, 64 in nTBLs). In dTBLs, “fatty acid metabolism”, “benzoate
265 degradation”, “propanoate metabolism” and “butanoate metabolism” were enriched,
266 suggesting increased SCFA production (**Figure 6**).

267 HIV-positive vs. negatives: The above SCFA-related pathways were enriched in HIV-
268 positive vs. -negative patients overall and, within dTBLs, in HIV-positives vs. -negatives

269 **(Figure 7A-B)**. Within nTBLs, HIV-positives were enriched in the “cell cycle – *Caulobacter*”,
270 “bacterial secretion system” and “oxidative phosphorylation” vs. -negatives **(Figure S7)**.

271 In different lymphotypes: When comparing lymphotypes’ inferred pathways in all
272 patients (overall including dTBLs and nTBLs), a similar core of pathways was enriched in
273 lymphotype 4. These included the “propanoate metabolism”, “tuberculosis”, “lipid
274 biosynthesis”, “butanoate metabolism”, “fatty acid metabolism” and “PPAR signalling
275 pathway” (most-to-least enriched) **(Figure 8A-B)**. In contrast, vs. lymphotype 4, lymphotype
276 1 was enriched in “epithelial cell signalling in *Helicobacter pylori* infection”, lymphotype 2 was
277 enriched in “carbohydrate digestion and absorption”, lymphotype 3 was enriched in “dioxin
278 degradation”, and lymphotype 5 was enriched in “carbohydrate digestion and absorption”
279 **(Figure S8A-H)**. When comparing the three dTBL lymphotypes, *Mycobacterium*-dominated
280 lymphotype 3 was, compared to each other dTBL lymphotypes, enriched in the similar core
281 pathways seen for the *Mycobacterium*-dominated lymphotype 4 overall in all patients
282 **(Figure 8C; Figure S9)**.

283

284 DISCUSSION

285 We characterised the local microbial environment in patients with lymphadenitis undergoing
286 investigation for TB in a HIV-endemic setting. Our key findings are: 1) lymphatic microbial
287 communities in dTBLs clustered into three distinct “lymphotypes” we termed “*Prevotella-*
288 *Corynebacterium*”, “*Prevotella-Streptococcus*”, and “*Mycobacterium*”, 2) the *Mycobacterium*
289 dTBL lymphotype was associated with HIV-positivity and other clinical features characteristic
290 of severe lymphadenitis, and 3) dTBLs relative to nTBLs were functionally enriched in fatty
291 acid-, amino acid-, and SCFA-related microbial metabolic pathways with known
292 immunomodulatory effects (the *Mycobacterium* lymphotype was most enriched in these
293 pathways than other dTBL lymphotypes). Finally, 4) dTBLs without *Mycobacterium* reads
294 and nTBLs with *Mycobacterium* reads were identified. These data show TBL at the site-of-
295 disease is not microbially homogenous and that distinct clusters of microbial communities
296 exist associated with different clinical characteristics. The long-term significance and
297 importance of these lymphotypes requires prospective evaluation.

298

299 We identified three lymphotypes within dTBLs termed “*Prevotella-Corynebacterium*”,
300 “*Prevotella-Streptococcus*”, and “*Mycobacterium*”, distinguished by different relative
301 abundances of these taxa (*Prevotella* co-occurred in the first two lymphotypes). These
302 individual taxa are enriched in respiratory secretions from pulmonary TB cases ^{29 30}.
303 Furthermore, within dTBLs , *Streptococcus* is associated with low BMI and extent of lung
304 damage ³⁰. *Prevotella* in bronchoalveolar lavage fluid also positively correlates with SCFA
305 concentrations and independently predicts incident TB in people without co-prevalent TB ³¹.
306 Compared to the other dTBL lymphotypes, “*Mycobacterium*” was associated with severe
307 disease and most frequently occurred in PLHIV, agreeing with diagnostics studies that show
308 stronger baseline mycobacterial PCR test readouts predict long term clinical outcomes in
309 pulmonary ³² and extrapulmonary TB ³³. Together, these data show distinct lymphotypes are
310 associated with different clinical characteristics and suggests that patients with the most
311 severe *Mycobacterium*-dominated lymphotype may initially progress through different site-of-

312 disease microbial states characterised by *Corynebacterium*-, *Streptococcus*- and/or
313 *Prevotella*-domination. Studies with longitudinal follow-up and repeat sampling are required
314 to examine whether these lymphotypes have potential for clinical staging.

315

316 Importantly, *Corynebacterium* and *Streptococcus* often dominated in dTBL patients.
317 Members of both taxa are causative agents of lymphadenitis and, even though these
318 patients have TB lymphadenitis confirmed via conventional diagnostics, *Corynebacterium*
319 and *Streptococcus* may therefore co-contribute to pathology and symptoms ³⁴⁻³⁶.
320 Coincidentally, these taxa fall within the anti-microbial spectrum of first-line TB treatment ¹⁶,
321 meaning that this regimen may, in part, cure lymphadenitis by killing *Corynebacterium* and
322 *Streptococcus* in addition to *Mycobacterium*.

323

324 Microbial pathways predicted to be most enriched in dTBLs involved fatty acid, amino acid,
325 and SCFAs (benzoate, propanoate) metabolism; all of which are associated with pulmonary
326 TB disease compared to sick patients without TB ^{37 38}. SCFAs in particular suppress immune
327 pathways involved in IFN- γ and IL-17A production and, *ex vivo*, limit macrophage-mediated
328 kill of *Mtb*. SCFA concentrations hence predict incident TB in patients ³¹. Our research
329 therefore suggests that the inflammation associated with lymphadenopathy is in part caused
330 by the presence of microbes including but limited to *Mycobacterium* that are able to produce
331 SCFAs that interfere with these immunological pathways; revealing potentially new
332 therapeutic targets to reduce lymphadenopathy.

333

334 We detected *Mycobacterium* in nTBLs. These reads could be from previous TB exposure or
335 disease. *Mtb* DNA has been found in the lymph nodes of healthy individuals and primates
336 exposed to TB, where the sites are hypothesised to serve as a *Mtb* growth and persistence
337 niche ⁷. dTBLs without *Mycobacterium* reads were also documented, however, 16S rRNA
338 sequencing has well known sub-optimal sensitivity for *Mycobacterium*, in part due to low 16s
339 RNA gene copy number ³⁹.

340 Our study has strengths and limitations. Patients were sampled once, as close as possible to
341 treatment initiation; animal models might permit repeat invasive sampling especially if
342 treatment is withheld. We did not co-analyse host immune signatures but plan to do so. The
343 programmatic context enabled large numbers of patients to be recruited, however, detailed
344 long-term follow-up, which could include imaging of lymph nodes and more detailed
345 measurements of differential responses to treatment, was not possible.

346

347 In conclusion, we show dTBL patients have a distinct microbiome at the site of disease,
348 characterized by three lymphotypes (*Mycobacterium*, *Prevotella-Corynebacterium*,
349 *Prevotella-Streptococcus*). This dysbiosis of the lymphatic microbiome likely contributes to
350 pathophysiology, including inflammatory state and clinical severity, which itself may reflect
351 the chronicity of TB disease. TB lymphadenitis does therefore not appear to be a microbially
352 homogenesis disease, and this reveals potentially new diagnosis, therapeutic, and
353 prognostic targets.

354

355 **ACKNOWLEDGMENTS:**

356 The authors thank study participants and Tygerberg Hospital FNA clinic staff especially Sr
357 Cupido. Additionally, we thank CLIME research group staff, especially Sr Ruth Wilson and
358 Roxanne Higgitt. GRN acknowledges funding from L'Oréal-UNESCO For Women in Science
359 Sub-Saharan Africa Young Talents Award, and the International Rising Talents Award. The
360 content is the sole responsibility of the authors and does not necessarily represent the
361 official views of the funders. Computations were performed using facilities provided by the
362 University of Cape Town's ICTS High Performance Computing team: hpc.uct.ac.za

363 **CONTRIBUTORSHIP:** GN, CCN and GT contributed to conceptualisation and design of the
364 study and supervised the study, funding acquisition, data collection, and wrote the
365 manuscript. GRN, CCN, IS, BGW, IS, JCC, LNS, and GT contributed to data and statistical
366 analysis, and figures. All authors contributed to interpretation of data and editing of the
367 manuscript. As the study guarantor, GT is responsible for the overall content of this
368 manuscript.

369 **FUNDING:** This work and authors were supported by the European & Developing Countries
370 Clinical Trials Partnership (EDCTP; project numbers SF1041, TMA2017CDF-1914-MOSAIC
371 and TMA2019CDF-2738-ESKAPE-TB), National Research Foundation (NRF), the South
372 African Medical Research Council (SAMRC), the Harry Crossley Foundation and
373 Stellenbosch University Faculty of Health Sciences, and the National Institute of Allergy and
374 Infectious Diseases of the National Institutes of Health under award numbers (R01AI136894;
375 U01AI152087; U54EB027049; D43TW010350).

376 **COMPETING INTERESTS:** None

377 **DATA AVAILABILITY:** Data are available upon reasonable request. De-identified patient
378 data, the study protocol, informed consent, and datasets generated in this study may be
379 requested from the corresponding author.

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FIGURE LEGENDS

380

381 **Figure 1: Study flow chart.** Fine needle aspirates, skin and saline controls were collected
382 from presumptive TBL patients. dTBLs: definite tuberculous lymphadenitis; nTBLs: non-
383 tuberculous lymphadenitis; pTBLs: probable tuberculous lymphadenitis; Smear: Smear
384 microscopy; MGIT960 Culture: Mycobacteria Growth Indicator Tube 960 liquid culture; Xpert:
385 Xpert MTB/RIF; Ultra: Xpert MTB/RIF Ultra.

386

387 **Figure 2: dTBLs have a distinct microbiome to nTBLs with *Mycobacterium***
388 **enrichment. (A)** Although α -diversity was similar, **(B)** β -diversity differed. *Mycobacterium*
389 was enriched in dTBLs compared to nTBLs based on **(C)** differential abundance testing and
390 **(D)** relative abundance. Discriminatory taxa appear above the threshold (red dotted line,
391 FDR=0.2). **(E)** dTBLs were more compositionally similar to each other than nTBLs.
392 Mycobacterial reads positively correlated with *Mtb* load: **(F)** Xpert and Ultra and **(G)** culture
393 (days-to-positivity). dTBLs: definite tuberculous lymphadenitis; nTBLs: non-tuberculous
394 lymphadenitis; Xpert: Xpert MTB/RIF; Ultra: Xpert MTB/RIF Ultra; MGIT960: Mycobacteria
395 Growth Indicator Tube 960 liquid culture; r_s : Spearman correlation coefficient; r_p : Pearson
396 correlation coefficient.

397

398 **Figure 3: Microbiome differences in HIV-positive dTBLs versus nTBLs but not in HIV-**
399 **negative dTBLs versus nTBLs. (A)** α -Diversity did not differ by HIV or TBL statuses, **(B)**
400 however, β -diversity differed between HIV-positives and -negatives overall (shaded circles
401 are dTBLs, empty circles are nTBLs). β -diversity differed by HIV status in **(C)** dTBLs but not
402 in **(D)** nTBLs. β -diversity differed by TBL status in **(E)** HIV-positives and **(F)** HIV-negatives.

403

404 **Figure 4: Five lymphotypes observed in presumptive TBL. (A)** LaPlace approximation
405 identified five clusters. **(B)** L5 had the highest α -diversity. **(C)** β -diversity differed between
406 each lymphotype (shaded circles dTBLs, empty circles nTBLs). **(D)** Stacked bar plots

407 showing L1 with a heterogenous mixture of genera, L2 dominated by *Corynebacterium*, L3
408 dominated by *Prevotella*, L4 dominated by *Mycobacterium*, and L4 dominated by
409 *Streptococcus*. Bolded taxa represent dominating taxa. **(E)** *Corynebacterium* was enriched in
410 L2; **(F)** *Prevotella* enriched in L3, **(G)** *Mycobacterium* enriched in L4, and *Streptococcus*
411 enriched in L5. Significantly more discriminatory taxa (bolded) appear closer to the left or
412 right, and higher above the threshold (red dotted line, FDR=0.2) as significance increases.
413 Relative taxa abundance is indicated by circle size. dTBLs: definite tuberculous
414 lymphadenitis; nTBLs: non-tuberculous lymphadenitis; L: lymphotype.

415

416 **Figure 5: Three lymphotypes identified in dTBLs.** **(A)** Best model fit based on LaPlace
417 approximation identified three clusters within dTBLs. **(B)** β -diversity differed between
418 lymphotypes. **(D)** Stacked bar plots showing L1 comprised of *Mycobacterium* and
419 accompanying heterogenous taxa, L2 dominated by *Prevotella* and *Streptococcus*, and L3
420 dominated by *Mycobacterium*. Bolded taxa represent dominating taxa. **(D)** No taxa were
421 enriched in L1, **(E)** L2 was enriched in *Streptococcus*, **(F)** and *Mycobacterium* was enriched in
422 L3. Significantly more discriminatory taxa (bolded) appear closer to the left or right, and
423 higher above the threshold (red dotted line, FDR=0.2) as significance increases. Relative
424 taxa abundance is indicated by circle size. dTBLs: definite tuberculous lymphadenitis; L:
425 lymphotype.

426

427 **Figure 6: Enriched microbial capacity for SCFA pathways in dTBLs versus nTBLs.**
428 Volcano plot depicting differentially abundant microbial pathways in dTBLs vs. nTBLs
429 inferred by PICRUST2. Key pathways of interest are bolded including aminobenzoate
430 degradation, benzoate degradation, and propanoate degradation. Significantly more
431 discriminatory pathways appear closer to the left or right, and higher above the threshold
432 (red dotted line, FDR=0.05) as significance increases. Relative gene abundance is indicated
433 by circle size. SCFA: short chain fatty acids; dTBLs: definite tuberculous lymphadenitis;
434 nTBLs: non-tuberculous lymphadenitis.

435

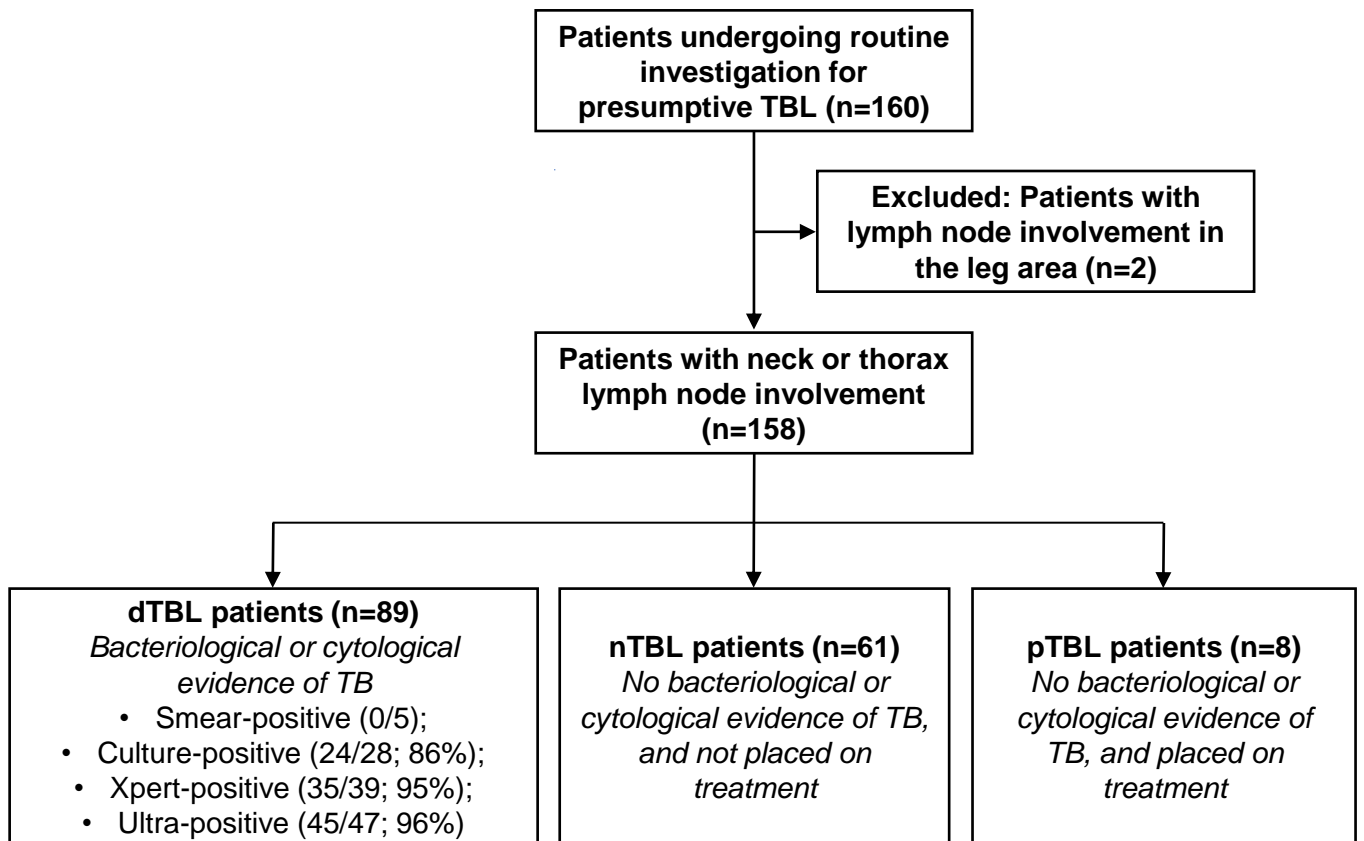
436 **Figure 7: Predicted metagenome function reveals increased capacity for SCFA**
437 **production in HIV-positive versus HIV-negative patients overall, and in dTBLs.**

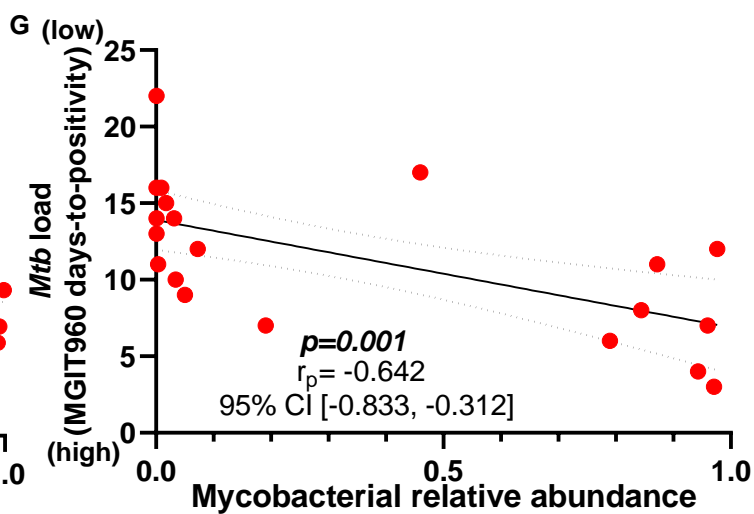
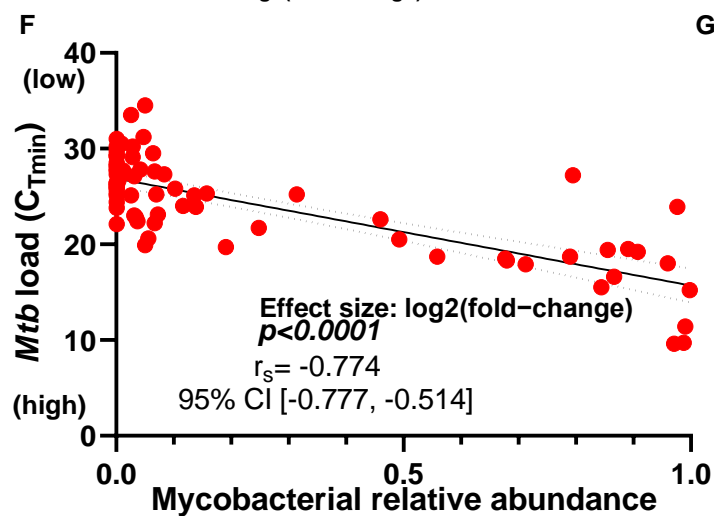
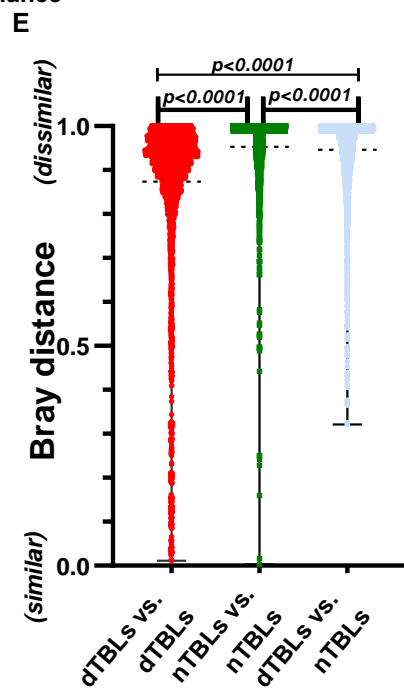
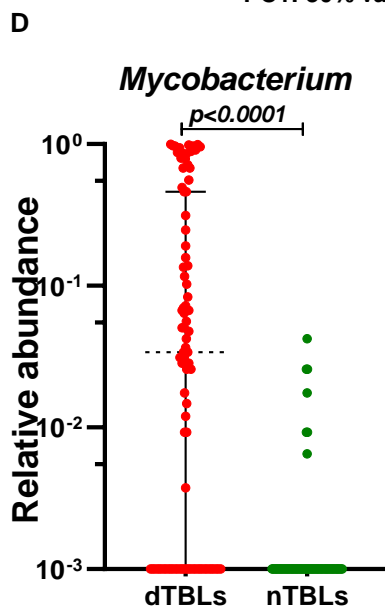
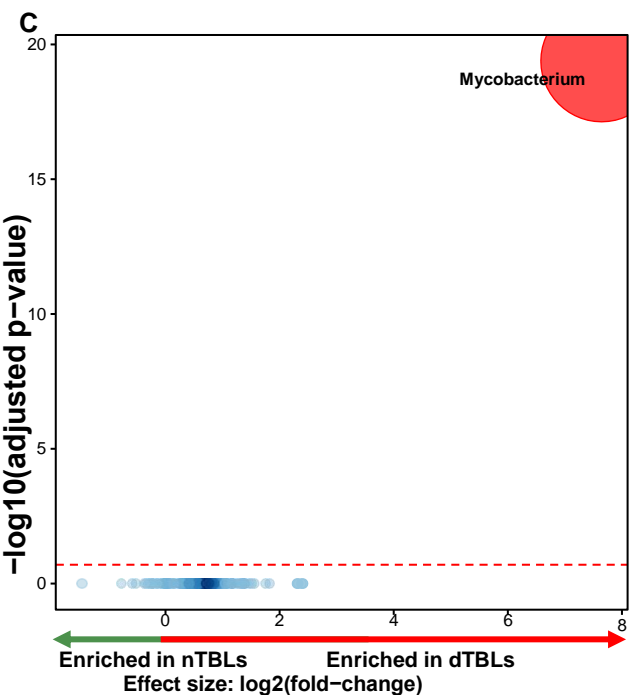
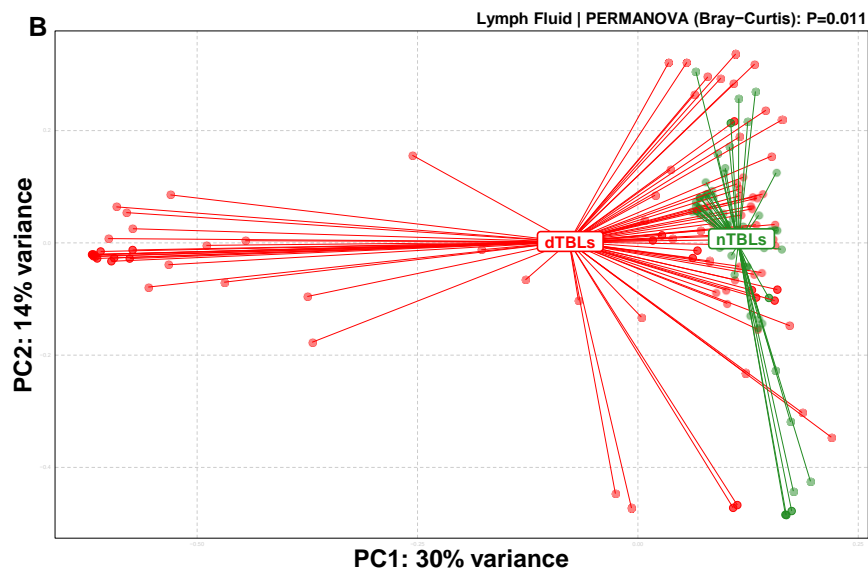
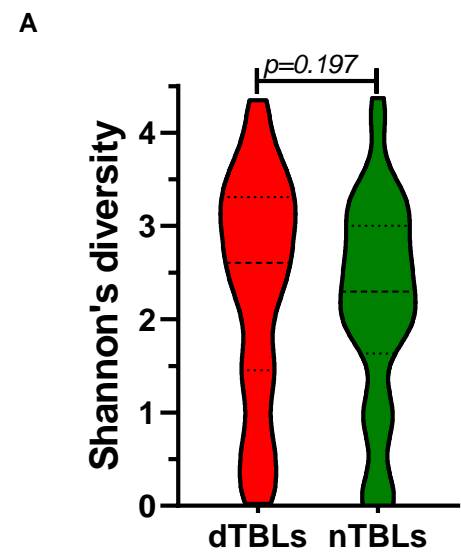
438 Volcano plot depicting functional pathways differing between **(A)** HIV-positive and HIV-
439 negative patients with presumptive TBL, and **(B)** in dTBLs. Key pathways of interest include
440 butanoate metabolism, propanoate metabolism and benzoate degradation. Significantly
441 more discriminatory pathways appear closer to the left or right, and higher above the
442 threshold (red dotted line, FDR=0.05). Relative gene abundance is indicated by circle size.
443 SCFA: short chain fatty acids.

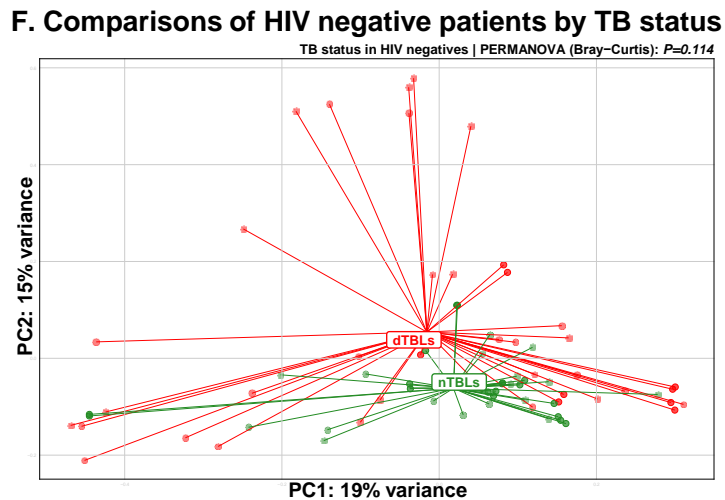
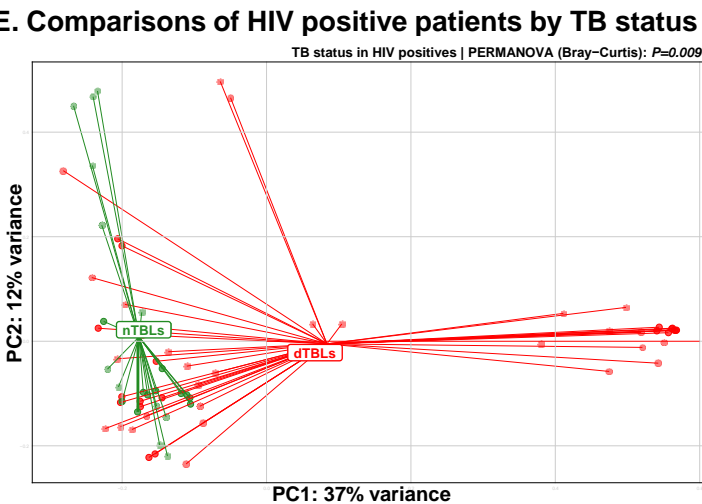
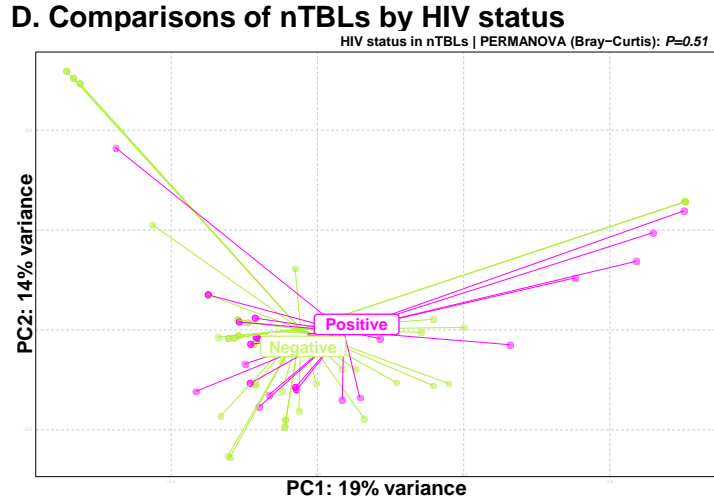
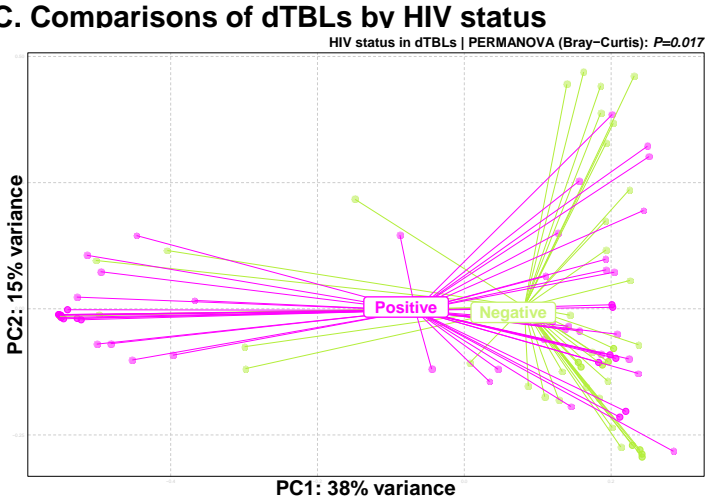
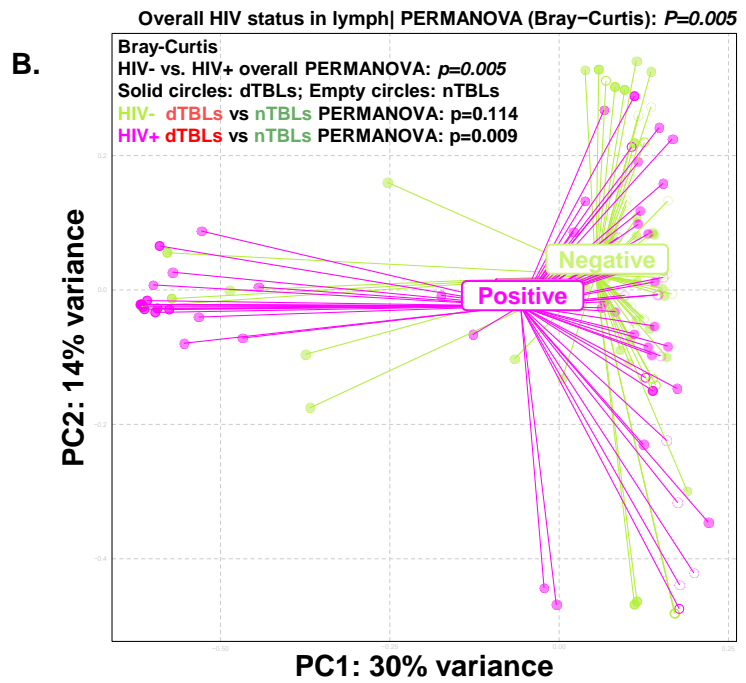
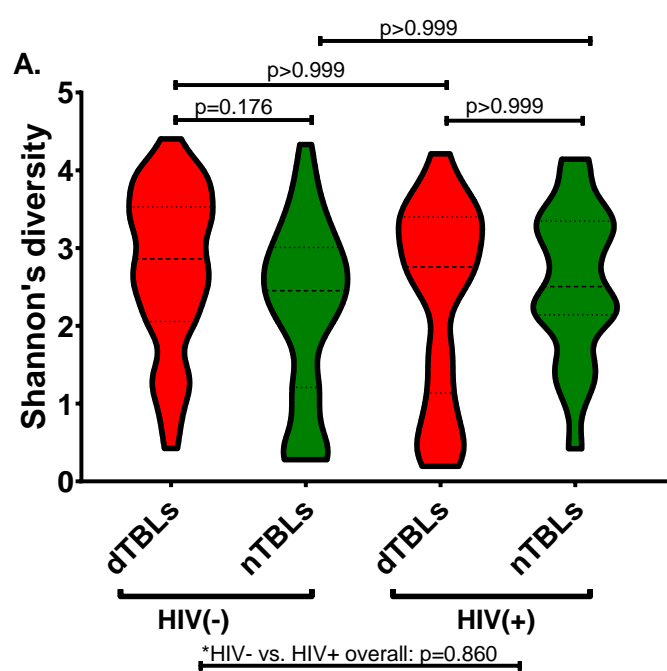
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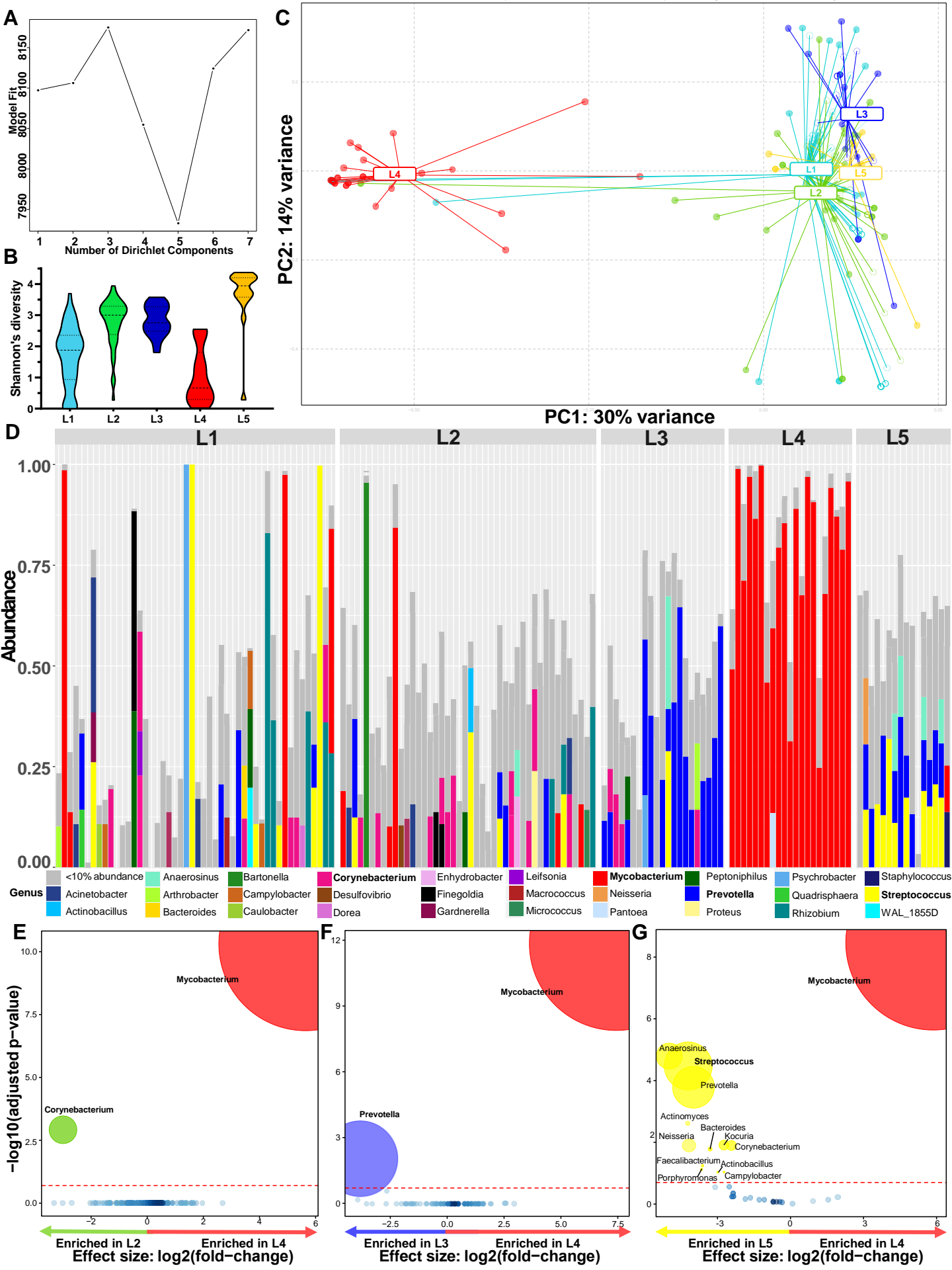
445 **Figure 8: Differential microbial pathways between lymphotypes showing similar core**
446 **pathways enriched in the *Mycobacterium*-dominated lymphotype..** (A) Volcano plot

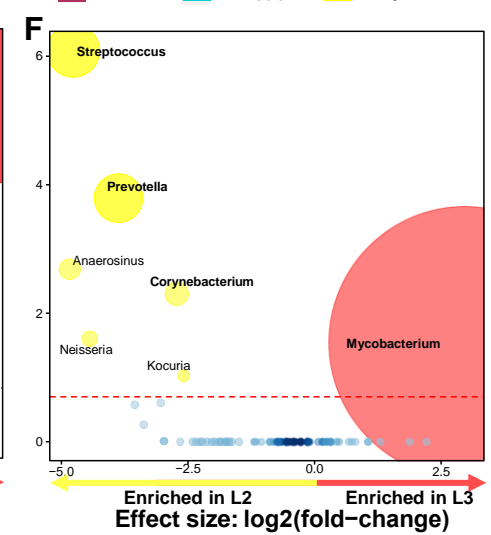
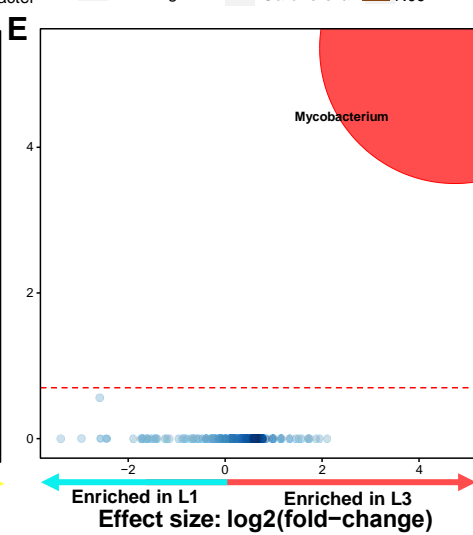
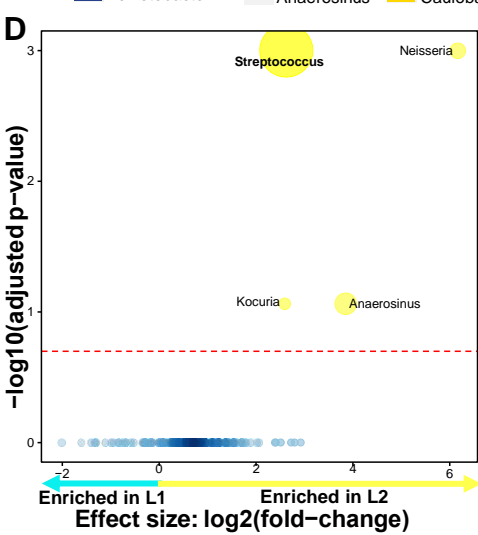
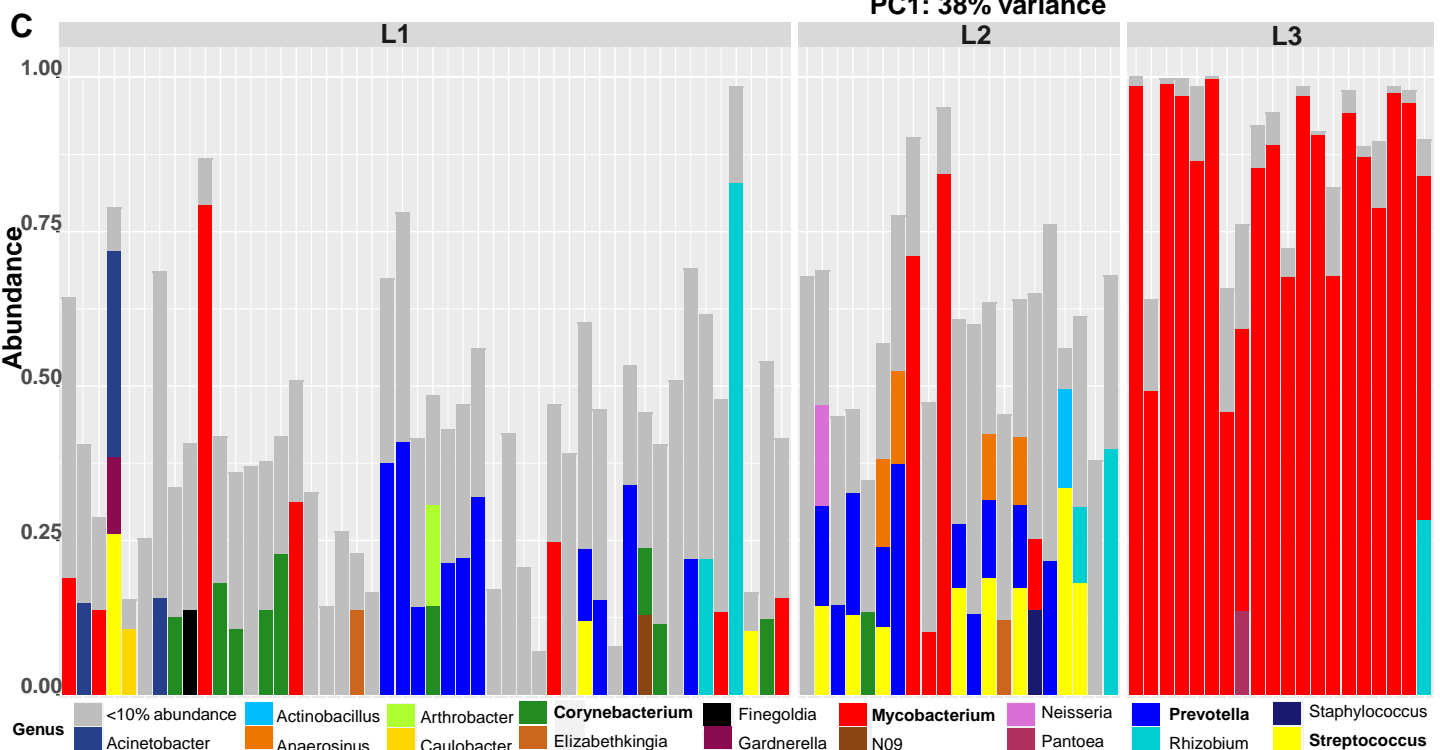
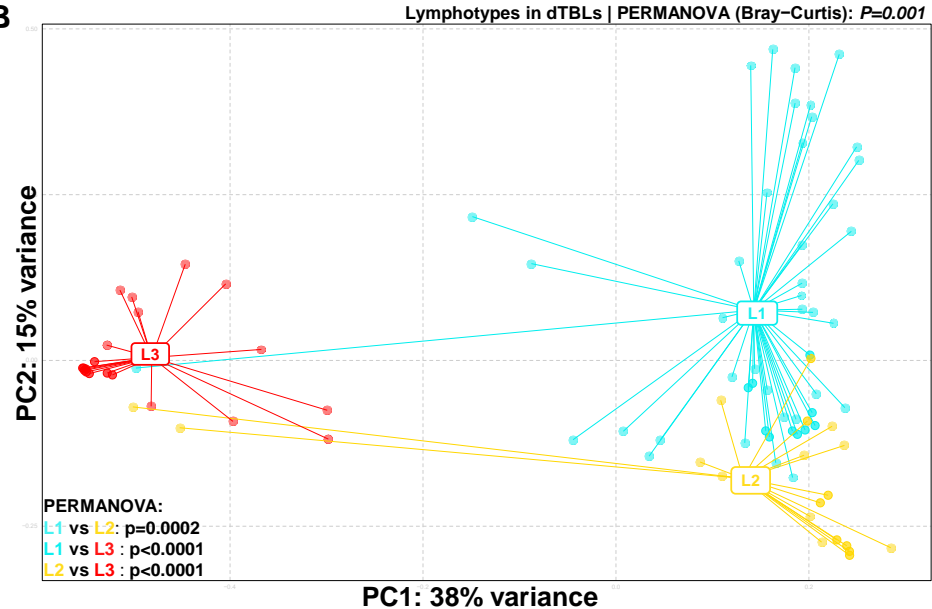
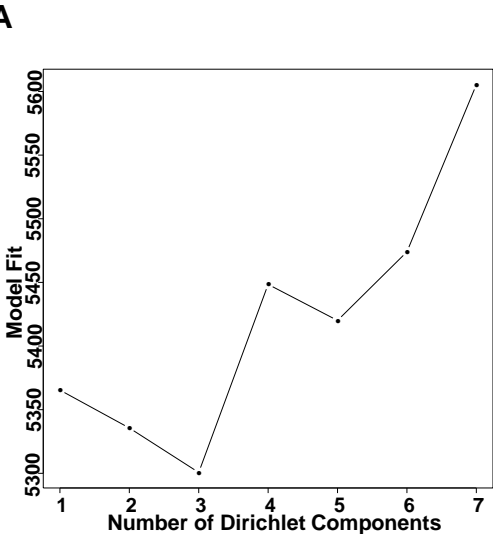
447 showing differentially abundant microbial pathways inferred by PICRUSt2 in lymphotype 2
448 vs. lymphotype 4 representing pathways enriched in lymphotype 4 compared to every other
449 lymphotype in all patients (overall including dTBLs and nTBLs). Significantly more
450 discriminatory pathways appear closer to the left or right, and higher above the threshold
451 (red dotted line, FDR=0.05) as significance increases. Relative gene abundance is indicated
452 by circle size. **(B)** 65.5% of all inferred pathways enriched L4 compared to each other overall
453 lymphotypes were common, whilst **(C)** 85.8% were common in L3 compared to each other
454 dTBL lymphotypes. Differentially enriched pathways common in all comparisons with the
455 *Mycobacterium* dominant lymphotype included pathways involving lipid biosynthesis, fatty
456 acids, and SCFA metabolism i.e. lipid biosynthesis proteins, propanoate metabolism,
457 benzoate degradation, and valine, leucine and isoleucine degradation. SCFA: short chain
458 fatty acids; dTBLs: definite tuberculous lymphadenitis; nTBLs: non-tuberculous
459 lymphadenitis; L: lymphotype.



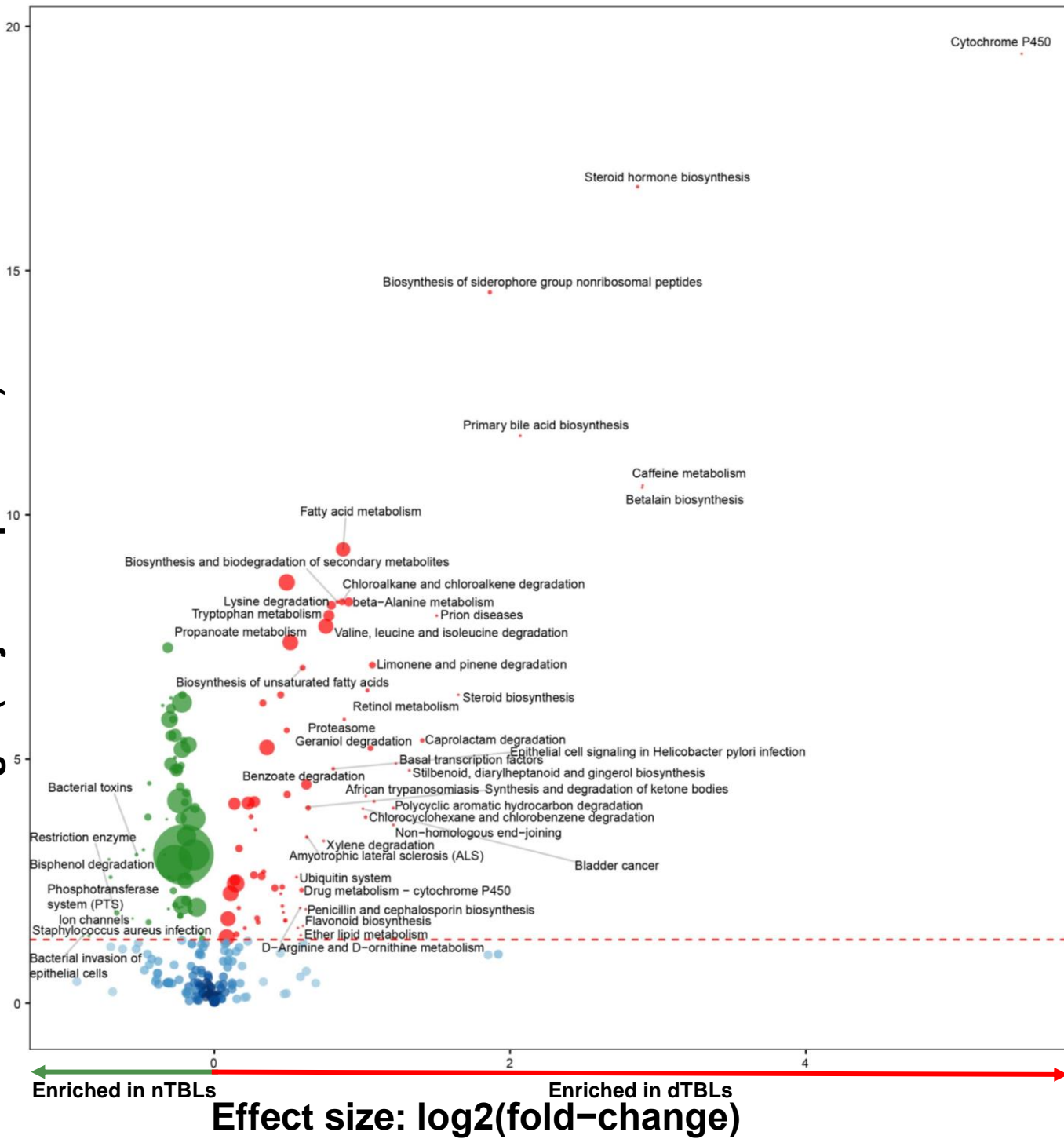


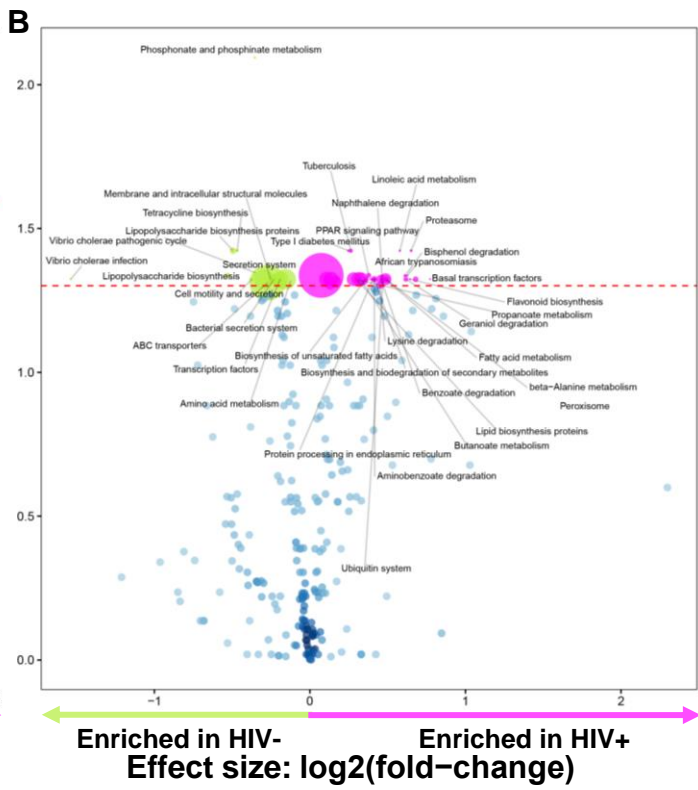
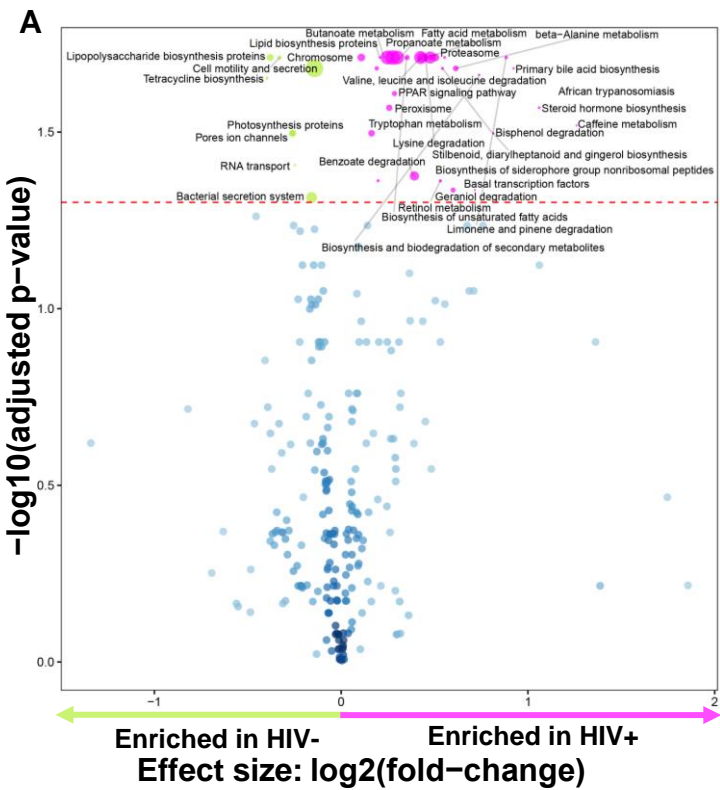


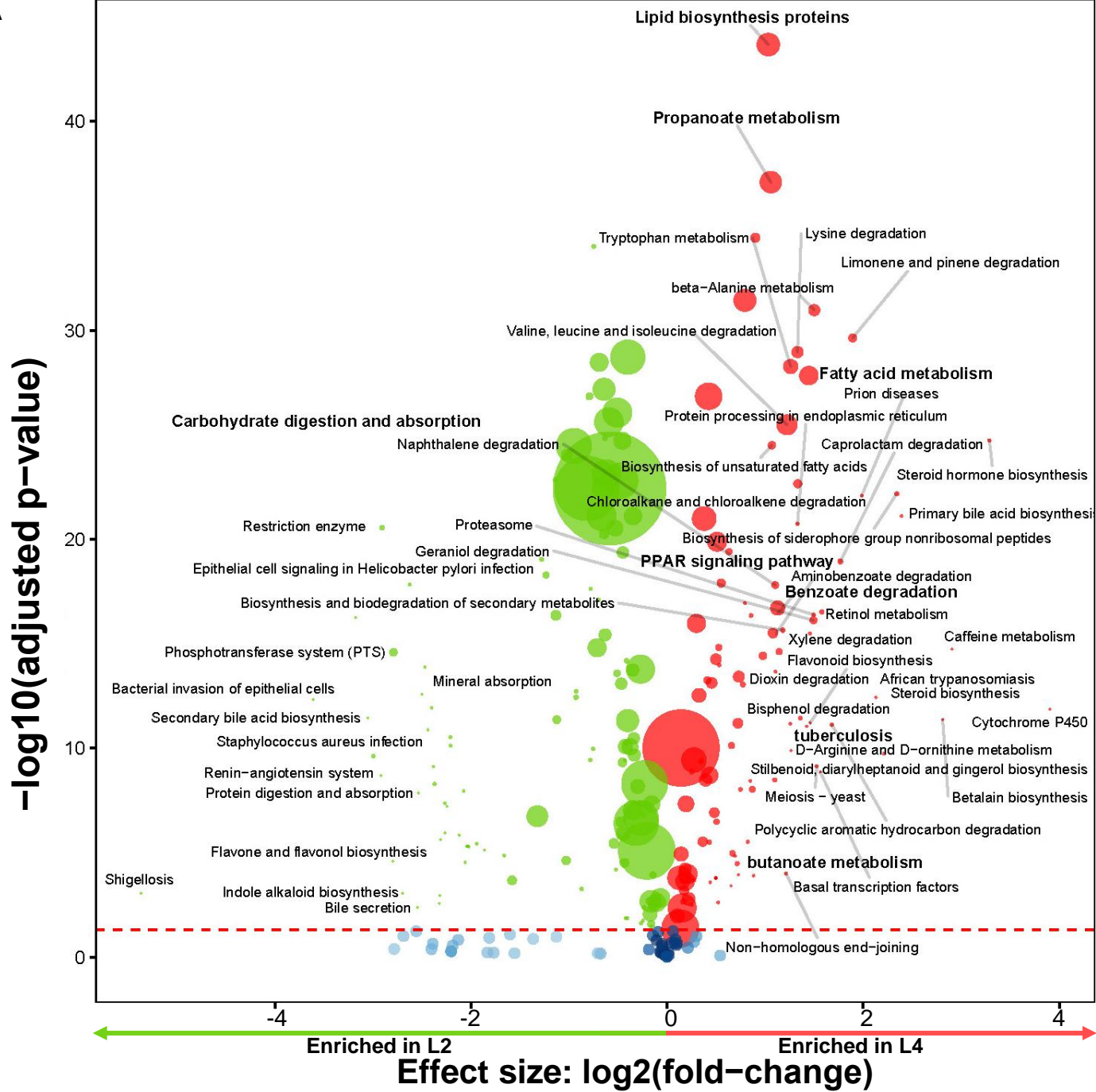
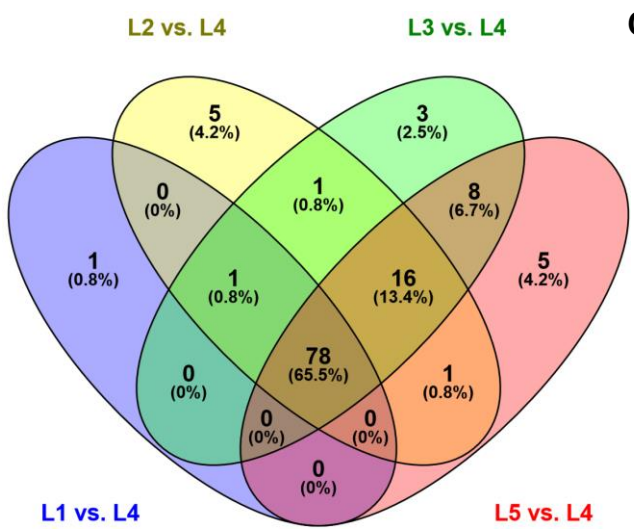




-log₁₀(adjusted p-value)





A**B****C**