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## Ultrasensitive detection of p24 in plasma samples from people with primary and chronic HIV-1 infection

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1 **Ultrasensitive detection of p24 in plasma samples from people with primary and**  
2 **chronic HIV-1 infection**

3

4 **Short title: Ultrasensitive detection of HIV-1 p24 in plasma**

5

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10

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27 **Abstract**

28 HIV-1 Gag p24 has long been identified as an informative biomarker of HIV replication,  
29 disease progression and therapeutic efficacy, but the lower sensitivity of  
30 immunoassays in comparison to molecular tests and the interference with antibodies  
31 in chronic HIV infection limits its application for clinical monitoring. The development  
32 of ultrasensitive protein detection technologies may help overcoming these  
33 limitations. Here we evaluated whether immune-complex dissociation combined with  
34 ultrasensitive digital ELISA Simoa technology could be used to quantify p24 in plasma  
35 samples from people with HIV-1 infection. We found that, among different immune-  
36 complex dissociation methods, only acid-mediated dissociation was compatible with  
37 ultrasensitive p24 quantification by digital ELISA, strongly enhancing p24 detection at  
38 different stages of HIV-1 infection. We show that ultrasensitive p24 levels correlated  
39 positively with plasma HIV-RNA and HIV-DNA and negatively with CD4+ T cells in the  
40 samples from people with primary and chronic HIV-1 infection. In addition, p24 levels  
41 also correlated with plasma D-dimers and IFN $\alpha$  levels. P24 levels sharply decreased to  
42 undetectable levels after initiation of combined antiretroviral treatment (cART).  
43 However, we identified a group of people who, 48 weeks after cART initiation, had  
44 detectable p24 levels despite most having undetectable viral loads. These people had  
45 different virologic and immunologic baseline characteristics when compared with  
46 people who had undetectable p24 after cART. These results demonstrate that  
47 ultrasensitive p24 analysis provides an efficient and robust mean to monitor p24  
48 antigen in plasma samples from people with HIV-1 infection, including during  
49 antiretroviral treatment, and may provide complementary information to other  
50 commonly used biomarkers.

51

52 **Importance**

53 The introduction of combined antiretroviral treatment has transformed HIV-1 infection  
54 in a manageable condition. In this context, there is a need for additional biomarkers to  
55 monitor HIV-1 residual disease or the outcome of new interventions, such as in the  
56 case of HIV cure strategies. The p24 antigen has a long half-life outside viral particles  
57 and it is therefore a very promising marker to monitor episodes of viral replication or  
58 transient activation of the viral reservoir. However, the formation of immune-  
59 complexes with anti-p24 antibodies makes its quantification difficult beyond acute  
60 HIV-1 infection. We show here that, upon immune-complex dissociation, new  
61 technologies allow the ultrasensitive p24 quantification in plasma samples throughout  
62 HIV-1 infection, at levels close to that of viral RNA and DNA determinations. Our results  
63 further indicate that ultrasensitive p24 quantification may have added value when  
64 used in combination with other classic clinical biomarkers.

65

66 **Key Words:** HIV/AIDS; HIV p24 antigen; Gag p24; Simoa; ELISA; biomarker; plasma

67

68

69

70 **Introduction**

71 Human Immunodeficiency Virus (HIV) capsid p24 is a 24-25kDa protein encoded by the  
72 *gag* gene and corresponds to the most abundant viral antigen. Detection of p24 is  
73 considered essential for the diagnosis of HIV infection, especially in individuals under  
74 pre-exposure prophylaxis and newly infected individuals (1). Fourth generation  
75 antibody-antigen assays detecting p24 in addition to anti-HIV antibodies are currently  
76 recommended for HIV testing (2), as they reduce the time between infection and HIV  
77 diagnosis allowing people with HIV (PWH) to start antiretroviral treatment as early as  
78 possible (3). Prior to the development of nucleic acid tests (NAT), p24 was used as a  
79 surrogate marker of HIV replication, disease progression and therapeutic efficacy in  
80 the clinical monitoring of PWH (4-10). At primary infection, p24 levels are increased  
81 and were shown to correlate with HIV-RNA (4, 11-16). At chronic HIV infection, PWH  
82 positive for p24 antigen showed a higher risk of developing AIDS than those who  
83 remain antigen negative (12, 17, 18). The quantification of plasma p24 has shown  
84 superiority to RNA viral determination in anticipating decline in CD4+ T cell counts in  
85 some studies (5, 18) and has shown to better correlate with CD8+ T cell activation in  
86 another (6). Moreover, p24 is sometimes detected dissociated from viral particles (i.e.  
87 in the absence of viral RNA), which might provide a more extensive estimation of the  
88 infection burden in the organism (6, 19).

89

90 However, although HIV-1 p24 is recognized as an informative biomarker, its detection  
91 by classical enzyme-linked immunosorbent assays (ELISAs) is less sensitive than HIV  
92 nucleic acid tests, in part due to the small sample volume that can be tested. In  
93 addition, after seroconversion, the development of anti-HIV humoral responses make

94 the detection of p24 inaccurate by immunoassays due to the formation of antigen-  
95 antibody immune-complexes (1). The development of Single-molecule array (Simoa)  
96 technology represents an important recent advance in ultrasensitive protein  
97 detection, reaching detection at femtomolar concentrations (20, 21). With this  
98 technology, single molecule HIV-1 Gag p24 detection is 1,000-fold more sensitive than  
99 current available ELISA tests. We have previously tested the sensitivity of this assay in  
100 HIV-infected cells and in culture supernatants. We showed that viral proteins produced  
101 by a single infected cell could be detected by an ultrasensitive p24 assay, which opens  
102 new opportunities for the study of HIV pathogenesis (22). To date, the applicability of  
103 an ultrasensitive HIV p24 assay in patients' samples was demonstrated only in serum  
104 from HIV-infected individuals during acute infection positive for HIV-RNA, but non-  
105 reactive for anti-HIV antibodies (23). More recently, the single molecule digital p24  
106 assay showed enhanced sensitivity when compared to the currently approved fourth-  
107 generation antigen/antibody combination and stand-alone p24 antigen assays in a  
108 panel of serially diluted RNA-positive/antibody-negative plasma-derived culture  
109 supernatants (24). The applicability and relevance of ultrasensitive HIV p24 detection  
110 in serum/plasma samples from chronically infected individuals remains unknown.

111

112 We show here that, upon immune-complex dissociation (ICD) with an approach  
113 compatible with the single molecule assay, p24 can be detected at ultrasensitive levels  
114 in plasma from most individuals during primary and chronic HIV-1 infection. The levels  
115 of p24 dropped sharply upon antiretroviral treatment initiation but remained  
116 detectable in some individuals despite undetectable RNA viral load. The increased  
117 sensitivity of this technique could bring new insights for the predictive value of p24 as

118 an informative biomarker of viral replication, inflammation and treatment efficacy in

119 people with primary and chronic HIV-1 infection.

120

121 **Results**

122 **Acid dissociation to disrupt antigen-antibody immune-complexes is compatible with**  
123 **ultrasensitive p24 quantification**

124 Immune-complex dissociation is required to increase the sensitivity of p24 antigen  
125 assays during chronic HIV-1 infection. We therefore assessed whether common  
126 methods for ICD are compatible with the digital ELISA technology. We tested (i) heat,  
127 (ii) heat with sodium dodecyl sulfate (SDS) and diethylenetriaminepentaacetic acid  
128 (DTPA), and (iii) acid dissociation with Glycine-HCl pH 1.8 or 2.5. Heat treatment  
129 denatures antibodies, but high and non-specific loss of antigen and protein coagulation  
130 due to this procedure was reported (25). The treatment with a solution containing SDS  
131 and DTPA for diluting samples prior to heat treatment aimed at reducing these  
132 problems. SDS reduces protein coagulation by giving them a negative charge and DTPA  
133 chelates iron and other ions, preventing further interference in the p24 assay (26).

134

135 We first evaluated the impact of these different methods on the ultrasensitive p24  
136 standard curve. Based on the average enzyme per bead (AEB) and the shape of the  
137 standard curves obtained after different treatments for ICD, we observed that heat  
138 alone or in combination with SDS+DTPA importantly interfered with p24  
139 determination, flattening the standard curves and increasing the background signal in  
140 the absence of p24 (Figures 1A and 1B). Higher AEB values were also observed when  
141 testing only the SDS+DTPA solution in the absence of p24 protein. The other reagents  
142 used for sample dilutions and virus inactivation did not shown any impact (Figure 1C).

143 We next evaluated the effect of heat-mediated with SDS and DTPA and acid  
144 dissociation methods in plasma from HIV-1 negative donors. We confirmed that heat-

145 mediated with SDS+DTPA treatment, but not acid dissociation, increases the  
146 background signal for p24 detection in plasma samples (Figure 1D). These results  
147 suggest that heat-mediated dissociation, with or without SDS+DTPA, is not compatible  
148 with the Simoa technology and may overestimate p24 levels in clinical samples. It is  
149 possible that heating itself alters the p24 conformation or that heating-mediated  
150 protein coagulation promotes non-specific binding of assay antibodies. Treatment with  
151 SDS/DTPA did not prevent these problems, conversely it contributed to an increase of  
152 non-specific signal.

153

154 In contrast, the curves obtained after acid-mediated dissociation with Glycine-HCl  
155 displayed the same profile than non-treated samples (Figures 1A, 1B and 1D). AEB  
156 values were comparable and standard curves obtained with non-treated samples and  
157 samples undergoing acid-mediated ICD presented equal distributions (Figures 1A and  
158 1B). Therefore, the acid-mediated ICD methods described here can be used for p24  
159 ultrasensitive quantification by digital ELISA, while other classical methods previously  
160 validated for ICD in classical ELISA assays are not compatible with this new technology.  
161 No differences were observed between Glycine pH 1.8 and pH 2.5, and we kept using  
162 the Glycine pH 1.8 protocol for further analyses.

163

#### 164 **Acid-mediated ICD combined with $_{us}$ p24 assay enhances p24 quantification in plasma** 165 **samples of people with HIV**

166 After defining the best experimental conditions to quantify the levels of p24 in the  
167 plasma, we evaluated the sensitivity of Simoa p24 detection in plasma samples from  
168 PWH. We first determined a cutoff to best discriminate between positive and negative

169 samples by analyzing plasma from HIV negative donors (n=15). Based on the results  
170 obtained, we determined 24 fg/mL (calculated as 2.5 standard deviations from the  
171 mean signal in plasma from HIV negative donors, see methods for further detail) as the  
172 cutoff to consider an ICD-treated plasma sample as positive for p24 in our experimental  
173 conditions (Figure 2A), and we used this cutoff in our analyses. This value is higher than  
174 the lower limit of quantification (10 fg/mL) and the lower limit of detection (LoD, 3  
175 fg/mL) proposed for the assay, likely due to interference by some blood components  
176 in protein quantification.

177

178 We next evaluated the levels of p24 in plasma samples from 25 PWH displaying a wide  
179 range of detectable viremia ranging from 1.7 to 7 HIV-1 RNA Log copies/mL. The  
180 treatment of plasma samples with Glycine pH 1.8 for ICD increased the p24 levels  
181 detected compared to non-treated samples ( $p=0.0026$ ) (Figure 2B). After ICD, 19/25  
182 samples had p24 levels above our experimental cutoff. While p24 was often detected  
183 in samples with VL  $>10^4$  copies/mL (18/19 positive samples), the sensitivity dropped  
184 sharply for samples with lower VL (1/6 positive samples). Indeed, we found a strong  
185 correlation between p24 values and the corresponding plasma viral loads (Figure 2C;  
186  $r=0.86$ ,  $p<0.0001$ ). The correlation was still statistically significant when we considered  
187 only the samples with p24 values above the cutoff ( $r=0.76$ ,  $p<0.0001$ ). This correlation  
188 with viral RNA was not observed when we used the p24 values obtained in samples  
189 not treated by ICD ( $r=0.41$ ,  $p=0.12$ ). These analyses showed that immune-complex  
190 dissociation enhanced the ultrasensitive quantification of p24 in the plasma of PWH.

191

192 **Ultrasensitive p24 detection in a cohort of PWH during primary HIV-1 infection**

193 To validate our approach, we analyzed the ultrasensitive p24 quantification in 92 plasma  
194 samples from a subcohort of people with primary HIV-1 infection from the ANRS CO6  
195 PRIMO cohort (27). Samples from 92 individuals displaying highly variable plasma RNA  
196 viral loads were analyzed (Figure 3A, median: 48,263 copies/mL; range: 24 –  
197 211,360,000). The median time since estimated HIV acquisition was 52 days (range:  
198 20-183 days). We detected  $_{us}p24$  values above the experimental cutoff in 72 of the 92  
199 samples (Figure 3A). There was a wide range of p24 antigen concentrations (median  
200 1.013 pg/mL; IQR [0.027 – 191.4]). As before, a strong correlation was observed  
201 between viral RNA and  $_{us}p24$  in the plasma samples analyzed (Figure 3B;  $r=0.7791$ ,  
202  $p<0.0001$ ). We also found a strong positive correlation between  $_{us}p24$  levels and the  
203 cell-associated HIV-DNA (Figure 3C,  $r=0.64$ ,  $p<0.0001$ ). These correlations were still  
204 statistically significant when we considered only the samples with  $_{us}p24$  values above  
205 the cutoff ( $r=0.69$ ,  $p<0.0001$  and  $r=0.61$ ,  $p<0.0001$  for HIV-RNA levels and HIV-DNA  
206 levels respectively). A weak negative correlation was observed between  $_{us}p24$  levels  
207 and the CD4+ T cell counts corresponding to these samples (Figure 3D;  $r=-0.22$ ,  
208  $p=0.03$ ), although only a trend was found with samples with above cutoff-p24 levels  
209 ( $r=-0.22$ ,  $p=0.07$ ).

210

211 These data showed that  $_{us}p24$  can be quantified after ICD in plasma samples from PWH  
212 in different stages of primary infection. Moreover, the p24 levels were associated with  
213 other classic biomarkers of HIV-1 infection.

214

215 **Ultrasensitive p24 quantification in PWH at chronic HIV-1 infection prior to and after**  
216 **cART initiation**

217 ICD should enable the quantification of p24 during chronic infection despite the  
218 presence of anti-p24 antibodies. We thus quantified  $_{us}p24$  in plasma samples from 137  
219 volunteers with chronic HIV-1 infection (viral loads are depicted in Figure 4A) who were  
220 enrolled in the ANRS REFLATE TB trial and initiated cART treatment. Before cART (W0),  
221 p24 values above cutoff were detected in 98 out of 137 samples (Figure 4A). There was  
222 again a statistically significant correlation between  $_{us}p24$  and the RNA viral load in the  
223 plasmas analyzed (Figure 4B,  $r=0.42$ ,  $p<0.0001$ ). We also found a positive correlation  
224 between  $_{us}p24$  levels and CD4+ T cell associated HIV-DNA levels (Figure 4C,  $r=0.32$ ,  
225  $p=0.0004$ ) and a trend for a weak negative correlation with CD4+ T cell counts (Figure  
226 4D,  $r=-0.15$ ,  $p=0.07$ ).

227

228 Next we aimed to analyze  $_{us}p24$  values after cART treatment. The participants at the  
229 ANRS REFLATE trial were co-infected with tuberculosis. They initiated antiretroviral  
230 treatment (raltegravir (400mg), raltegravir (800mg) or efavirenz (600mg), with  
231 tenofovir and lamivudine) 2-8 weeks after treatment for TB was initiated. We  
232 compared the  $_{us}p24$  values prior to cART initiation to those 24 and 48 weeks after cART  
233 initiation in the 108 participants with samples available throughout the follow up (W0,  
234 W24 and W48). There was a dramatic decrease of  $_{us}p24$  levels at weeks 24 and 48 after  
235 cART initiation, in line with the decrease in RNA viral load levels observed at the same  
236 time points (Figure 5A and (28)). The  $_{us}p24$  became undetectable for 89/108  
237 participants after 48 weeks on cART (Figure 5A). Among the 19 individuals with  $_{us}p24$   
238 above the cutoff at W48, 10 also had detectable  $_{us}p24$  at W24. Five had detectable viral  
239 RNA loads at W48. Overall, 14/19 samples with detectable  $_{us}p24$  levels at W48 were  
240 characterized by persistent levels of antigen and/or transient viral RNA relapse on

241 cART. We observed several differences when we compared the characteristics before  
242 cART initiation of these 19 participants with the characteristics of the other  
243 participants whose p24 levels were detectable before cART and dropped to  
244 undetectable levels with treatment (n=59). Although both subgroups of participants  
245 had similar RNA viral loads and  $_{us}p24$  levels before treatment, we found a trend for a  
246 lower HIV-RNA/ $_{us}p24$  ratio in the participants who maintained detectable  $_{us}p24$  after  
247 48 weeks of treatment (Figure 5B). Moreover, these participants had, before  
248 treatment, lower cell-associated HIV-DNA levels (Figure 5C) and higher CD4+ T cell  
249 counts (Figure 5D) than the participants with undetectable  $_{us}p24$  levels after cART. No  
250 differences were observed for the same parameters at W48. The baseline  
251 demographic and clinical characteristics of the participants with positive or negative  
252  $_{us}p24$  at W48 after cART initiation are shown in Table 1.

253  
254 We analyzed the association of  $_{us}p24$  levels with some of the main plasma markers  
255 associated with inflammation and coagulation in PWH measured in the same samples  
256 (28). At W0, a weak significant correlation was observed between  $_{us}p24$  levels with D-  
257 dimers ( $r=0.24$ ,  $p=0.006$ ) and IFN $\alpha$  ( $r=0.38$ ,  $p<0.001$ ) (Figure 6A), and these correlations  
258 were still statistically significant when we took into consideration only the  $_{us}p24$  values  
259 above the cutoff ( $r=0.23$ ,  $p=0.03$ ; and  $r=0.25$   $p=0.02$ , for D-dimers and IFN- $\alpha$   
260 respectively). We did not find correlations between  $_{us}p24$  and CRP, IL-6 or sCD14. No  
261 correlations were found between  $_{us}p24$  and soluble plasma markers at W48. We then  
262 studied the subgroup of participants who remained detectable for p24 at W48. They  
263 also differed for the levels of some inflammatory markers before treatment initiation.  
264 While the participants whose  $_{us}p24$  levels became undetectable with cART had higher

265 IFN $\alpha$  levels at W0 (Figure 6B), the participants whose  $_{us}p24$  levels remained detectable  
266 were characterized by higher plasma levels of CRP (Figure 6C), and tended to have  
267 higher levels of D-dimers (Figure 6D) and IL-6 (Figure 6E). These differences were lost  
268 after 48 weeks of cART (not shown). Altogether, these results show that  $_{us}p24$  can be  
269 monitored throughout HIV-1 infection and may have a complementary value to other  
270 biomarkers. Moreover, the detection of p24 in the plasma of PWH under cART may  
271 reveal the presence of a subset of people with particular immunovirological  
272 characteristics.

273 **Discussion**

274 Plasma HIV-RNA and CD4+ T cell counts are commonly used biomarkers to monitor  
275 HIV-1 infection. However, these markers do not always reflect the evolution of  
276 infection, in particular the occurrence of comorbidities or viral reactivation events  
277 during antiretroviral treatment. Here, we developed a method that allowed  
278 quantification of  $_{us}p24$  despite the presence of anti-p24 antibodies. We show that  
279 ultrasensitive p24 quantification is a robust virologic marker associated with HIV-1 RNA  
280 and DNA during primary and chronic HIV-1 infection, which recapitulates viral  
281 suppression observed upon cART, but may also provide added value regarding events  
282 dissociated from viral replication.

283

284 In the present work we report a higher frequency of p24 detection in samples with  
285 viral loads higher than  $10^3$  copies/mL, which correspond to an increment of at least 1  
286 Log when compared to conventional ELISA methods (23, 24, 29). Overall, we estimate  
287 that current standard immunoassays would allow p24 detection in a very small fraction  
288 (15%) of the samples included in this study. Here, the quantification of p24 was  
289 possible in > 70% of tested samples. As compared to nucleic acid quantification, the  
290 sensitivity of the  $_{us}p24$  assay is still limited, due in part to the amount of plasma that  
291 can be tested. The inclusion of a prior ultracentrifugation step represents an increase  
292 in the hands-on time, but efficiently increases the sensitivity of immunoassays for  
293 quantification of p24 in plasma samples with RNA viral loads at the magnitude of  $10^2$   
294 copies/mL (not shown).

295

296 Numerous studies have shown that chronic HIV-related immune activation and  
297 inflammation are associated with increased risk of morbidity and mortality in PWH (30-  
298 35). Moreover, some plasma markers better predict evolution of HIV-1 infection than  
299 viral load or CD4+ T cell counts (36-38). We found that  $_{us}p24$  was correlated with  
300 plasma levels of the coagulation marker D-dimer during chronic infection. We also  
301 found a correlation between  $_{us}p24$  and  $IFN\alpha$ , which could also be quantified by the  
302 ultrasensitive digital assay in the same samples. Some recent studies have shown that  
303 markers of HIV-1 transcription may be better associated with systemic inflammation  
304 than viremia or the levels of integrated proviruses (39-42). The relative association of  
305 available biomarkers of infection with the levels of inflammation may depend on the  
306 various factors that sustain chronic inflammation (33, 43).

307

308 Strong correlations were found between HIV-RNA and  $_{us}p24$  in the samples obtained  
309 during primary and chronic infection, and the overall  $_{us}p24$  detection rate was similar  
310 in the samples from the chronically infected cohort than in those from the PHI cohort  
311 (72% vs 78%). However, while in the PHI cohort we could detect p24 in most samples  
312 with  $VL > 10^4$  RNA copies/mL (64/68 samples with detectable  $_{us}p24$ ), the  $_{us}p24$  detection  
313 rate was lower in the chronic samples with equivalent VL (94/121 samples with  
314 detectable  $_{us}p24$ ,  $p=0.008$ ). The relative half-lives of HIV-1 p24 and RNA diverge during  
315 the course of infection (44) and the difference of antibodies abundance and avidity  
316 between PHI and chronic infection could also impact the ICD efficacy (26). It was  
317 indeed previously described that ICD can be incomplete in some individuals, depending  
318 on the concentration and avidity of specific anti-HIV antibodies in the samples (45). It  
319 is also important to notice that the samples corresponding to chronic infection came

320 from participants co-infected with HIV-1 and TB, which were characterized by higher  
321 inflammation levels than PWH with no TB co-infection, even after 48 weeks of  
322 suppressive antiretroviral treatment (28). Additional studies including PWH without  
323 co-infections should be done in the future to elucidate whether  $_{us}p24$  may in some  
324 cases better reflect viral activity in blood and/or in lymphoid tissues than viral RNA.  
325 Further studies might also analyze its potential contribution to chronic inflammation.  
326 In any case, HIV-1  $_{us}p24$  and RNA levels may provide complementary readouts to  
327 monitor infection. It is reasonable to imagine that p24 levels during chronic infection  
328 can indicate the presence of viral particles, in particular without cART, but also antigen  
329 production by cells carrying non-productive viruses, which frequency increases with  
330 duration of infection (46-48). The latter might particularly be the case during  
331 antiretroviral treatment. The half-life of p24 outside viral particles was shown to be  
332 around 42 days, and thus longer than for viral RNA, which may allow the detection of  
333 p24 even after viral replication is suppressed (13). It was indeed intriguing to observe  
334 that in some samples p24 remained detectable under cART despite undetectable viral  
335 RNA levels. These samples came from participants with marked immunological and  
336 virological differences before treatment initiation. The observation that in these  
337 individuals there was a trend towards lower RNA/p24 ratio before ART initiation and  
338 that in most cases  $_{us}p24$  could be detected both in the W24 and W48 samples, invites  
339 to think that these individuals might carry infected cells with higher antigen  
340 production. It is tempting to speculate that this may have led to enhanced stimulation  
341 of immune responses before treatment initiation (and perhaps at a lesser extent also  
342 under cART), which may explain higher levels of inflammatory markers at lower  
343 frequency of infected cells at that point. Unfortunately, no analyses of T or B cell

344 activation, nor of HIV-specific responses were available for this study. The use of <sub>us</sub>p24  
345 as a biomarker in HIV infection and persistence will require validation in other cohorts  
346 of PWH.

347

348 In conclusion, this study shows that digital ELISA combined with acid-ICD allows the  
349 monitoring of <sub>us</sub>p24 in individuals with primary and chronic HIV-1 infection. This novel  
350 tool may provide helpful information to monitor the burden of infection during cART.

351 Moreover, this marker could be helpful to investigate the presence of HIV antigens in  
352 samples from participants undergoing interventions aiming at HIV remission or  
353 eradication, such as HIV reservoir reactivation in “shock and kill” strategies (49).

354 **Methods**

355 **Cohorts and ethics statement**

356 Blood samples from HIV-negative controls were obtained from the French Blood Bank  
357 (Etablissement Français du Sang) in the context of a collaboration agreement with the  
358 Institut Pasteur (C CPSL UNT, number 15/EFS/023). Plasma samples from people with  
359 acute and chronic HIV-1 infection monitored for virologic markers at the Hospital  
360 Necker-Enfants malades (France) were used to determine the efficacy of ICD and the  
361 sensitivity of the Quanterix Simoa p24 assay in plasma samples. Plasma samples from  
362 the French National Agency for Research on AIDS and Viral Hepatitis (ANRS) CO6  
363 PRIMO cohort (50) and 12180 Reflate TB trial (51) were used to validate the protocol  
364 for ultrasensitive p24 detection combined with ICD. People with primary HIV infection  
365 (PHI) were enrolled in the ANRS CO6 PRIMO following the inclusion criteria as  
366 described in (50). The cohort was approved by the Ile-de-France-3 Ethics Committee  
367 and all patients give their written informed consent. The ANRS 12180 Reflate TB trial  
368 was carried out in accordance with the ANRS Ethical Chart for Research in Developing  
369 Countries, the Brazilian regulatory requirements for clinical trials and the Declaration  
370 of Helsinki. The protocol was approved by national and local ethics committees in Brazil  
371 (Comissão Nacional de Ética em Pesquisa [CONEP] and Comitê de Ética em Pesquisa  
372 [CEP] at IPEC/FIOCRUZ) and France (Comité de Protection des Personnes de Paris Ile-  
373 de-France-I). The experiments were conducted with the understanding and the written  
374 informed consent of each participant.

375

376 **Samples**

377 EDTA blood samples were collected from each individual analyzed. Plasmas were  
378 collected after centrifugation at 1800 rpm for 20 minutes and stored at -80°C.

379

#### 380 **Immune-complex dissociation (ICD)**

381 Heat-mediated dissociation (52), with SDS and DTPA (26) and acid dissociation (53-55)  
382 methods were tested for compatibility with ultrasensitive HIV-1 Gag p24 detection.

383 *Heat-mediated immune-complex dissociation:* Plasma samples (100 µL) were diluted  
384 1/3 with distilled water and incubated for 5 minutes at 95°C in a water bath, then  
385 cooled to room temperature prior to p24 quantification.

386 *Heat-mediated immune-complex dissociation with SDS and DTPA:* Plasma samples (100  
387 µL) were diluted 1/3 with a solution of 7mM sodium dodecyl sulfate (SDS) and 1.5 mM  
388 diethylenetriaminepentaacetic acid (DTPA), pH 7.2. Samples were incubated for 5  
389 minutes at 95°C in a water bath, then cooled to room temperature prior to p24  
390 quantification.

391 *Acid dissociation:* Plasma samples (100 µL) were diluted 1:1 with 1.5 M glycine-HCl pH  
392 1.8, incubated 60 minutes at 37°C, and then neutralized with 1.5 M Tris-HCl pH 9.0. In  
393 parallel we tested 1.5 M glycine-HCl pH 2.5 and neutralization with 1.5 M Tris-HCl pH  
394 7.5.

395

#### 396 **Ultrasensitive p24 (<sub>us</sub>p24) digital immunoassay**

397 Plasma concentration of HIV-1 Gag p24 was determined on a Simoa HD-1 analyzer  
398 using the Simoa HIV p24 kit (Quanterix, USA) following manufacturer's instructions.

399 Plasma samples were thawed at room temperature, centrifuged at 3000rpm for 5  
400 minutes, and then inactivated with Triton X-100 (final concentration 2%) prior to p24

401 quantification. Four-parameter logistic (4PL) regression fitting was used to estimate  
402 the concentration of p24. Samples below the limit of quantification were given an  
403 arbitrary value of 0.024 pg/mL based on the established cutoff (cutoff was determined  
404 based on the p24 AEB signal in plasma from HIV negative donors. It was calculated as  
405 2.5 standard deviations from the mean of p24 AEB signal. Figure 2A represents the  
406 range of background p24 signal in HIV negative samples, all below the established  
407 cutoff of 0.024 pg/mL).

408

#### 409 **IFN $\alpha$ ultrasensitive digital immunoassay**

410 Plasma samples from the ANRS CO6 PRIMO cohort and the ANRS Reflate TB trial were  
411 assayed for IFN $\alpha$ . Plasma samples were thawed at room temperature, centrifuged at  
412 3000rpm for 5 minutes, and then inactivated with Triton X-100 (final concentration  
413 2%) prior to IFN $\alpha$  quantification. Plasma concentration of IFN $\alpha$  was determined on a  
414 HD-1 analyzer using the Simoa human IFN $\alpha$  kit (Quanterix, USA) following  
415 manufacturer's instructions. Four-parameter logistic (4PL) regression fitting was used  
416 to estimate the concentration of IFN $\alpha$ .

417

#### 418 **Clinical parameters and inflammation biomarkers**

419 ANRS PRIMO cohort viral RNA loads in plasma were determined with the COBAS  
420 AmpliPrep/COBAS TaqMan HIV-1 Test, v2.0 (Roche Diagnostics, Germany). Total cell-  
421 associated HIV-1 DNA was quantified with the Generic HIV-1 DNA Cell kit (Biocentric,  
422 Bandol, France). CD4+ T-cell counts were determined by flow cytometry as previously  
423 described (50, 56). The date of infection was estimated based on the date of symptom  
424 onset minus 15 days, or, in asymptomatic patients, the date of the incomplete Western

425 blot finding minus 1 month or the midpoint between a negative and a positive ELISA  
426 result (57).

427

428 The clinical parameters and inflammation biomarkers for the participants to the ANRS  
429 Replate TB trial were determined as described in (51) and (28). Briefly, CD4+ T-cell  
430 counts were determined by flow cytometry. Plasma HIV-RNA was determined either  
431 with the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, v2.0 (Roche Diagnostics,  
432 Germany) or the VERSANT HIV-RNA 3.0 assay (bDNA; Bayer, USA). Total cell-associated  
433 HIV-1 DNA was quantified with the Generic HIV-1 DNA Cell kit (Biocentric, Bandol,  
434 France). hsCRP and IL-6 levels were measured with the high-sensitivity Tina-quant C-  
435 Reactive Protein Gen.3 and the Elecsys IL-6 Immunoassay kits, respectively. D-Dimers  
436 were quantified using the STA -Liatest D-Di Plus. Human sCD14 was measured with the  
437 hCD14 Quantikine ELISA Kit.

438

#### 439 **Statistical analyses**

440 Statistical analyses were performed using GraphPad Prism, version 8.3.1. Comparisons  
441 between groups were based on the nonparametric Mann-Whitney test. Correlations  
442 were performed using the Spearman test. Differences were considered significant at a  
443 *P* value <0.05.

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448

449 **DECLARATION OF INTERESTS**

450 The authors declare no competing interests.

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660 **Figure legends**

661 **Figure 1. Acid dissociation to disrupt antigen-antibodies immune-complexes is**  
662 **compatible with ultrasensitive p24 quantification. A)** Bars denote the average  
663 enzymes per beads (AEB) for: 0; 0.01; 0.02; 0.07; 0.24; 0.67; 2.39 and 13.17 pg/mL of  
664 p24 standards tested in different experimental conditions for immune-complex  
665 dissociation, as follows: non-treated (black), heat-mediated (red), heat-mediated with  
666 SDS and DTPA (orange) and acid dissociation with Glycine-HCl pH 1.8 (blue) or pH 2.5  
667 (violet). **B)** Four-parameter logistic regression curves obtained from different  
668 experimental conditions for immune-complex dissociation, as follows: non-treated  
669 (black), heat-mediated (red), heat-mediated with SDS and DTPA (orange) and acid  
670 dissociation with Glycine-HCl pH 1.8 (blue) or pH 2.5 (violet). **C)** AEB values obtained  
671 for Standard A (p24 = 0 pg/mL, negative control) (dark gray), Standard A + Triton X-100  
672 2% (light gray), Sample diluent manufactured by Quanterix (dark blue), PBS 1x (light  
673 blue) and the solution of 7mM SDS + 1.5 mM DTPA pH 7.2 (orange). Dashed line  
674 indicates the median value obtained for Standard A condition. **D)** AEB values obtained  
675 from plasma samples from HIV-1 negative donors non-treated (gray), after heat-  
676 mediated dissociation with SDS and DTPA (orange) and after acid dissociation with  
677 Glycine-HCl pH 1.8 (green). Dashed line indicates the median value obtained for non-  
678 treated condition.

679

680 **Figure 2. Sensitivity of ultrasensitive p24 quantification in plasma samples. A)** p24  
681 signal in plasma samples of HIV-1 negative donors. Samples received acid dissociation  
682 treatment with Glycine-HCl pH 1.8. Median is indicated by black line. Dashed line  
683 denotes the cutoff value of 0.024 pg/mL calculated as 2.5 standard deviations from the

684 mean of p24 signal in the plasma from HIV negative donors. **B)** p24 levels detected in  
685 plasma samples from HIV-1 infected individuals non-treated for immune-complex  
686 dissociation (NT, open symbols) and after acid dissociation with Glycine-HCl pH 1.8  
687 (ICD, green dots). **C)** Relationship between positive p24 and HIV-RNA in plasma  
688 samples from HIV-1 infected individuals. Correlation was calculated using a  
689 nonparametric Spearman test.

690

691 **Figure 3. Ultrasensitive p24 detection in a cohort of acutely HIV infected individuals.**

692 **A)**

693 *Left panel:* p24 levels in plasma samples from 92 individuals acutely infected with HIV-  
694 1 (ANRS PRIMO Cohort). Acid dissociation with Glycine-HCl pH 1.8 was used to disrupt  
695 immune-complexes. Samples below the limit of quantification were given an arbitrary  
696 value of 0.024 pg/mL based on the established cutoff. *Right panel:* HIV-RNA levels in  
697 plasma samples from the same 92 individuals acutely infected with HIV-1. Median is  
698 indicated by black line. Relationship between **B)** p24 and HIV-RNA, **C)** p24 and HIV-DNA  
699 and **D)** p24 and CD4+ T cell counts in plasma samples from individuals acutely infected  
700 with HIV-1. Correlations were calculated using a nonparametric Spearman test.

701

702 **Figure 4. Ultrasensitive p24 detection in a cohort of chronically HIV infected**

703 **individuals.** *Left panel:* p24 levels in plasma samples from 137 individuals chronically  
704 infected with HIV-1 (ANRS 12180 Replate TB trial). Acid dissociation with Glycine-HCl  
705 pH 1.8 was used to disrupt immune-complexes. Samples below the limit of  
706 quantification were given an arbitrary value of 0.024 pg/mL based on the established  
707 cutoff. *Right panel:* HIV-RNA levels in plasma samples from the same 137 individuals

708 chronically infected with HIV-1. Median is indicated by black line. Relationship  
709 between **B)** p24 and HIV-RNA, **C)** p24 and HIV-DNA and **D)** p24 and CD4+ T cell counts  
710 in plasma samples from individuals chronically infected with HIV-1. Correlations were  
711 calculated using a nonparametric Spearman test.

712

713 **Figure 5. p24 levels in patients chronically infected with HIV prior to and after cART**

714 **initiation. A)** p24 levels in plasma samples from 108 individuals chronically infected  
715 with HIV-1 (ANRS 12180 Replate TB trial) prior to antiretroviral treatment initiation  
716 (W0) and longitudinally monitored at weeks 24 and 48 after cART. Baseline (W0)  
717 differences between patients who presented detectable or undetectable p24 levels 48  
718 weeks after cART initiation: **B)** HIV-RNA levels (left panel), p24 (middle panel) and the  
719 ratio HIV-RNA/p24 (right panel), **C)** HIV-DNA, **D)** CD4+ T cell counts. Median is indicated  
720 by black lines. \*\* <0.01, \*\*\*\* < 0.0001.

721

722 **Figure 6. Association of p24 levels and inflammation markers in patients chronically**

723 **infected with HIV prior to and after cART initiation. A)** Relationship between p24 and  
724 D-dimers (Left panel) and IFN $\alpha$  (Right panel) in plasma samples from 137 individuals  
725 chronically infected with HIV-1 (ANRS 12180 Replate TB trial) prior to cART initiation  
726 (W0). Correlations were calculated using a nonparametric Spearman test. Baseline  
727 (W0) differences between 108 patients who presented detectable or undetectable p24  
728 levels 48 weeks after cART initiation: **B)** IFN $\alpha$ , **C)** CRP, **D)** D-dimers, and **E)** IL-6. Median  
729 is indicated by black lines. \* <0.05, \*\* <0.01.

730 **Table 1.** Baseline demographic and clinical characteristics of the participants\* enrolled in the ANRS 12180 Reflate TB trial with <sub>us</sub>p24 positive or negative at W48 after cART  
 731 initiation

Characteristics		Frequency		p-value
		p24+ at W48 (n=19)	p24- at W48 (n=89)	
Age (median and IQR)		32 [27-42]	38 [31-44]	0.1607
Gender	Male	13 (68.4%)	63 (70.8%)	0.8376
	Female	6 (31.6%)	26 (29.2%)	
cART arm	Efavirenz	8 (42.1%)	32 (36.0%)	0.5580
	Raltegravir 400mg	4 (21.1%)	30 (33.7%)	
	Raltegravir 800mg	7 (36.8%)	27 (30.3%)	
Response to TB treatment	Cure	3 (15.8%)	13 (14.6%)	0.8921
	Treatment completed	16 (84.2%)	75 (84.3%)	
	Treatment failure	0 (0%)	1 (1.1%)	
IRIS	Yes	1 (5.3%)	9 (10.1%)	0.5080
	No	18 (94.7%)	80 (89.9%)	
Resistance to ARV treatment (follow up)	Yes/Potential	3 (15.8%)	11 (12.4%)	0.6862
	No	16 (84.2%)	78 (87.6%)	
Resistance to ARV treatment (W48)	Yes/Potential	1 (5.3%)	2 (2.2%)	0.4677
	No	18 (94.7%)	87 (97.8%)	
AUC viral load (median and IQR)		255,302 [77,868-1,002,300]	180,736 [73,990-745,161]	0.6597
Frequency detectable viral load (>50 copies/mL)	W4	9 (47.4%)	50 (56.2%)	0.4837
	W12	4 (21.1%)	18 (20.2%)	0.9352
	W24	3 (16.7%)	12 (13.5%)	0.7227
	W48	6 (31.6%)	8 (9%)	<b>0.0078</b>

732 \* Participants for whom samples prior to cART initiation and at 24 and 48 weeks after cART initiation were available (108 participants).

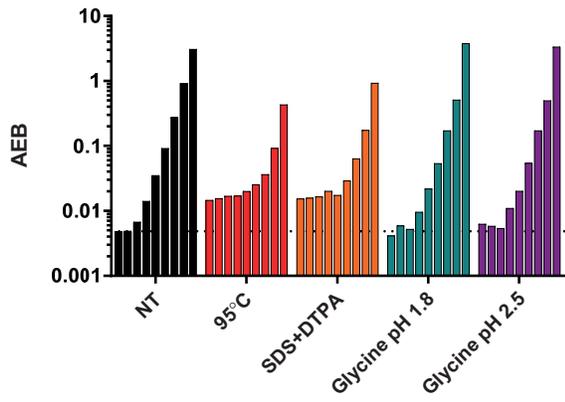
733 Response to TB treatment was defined as: Cure: Patient who is sputum smear-negative in the last month of treatment and on at least one previous occasion; Treatment completed:

734 Patient who has completed treatment but who does not meet the criteria to be classified as a cure or a failure; Treatment failure : patient who is sputum smear-positive at 5 months or

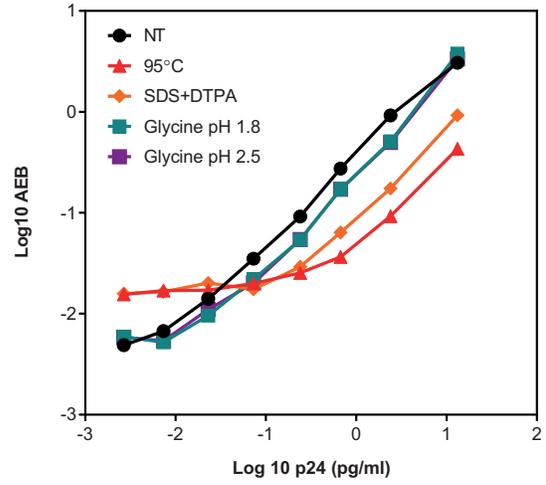
735 later during treatment and also a patient who was initially smear-negative before starting treatment and became smear-positive after completing the initial phase of treatment.

Figure 1

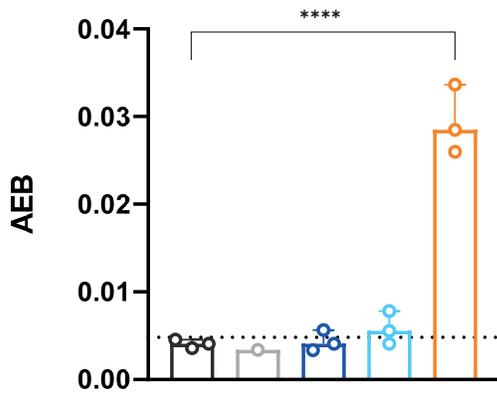
A



B

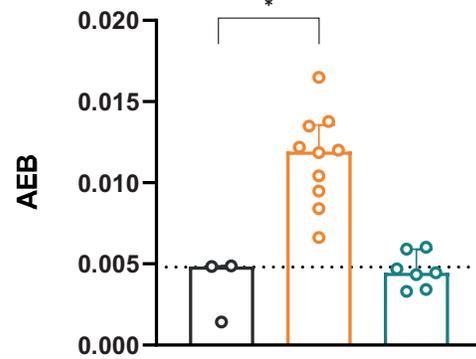


C



- STD A
- STD A + Triton X-100 2%
- Sample diluent
- PBS
- SDS+DTPA

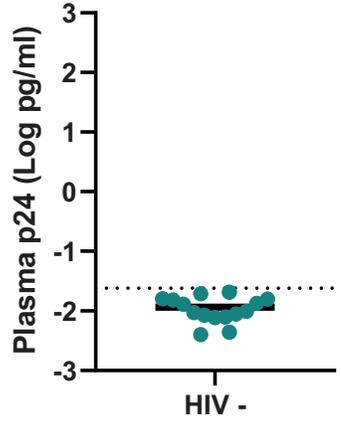
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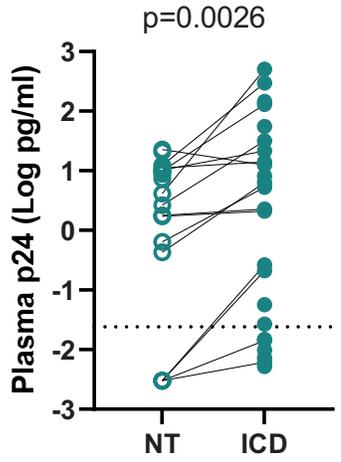
- HIV- non-treated
- HIV- SDS+DTPA
- HIV- Glycine pH1.8

Figure 2

A



B



C

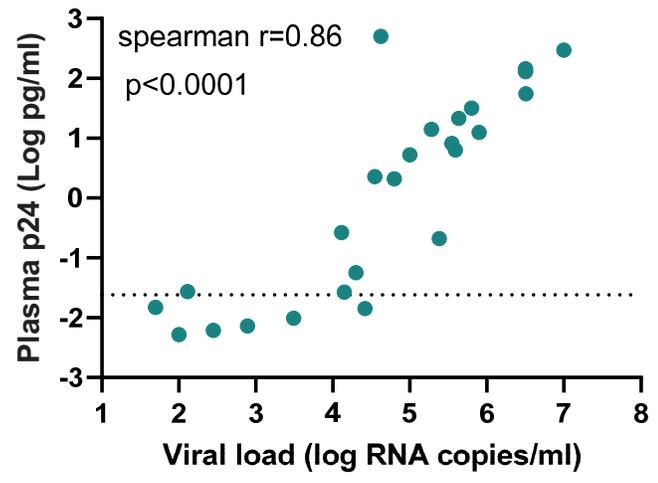


Figure 3

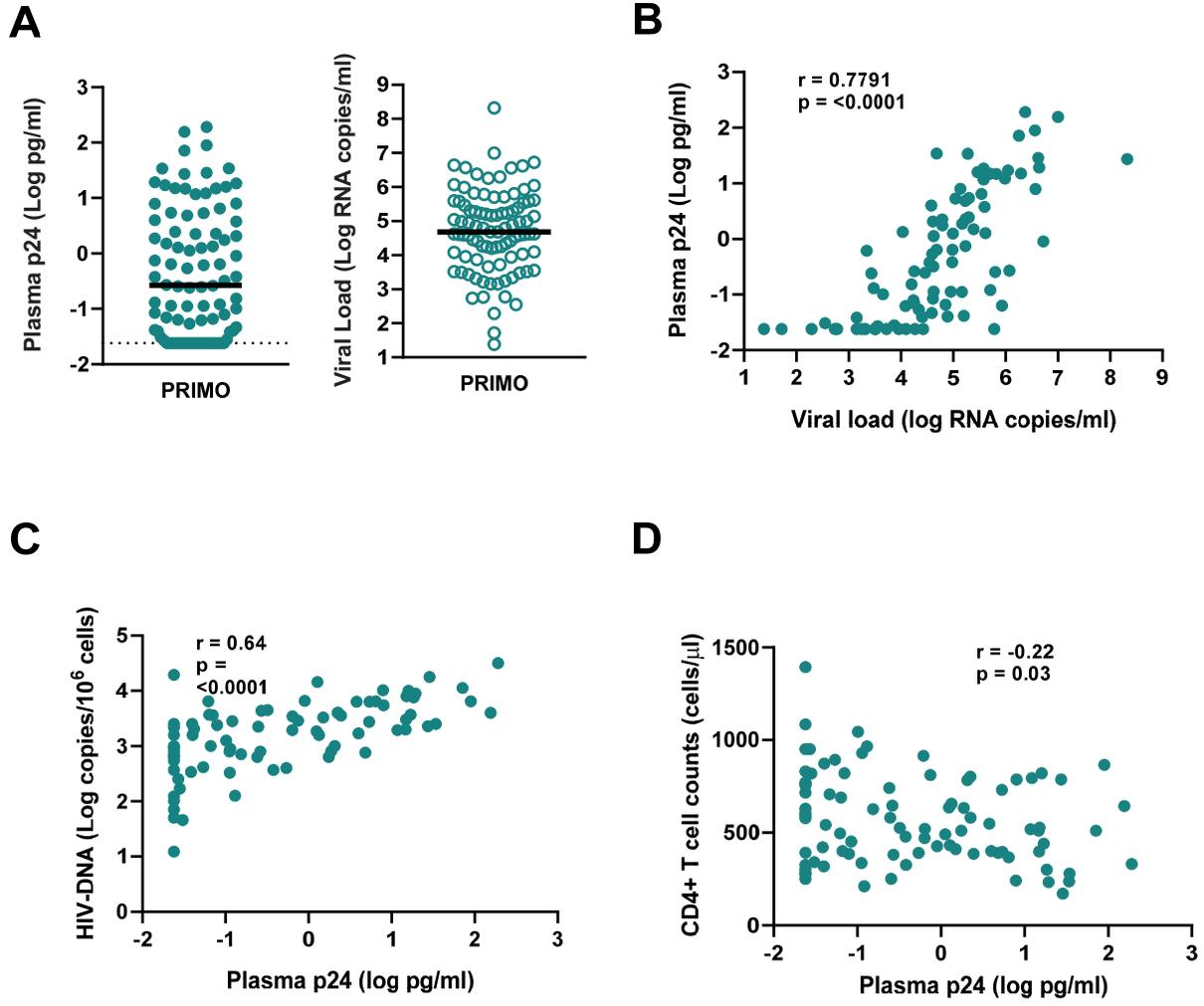
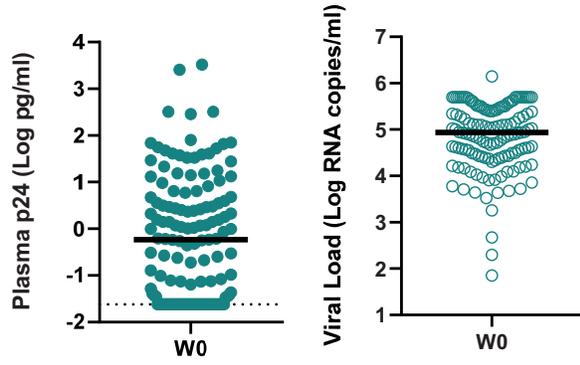
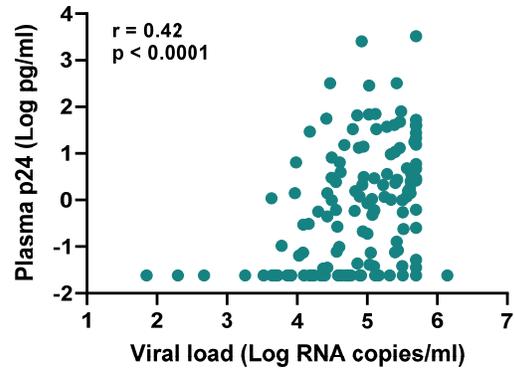


Figure 4

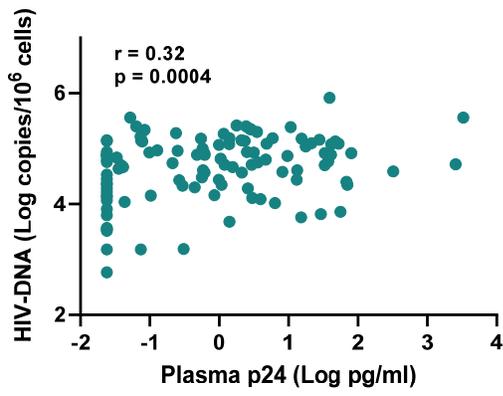
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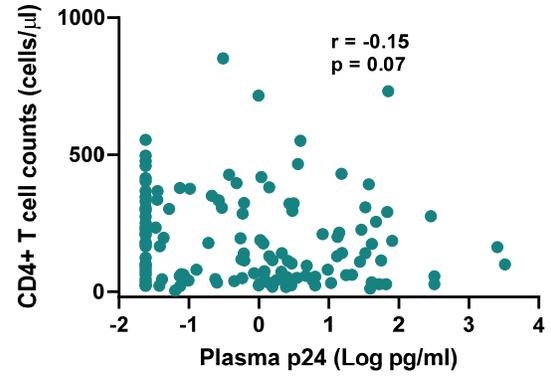
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C

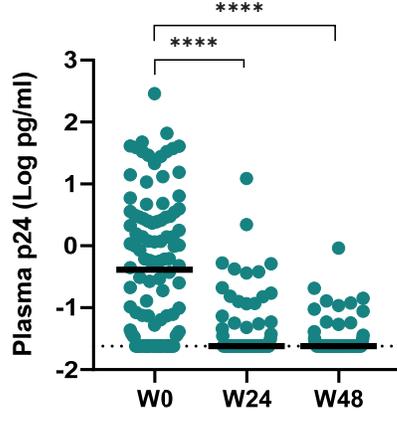


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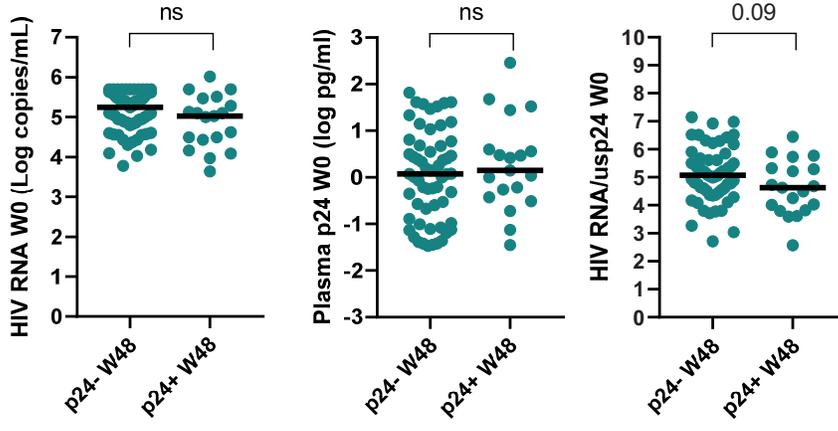


## Figure 5

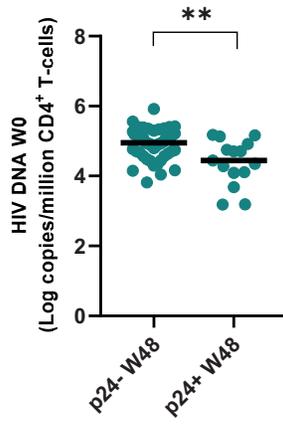
A



B



C



D

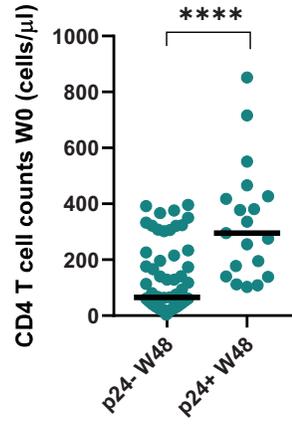
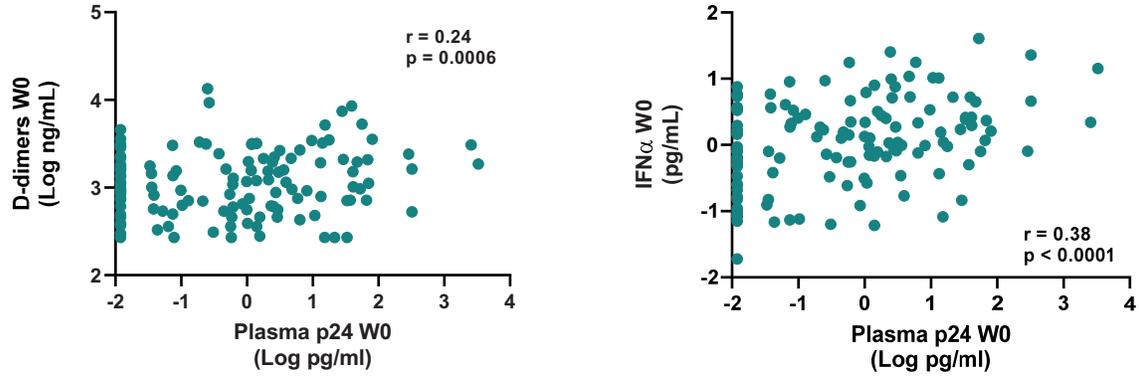
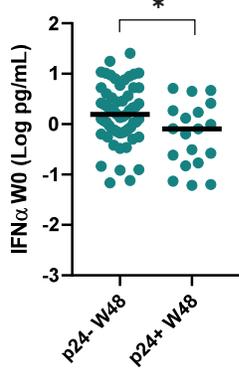


Figure 6

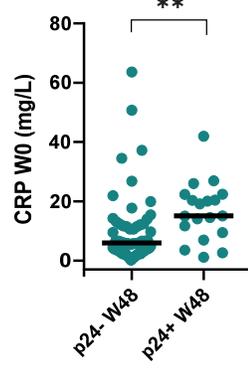
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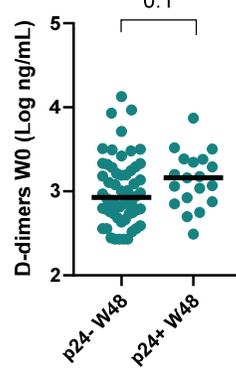
B



C



D



E

