

1 **Platelets from cART-suppressed HIV-infected patients with poor CD4+T cell**
2 **recovery carry infectious HIV**

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29 **One-sentence summary: HIV sheltered in Platelets from cART-suppressed patients in**
30 **immunological failure**

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32 **ABSTRACT**

33

34 Beside hemostasis, human platelets exert several immune functions and interact with infectious
35 pathogens including HIV, although for uncertain reasons. Here, we demonstrate that platelets from
36 HIV-infected patients shelter infectious HIV *in vivo*, despite successful viral suppression by the
37 combined antiretroviral therapy (cART) and in strong correlation with low blood CD4⁺T-cell
38 counts (<350cells/microL) and nadir (<200cells/microL). Patient platelets carrying HIV can
39 propagate infection to macrophages *in-vitro* in a process prevented by blocking platelet-macrophage
40 interaction with the therapeutical anti-platelet agent Abciximab (anti-integrin α IIb/ β 3 Fab).
41 Moreover, 88% of virally suppressed patients sheltering HIV in platelets are immunological
42 nonresponders and fail to restore a proper immune status during cART with a >50-fold higher
43 likelihood than patients without HIV in platelets (OR: 56, 95%CI: 4.3-719.2, $p=0.002$). Altogether,
44 our results show that platelets act as transient infectious HIV carriers despite cART, thus
45 establishing an alternative pathway for HIV dissemination in correlation with immunological
46 failure.

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60 **MAIN TEXT**

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62 Human platelets, which primarily function in hemostasis, also contribute to important
63 immunological functions (1). Platelets are capable of engulfing viral and non-viral infectious
64 pathogens (2), thus participating in innate immune responses: platelets directly interact with and
65 internalize human immunodeficiency virus (HIV), Dengue virus, Hepatitis C virus, Influenza virus,
66 *Staphylococcus aureus* and other pathogens at least *in vitro* (3-11). We and others have shown a
67 direct interaction between HIV, platelets and megakaryocytes, the platelet precursors (5, 7, 10-13),
68 mainly obtained *in vitro* in conditions requiring large quantities of laboratory-adapted cell-free virus
69 to interact with platelets, these experimental conditions compromising biological relevance (7, 10),
70 but also *in vivo* in studies performed at a pre-cART era and detecting exclusively viral RNA but not
71 replication-competent viruses (12, 13). Evidence for platelets containing infectious HIV *in vivo*,
72 especially in cART-treated patients, the significance and clinical implication of the interaction
73 between HIV and platelets and their role in AIDS remain elusive.

74 To address the relevance of HIV-containing platelets *in vivo*, we isolated platelets from
75 HIV-infected patients under cART with undetectable, namely below the limit of detection (LOD) of
76 $1.6 \log_{10}$ HIV RNA copies/ml, or detectable viral load at the date of sampling (Tables S1-2). We
77 first evaluated the presence of HIV in platelets by qPCR in 39 of these patients (Table S2, Figure
78 1A). In patients with undetectable viral load in the blood plasma, a subgroup of patients, referred to
79 as the positive group (in orange), has platelets containing HIV RNA (9.92 [CI: 6.5-17.8] copies per
80 million platelets in the positive group) although at lower amount as compared with viremic patients
81 (122.3 [CI:65-1111] copies per million platelets in the positive group) (Figure 1A). Longitudinal
82 clinical data obtained from these positive patients with undetectable viral load at date of sampling
83 indicate that viral load from 6 out of 9 of them was consistently undetectable (below LOD) for more
84 than 1 year (14 [CI:0-61] median months prior sampling, patients marked with * in Table S3), thus
85 precluding detection of blood viruses non-specifically attached to platelets. Furthermore, platelet-

86 rich plasma (PRP) samples contain less than 1 lymphocyte per sample tested, corresponding to 2.16
87 [CI:1.4-2.7] CD3⁺ cells per million platelets (Figure S2A-C). Contamination by HIV-infected T-
88 cells is even much lower considering that in cART-treated patients with undetectable below LOD
89 viral load, infected cells represent 0.01 to 0.1% of total blood CD4⁺ T-cells with 100 to 1000 HIV
90 DNA copies per million CD4⁺ T-cells (14). Therefore, contamination by HIV-infected CD4⁺ T-cells
91 and their fragments in platelet preparations is negligible, and contamination values are too low to
92 account for the HIV signal detected in platelets.

93 To identify intact viral particles in platelets and not only cytosolic HIV RNA, detection of
94 HIV RNA by fluorescence *in situ* hybridization (FISH) was combined with that of viral protein
95 p24-Gag by immunofluorescence, both labeling being quantified with a single-cell approach by
96 flow cytometry (Figure 1B and S1A-C), using the recently developed FISH-flow technique (15).
97 FISH-flow is able to detect the very low amount of latent HIV reservoir cells in cART-treated
98 patients, which comprises around 1 infected cell per million CD4⁺ T-cells (15-17). HIV RNA⁺/Gag⁺
99 platelets comprise 0.1% [CI: 0.047-0.15] of total platelets (Figure 1B, left boxplot, orange)
100 circulating in the blood of patients found to contain HIV in platelets. When stratified according to
101 undetectable (below LOD) or detectable viral load at date of sampling (Figure 1B, right boxplot),
102 patients harboring HIV in platelets (in orange) do not differ in the number of HIV RNA⁺/Gag⁺
103 platelets per 10⁶ platelets (1186.99 [CI: 631-1600] and 825.8 [CI: 387-2676], respectively, p>0.05)
104 (compared orange boxplots in Figure 1B, right panel). The HIV RNA⁺/Gag⁺ signal detected in
105 platelets do not originate from HIV-infected CD4⁺ T-cell contaminants, as these cells obtained from
106 cART-treated patients do not transcribe HIV RNA at quantities detectable by FISH-flow unless
107 they are activated with mitogens (Figure S2D), as also described by others (16).

108 HIV is exclusively contained within platelets as shown by p24-Gag immunolocalization
109 under confocal microscopy (Figure 1C, Figure S3A-B) in parallel with flow cytometry (Figure
110 S1D) and electron microscopy (Figure 1D and S3C-D). The viruses are confined within closed
111 vacuoles distinct from the surface-connected canalicular system (SCCS) (Figure 1C-D,

112 arrowheads), as we previously reported *in vitro* (5, 10). To validate our microscopy-based
113 immunostaining approaches, p24-Gag⁺ immunostaining was evaluated by flow cytometry that
114 results into a clear discrimination between patients displaying or not HIV in platelets based on p24-
115 Gag⁺ mean fluorescence intensities (Figure 1C right graph, Figure S1D, negative group in grey,
116 positive group in orange). Again, HIV proteins are detected in platelets from a subset of cART-
117 treated patients either viremic or with viral load below LOD at date of sampling.

118 HIV detected in platelets by microscopy are compartmentalized in vesicles distinct from
119 platelet canalicular networks but resembling the virus-containing compartments typically formed in
120 HIV-infected myeloid cells *in vitro* (18) but also *in vivo* (19, 20); these compartments are
121 functionally distinct from the cell endosomal system, providing thus a shelter for the virus against
122 the direct action of neutralizing antibodies, cytoplasmic innate immunity sensors and late
123 endosomal/lysosomal degrading environment (18). Accordingly, platelets sheltering HIV in these
124 privileged compartments would preserve virus integrity and infectiousness throughout their 8-10
125 days lifespan.

126 We next investigated the infectiousness of HIV localized into platelets using
127 CD4⁺CCR5⁺CXCR4⁺ indicator reporter cell lines (21, 22) (Figure 1E, image). These experiments
128 revealed that in patients with undetectable (below LOD) or detectable viral load at date of sampling,
129 a subgroup of patients, referred to as the positive group (in orange), contains platelets with similar
130 amount of infectious viral particles (2053 [CI: 1195-6013] and 1353 [CI: 533-4671] infectious units
131 (IU)/10⁶ platelets, respectively, p>0.05) (compared orange boxplots in Figure 1E, graph). Among
132 those patients containing infectious HIV in platelets and displaying viral load below LOD at date of
133 sampling, 7 out of 10 were consistently virally suppressed for 19.5 [CI:1-50] median months prior
134 sampling (patients marked by + in Table S3), ruling out a potential contamination with infectious
135 cell-free virus in indicator cell assays and confirming the presence of intact, infectious HIV
136 sheltered by platelets. We also ruled out contamination of PRP samples with cell-free virus from
137 residual viremia (23): in a subset of patients that were virally suppressed below LOD at date of

138 sampling, no infectious viruses were detected in platelet-poor plasma in pairwise comparisons with
139 full corresponding PRP samples that were positive for HIV detection (Figure S4A).

140 Platelets sheltering HIV could originate from direct endocytosis of viruses by platelets - as
141 occurs *in vitro* (7, 10) and speculatively in the blood of viremic patients. However, the presence of
142 intact, replication-competent virus in platelets from individuals with consistent, undetectable viral
143 load below LOD for long periods during cART indicates that platelets associate with HIV in an
144 active viral replication niche. Bone marrow megakaryocyte precursors from long-time cART-
145 suppressed patients have been recently shown to contain replication-competent viral DNA (24) and
146 infected megakaryocytes might produce viruses that could be sequestered by platelets during
147 thrombopoiesis (11, 24, 25). Accordingly, HIV DNA (Figure S6A-C) and RNA (Figure S6F) were
148 detected by *in situ* hybridization on bone marrow smears from a cART-treated patient in cells
149 morphologically identified as megakaryocytes (MK). In addition, HIV integrated proviral DNA was
150 detected in megakaryocytes purified from bone marrow samples from 9 out of 11 cART-suppressed
151 patients (Table S7) with an estimate of 74.7 [CI:140.6-39] HIV DNA integrated copies per million
152 megakaryocytes (Figure S6D-E). PRP samples from 4 of these patients with infected
153 megakaryocytes could be obtained. In all 4 PRP samples, platelets containing HIV RNA and
154 proteins were detected (Figure S6G). Altogether, these results indicate that infected megakaryocytes
155 are the potential source of infectious viruses confined within platelets, which would form an
156 important HIV compartment under cART.

157 Accordingly, phylogenetic analyses following deep sequencing of HIV *env* V3 sequences
158 were obtained from plasma before viral suppression and paired samples of peripheral blood
159 mononuclear cells (PBMC) and platelets after 69 months of cART suppression. These analyses
160 demonstrate that platelet-associated HIV is compartmentalized from PBMC-derived provirus under
161 virological control and also from HIV detected in plasma before cART suppression (Figure S7). In
162 addition, translated sequences revealed a shift in HIV tropism from R5 variants in plasma to X4
163 variants in PBMC and platelets detected under consistent suppression. The independent grouping of

164 platelets-derived X4 variants (Figure S7A, orange) in the phylogenetic analysis demonstrates that
165 viruses contained in platelets do not originate from a latent reservoir established prior to therapy,
166 nor from the CD4⁺ T-cell reservoir. More longitudinal, in-depth phylogenetic studies on HIV
167 genome sequences obtained from platelets, bone marrow and CD4⁺ T-cells are required to confirm
168 whether platelets associate with HIV in ongoing viral replication sites or, alternatively, originate
169 from a latent reservoir established prior to therapy, whose proviruses are reactivated for viral
170 production (26, 27). Nevertheless, our results strongly suggest that viruses contained in platelets are
171 product of ongoing active replication in cART-suppressed patients in a compartment different from
172 blood.

173 Platelets end their life being phagocytosed by cells of the mononuclear phagocytic system
174 mainly in the liver and spleen (28). Macrophages (MΦ) are central in the process, specialized in
175 recognizing senescent (aged or apoptotic) platelets through integrins and scavenger receptors (29,
176 30), being thus the main scavenging system responsible for platelet uptake and clearance in tissues
177 (28, 31) and the most likely target cells for infection with HIV enclosed in platelets.

178 Therefore, we next investigated whether HIV-containing platelets can propagate viral
179 infection to HIV-target cells such as tissue macrophages, as schematized in Figure 2A. HIV-
180 containing platelets from individuals with viral load below LOD at date of sampling and
181 consistently below LOD for 52.5 [CI:12-75] months interact with tissue-like macrophages (Figure
182 2B-C, Movie 1), resulting in productive infection of macrophages. HIV proviral DNA integrated
183 the genome of macrophages (48.25 [CI: 5.24-183.9] proviral HIV DNA copies per million
184 macrophages) (Figure 2D), that in turn produced infectious viruses (Figure 2E). The process could
185 be inhibited by preventing platelet-macrophage interaction with platelet-specific anti-integrin
186 α_{IIb}/β₃ Fab (Abciximab) (Figure S4B-D). Altogether, these results show that infectious viruses
187 sheltered in platelets spread infection to macrophages *in vitro*, demonstrating that platelets can act
188 as carriers of HIV throughout the body, in a process inhibited by Abciximab, a common anti-
189 platelet agent used for clinical purposes (32).

190 We compared clinical parameters at date of sampling from patients classified as positive (in
191 orange) or negative (in gray) for detection of HIV-containing platelets. Correlation with HIV-
192 containing platelet content, as detected by all four different techniques used, revealed that patients
193 with platelets carrying HIV had a 3-fold lower CD4⁺ T-cell count (<350/ μ l) as compared with
194 patients in which HIV-containing platelets were not detected (Figure 3A). In contrast, presence or
195 infectivity of HIV-containing platelets were not associated with total lymphocyte or platelet count,
196 age, years since diagnosis, years under cART (Figure S5A-C), or cART regimen (Table S5). We
197 have performed multivariate analysis of patients' clinical parameters at sampling date of samples
198 with positive or negative detection of HIV in platelets. Principal component analysis clusters away
199 platelet samples positive from those negative for HIV in platelets (Figure 3B). This distribution is
200 mainly driven by the principal component (dimension) 2 to which CD4⁺ T-cell count is the stronger
201 contributor (Figure S5D) and inversely correlates with the presence of HIV in platelets (Figure 3B).
202 Furthermore, a multinomial logistic regression indicates that among clinical parameters, excluding
203 nadir due to colinearity, CD4⁺ T-cell count is the only parameter with predictive value for the
204 presence of HIV in platelets (p=0.007). Altogether, these multivariate analyses confirmed that low
205 CD4⁺ T-cell count is strongly correlated with HIV association with platelets indicating an important
206 predictive component, although not necessarily causative. In addition, the presence and infectivity
207 of HIV-containing platelets in exclusive association with low CD4⁺T-cell counts at the sampling
208 date indicates that localization of infectious virus in platelets is related to bad clinical prognosis and
209 poor immunological recovery.

210 To address clinical implications of the interaction between HIV and platelets and their role
211 in Acquired Immunodeficiency Syndrome (AIDS), a subset of 35 out of all patients analyzed were
212 selected (Table 1). Selection was based on the two following criteria: first, having HIV detected in
213 platelet according to at least two different techniques used above (Tables S2); and second, having
214 precise and unambiguous clinical data available on at least 1 year prior the date of sampling. In
215 these patients, the historical CD4⁺ nadir, a clinical parameter in patient medical history critical for

216 recovery of an immunocompetent status (33), is significantly lower (<200 cells/ μ l) in patients
217 harboring HIV in platelets as compared with patients lacking HIV in platelets ($p=0.006$, Figure 4A),
218 although a causative relation remains to be established. Low CD4⁺T-cell count (<350 cells/ μ l),
219 irrespective of virological control, persists in patients displaying HIV-containing platelets, as
220 revealed by analysis of mean patients' CD4⁺T-cell counts at date of sampling and during a
221 retrospective and prospective 18-month period before and after sampling, respectively (Figure 4B).
222 These results indicate that the presence of HIV in platelets corresponds with immunological failure,
223 defined as HIV-infected cART patients that fail to recover CD4⁺ T-cell count above 350 cells/ μ l
224 (34).

225 Accordingly, 80% of the cART-treated patients with HIV-containing platelets (Figure 4C, in
226 orange) are in immunological failure, as compared to only 15% lacking HIV in platelets (Figure 4C,
227 in grey). Patients whose platelets carry HIV are endowed with a >20-fold higher chance of failing
228 to recover immunologically above 350 CD4⁺ T-cells/ μ l, despite cART, compared to patients
229 without HIV in platelets (OR:22.6, [CI:3.8-132.1], $p<0.001$). Having defined virological
230 suppression as patients with at least 1 year of viral load continuously below LOD prior the date of
231 sampling, the likelihood of immunological failure for patients whose platelets carry HIV is higher
232 and statistically significant in patients with controlled viremia (OR:56, [CI:4.3-719.2], $p=0.002$), as
233 compared to those with non-controlled viremia, i.e., patients viremic at date of sampling or
234 displaying less than 12 months of continuous suppression (OR:6 [CI:0.3-101.5]) (Tables 1 and S4).
235 These results indicate that the presence of HIV in platelets is strongly associated with
236 immunological failure during virological control, a defining feature of immunological
237 nonresponders (InRs) (34-36), i.e. patients that fail to reconstitute their competent immune status
238 despite treatment observance with prolonged below LOD viral load (35), and for whom no efficient
239 treatment is available. A systematic clinical study is required to demonstrate the prospective
240 predictive value of detecting HIV-containing platelets for InR diagnosis and anticipation of
241 appropriate therapeutic strategy.

242 Although our study reveals a platelet-mediated pathway for viral dissemination strongly
243 correlated with immunological failure, the causative nature of this correlation remains to be
244 established. The poor immunological recovery in cART-treated patients is mainly driven by
245 persistent inflammation associated with T-cell immune dysfunctions (37-39). Residual
246 inflammation blood markers, such as d-dimers and IL-6 detected in the plasma, are not correlated
247 with immunological failure (40) and thus unlikely to impact on HIV association with platelets. Such
248 inflammation-driven T-cell dysfunction could result from the low residual production of HIV by
249 tissue reservoirs such as macrophages (19, 41) that could be fueled by the transfer of infection from
250 HIV-sheltering platelets to tissue-macrophages, as reported herein. Further studies are necessary to
251 approach the molecular and cellular mechanisms connecting immunological failure and HIV-
252 containing platelets, and to confirm the reported findings on different cohorts of cART-suppressed
253 patients in other laboratories.

254 Here, we demonstrate that platelets from HIV-infected cART-treated patients shelter
255 infectious, replication-competent HIV *in vivo* and do so despite cART-mediated viral control below
256 LOD, correlated with immunological failure. Observations we report herein reveal a neglected
257 transient shelter for HIV in the blood of HIV-infected cART-treated patient. Such HIV shelter
258 differs from the blood T-cell reservoir. While T-cell reservoirs are long-lived and harbor integrated
259 HIV DNA representing a virus factory able to be reactivated, platelets only transport HIV, carry
260 HIV RNA and cannot replicate the virus. Platelets are short-lived and anuclear blood components,
261 lacking the nuclear machinery to meet the recent criteria used to define HIV reservoirs (27) which
262 include i) display of integrated proviral DNA, and ii) rebound viral production upon therapy
263 interruption. Instead, platelets and other blood cells (42, 43) may participate to HIV transmission
264 and persistence by hiding and keeping virus away from factors of the immune response. Also, as
265 shown here for platelets, they could promote infection by delivering virus to target cells such as
266 macrophages.

267 In conclusion, our study sheds light on HIV pathophysiology, describing an alternative
268 pathway for viral dissemination in which platelets act as transient carriers of infectious viruses and
269 as new players in cell-to-cell HIV transmission. The presence of HIV-containing platelets
270 characterizes poor immunological recovery in HIV-infected cART-treated patients, and offers a
271 potential predictive marker for the design of efficient therapeutic strategies against immunological
272 failure.

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276 **MATERIALS AND METHODS**

277

278 *Study design*

279 The study investigated the presence of replication-competent HIV within platelets from
280 HIV-infected patients under cART. Ethical statement: in this study, patients belong to a large
281 prospective cohort study of HIV-infected individuals aged at least 15 years from the French
282 Hospital Database on HIV (FHDH) created in 1989. These patients receive care at the Ambroise
283 Paré and Raymond Poincaré Hospitals (near Paris, France) that are two among 70 participating
284 centers in the FHDH. The only enrollment criteria are documented HIV-1 or HIV-2 infection and
285 all included patient had signed a written informed consent. Data submitted by the participating
286 centers were anonymized, then encrypted. The FHDH was approved by the French national
287 institutional ethic committees, namely the “Commission Nationale de l’Informatique et des Libertés
288 (CNIL)” on 27 November 1991 (Journal Officiel, 17 January 1992). Furthermore, the use of human
289 samples in this study was ruled by the World Medical Association Declaration of Helsinki. The
290 ethical authorization for the study of post-mortem bone marrow patient samples was given to The
291 State University of New Jersey (Rutgers) and The University of Texas Medical Branch (UTMB)
292 under the Institutional Review Board (IRB) authorization number Pro20140000794 and

293 Pro2012001303 from Rutgers and 18-0136 and 18-0134 from UTMB. Both IRB include consent for
294 whole-body donation.

295 Platelet-rich plasmas (PRP) employed in this study were obtained during routine blood tests
296 of 78 HIV-infected cART-treated patients belonging to the FDH and receiving care at the Ambroise
297 Paré and Raymond Poincaré Hospitals. The enrollment criteria for patients PRP collection was
298 cART initiated for at least one year at the time of blood sampling. Viremia is regularly tested in
299 human plasma using the Abbott RealTime HIV-1 assay on an automated *m2000* system (Abbott
300 *m200sp/rt*), allowing for the detection of HIV RNA copies/ml of plasma in the range of 1.6 log₁₀
301 copies (40 copies) to 7 log₁₀ copies. Values below this limit of detection (LOD) of 1.6 log₁₀ copies
302 represent “undetectable viremia”. Clinical information of patients whose PRP were employed in the
303 present study are available in Tables S1 and S2, which contain data on viral load (log₁₀ HIV copies
304 per ml of blood), total lymphocyte counts (10⁶ per ml of blood), CD4⁺T-cells count and historical
305 nadir (cells per µl of blood), platelet counts (10⁶ per ml of blood) and other parameters. Regimen of
306 cART taken by patients is shown in Table S5.

307 Noteworthy, the patients from this study were seen for clinical purposes, because part of
308 these patients has no referent general practitioner, therefore directly consulting the hospital
309 department. Thus, the set of cART-patients studied here is not representative of the overall HIV-
310 infected clinic population and the rate of immunological failure within the set of patients we
311 analyzed is not representative of the global rate of immunological failure observed in the overall
312 COREVIH included (and thereafter treated) patients.

313 Human peripheral blood and PRP from healthy HIV-seronegative donors were obtained
314 from the French blood collecting center (Etablissement Français du Sang, Paris, France). PRP from
315 healthy donors were used as negative controls for all conducted experiments and were obtained and
316 processed using the same method applied for PRP from HIV-infected patients.

317 Bone marrow samples were obtained post-mortem through whole-body donation from
318 cART-treated HIV-infected and non-infected patients.

319

320 ***Platelet-rich Plasma (PRP) collection***

321 Peripheral blood samples were obtained from donors by venipuncture into plastic tubes
322 containing anticoagulant EDTA. Samples were centrifuged for 10 minutes at 160g and 22°C for
323 blood fractionation allowing for clear separation of PRP from whole blood. To avoid potential
324 contamination by rare leukocytes, the upper two thirds of the PRP fraction were carefully collected
325 and transferred to new plastic tubes as recommended for functional analyses (44). Platelet-poor
326 plasma was obtained after centrifugation of PRP at 1100g, 10 minutes, room temperature and
327 collect supernatants.

328 PRP fractions were frozen at -80°C until employed in the experiments (45). We verified by
329 flow cytometry that: i) viable CD41⁺ platelets represent >70% percent of total events recorded in
330 PRP; ii) >90% of CD41⁺ platelets conserved CD42b⁺ membrane staining after thawing; and iii)
331 thawing does not increase the number of activated platelets characterized by CD62P⁺
332 immunostaining, with PRP samples displaying 49.2% and 51% of activated platelets
333 (CD41⁺CD62P⁺) before and after thawing, respectively.

334

335 ***Quantitative PCR***

336 Quantitative Real-Time PCR (qPCR) of HIV-1 LTR RNA sequences was performed from
337 platelet pellets obtained after centrifugation of 500 µl of PRP at 1100g, 10 minutes, at room
338 temperature. Platelet RNA was obtained as described (46), and processed for qPCR TaqMan assay
339 using LTR-specific primer (Vi03453409_s1, Thermo Fisher Scientific Inc.) labeled with a FAM-
340 MGB probe on a Applied Biosystems® TaqMan® RNA-to-Ct™ 1-Step Kit protocol (Thermo
341 Fisher Scientific Inc.). RNA from PRP samples were eluted into 15 µl of nuclease-free water and 5
342 µl of this eluate was used per qPCR reaction. PRP RNA samples display an average A260/280 ratio
343 of 1.82 [CI:1.65-2.01], indicating good RNA quality (high quality= \sim 2 A260/280 ratio according to
344 technique standards). The mean amount of RNA applied per reaction was 63.39 [CI:42.5-85.2] ng,

345 which fits into the range of specific detection of the qPCR reagents. Reaction and data acquisition
346 were performed using a LightCycler 480 II (Roche). The number of HIV LTR copies per million
347 platelets was calculated based on PRP platelet count and qPCR Crossing Point (Cp) values obtained
348 from a standard curve using a full-length molecular clone of HIV-1 pNL4-3 strain.

349 Contamination of PRP samples with lymphocytes was assessed by detection of the
350 lymphocyte-specific CD3 δ transcript (47) in PRP by qPCR TaqMan assay using CD3 δ primer
351 (Hs00174158_m1, Thermo Fisher Scientific Inc.) labeled with FAM- MGB probe on a Applied
352 Biosystems® TaqMan® RNA-to-Ct™ 1-Step Kit protocol (Thermo Fisher Scientific Inc.). The
353 number of lymphocytes was calculated based on Cp values obtained from a standard curve using
354 serial dilutions of RNA obtained from known amounts of CD4⁺ T-cells.

355
356 ***Flow cytometry after fluorescence in situ hybridization (FISH-flow)***

357 Paraformaldehyde (4% v/v final concentration) was added to 25 μ l of PRP to fix samples,
358 then platelets were centrifuged at 1100g, 10 minutes, 22°C, and washed twice in phosphate buffered
359 saline without Ca⁺²/Mg⁺² (PBS). Platelets were then processed for fluorescence *in situ* hybridization
360 (FISH) using Cy5-labeled single molecule-FISH probe set for HIV Gag mRNA (HIV-1 vector
361 pNL4-3 GenBank AF324493.2) designed by Stellaris Probe Designer program
362 (<http://www.singlemoleculfish.com>) as we described (48). Probe set is detailed in Table S6. Prior
363 to hybridization, platelets were immunostained with anti-p24 antibody (1:20, KC57 clone, Beckman
364 Coulter Inc.) coupled to FITC and anti-CD41/CD61 antibody coupled to PE (1:20, A2A9/6 clone,
365 Biolegend, Inc.).

366 Flow cytometry analyzes of platelets processed for FISH (FISH-flow) were performed under
367 the Guava easyCyte high-throughput system version 6HT2L (Merck Millipore, Merck, KGaA) set
368 to very low flow rate (0.12 μ l/s) and laser gains of 3.5 (GRN-B laser line), 4.3 (YEL-B laser line),
369 and 7.6 (RED-R laser line) in a range of 1 to 1024 units.

370 The gating strategy to retrieve HIV RNA⁺/Gag⁺ double positive platelets (Figure S1A-C)
371 was established in InCyte (Merck Millipore, Merck, KGaA) software consisting in: i) gating the
372 population of single platelets based on forward and side-scatter (in log/log scale, FSC threshold
373 value=100); ii) then gating on CD41⁺ events after applying a threshold based on isotype controls;
374 and iii) calculating CD41⁺ events according to p24 immunostaining and HIV RNA probe
375 hybridization as described (15, 48). Data normalization was performed by subtracting the maximum
376 percentage acquired in n=11 healthy donor samples from the percentages acquired in patient
377 samples per experiment.

378 FISH-flow method was also applied as described (16) for detection of HIV RNA⁺/Gag⁺
379 peripheral blood mononuclear cells (PBMC), isolated from blood buffy coats of cART-treated or
380 chronically infected non-treated HIV-infected patients after Ficoll-Hypaque density gradient
381 centrifugation.

382

383 ***Retrospective and prospective analysis of clinical parameters***

384 We categorized cART-treated HIV-infected patients in two groups according to the presence
385 (positive) or absence (negative) of HIV in platelets. Patients were classified as positive or negative
386 based on similar results obtained in at least two different techniques used for detection of HIV-
387 containing platelets. CD4⁺ T-cell historical nadir, age, years since HIV diagnosis and years since
388 treatment of patients from the positive and negative groups were compared using statistical methods
389 as described below. From this group of patients classified as positive or negative for HIV in
390 platelets, we selected those 35 individuals for which we could collect longitudinal CD4⁺ T-cell
391 count and viral load measurements (Table 1). Clinical parameters of these 35 individuals with
392 consistent detection of HIV in platelets were used for retrospective and prospective analyses
393 presented in Figure 4.

394 The retrospective and prospective analyses of patient clinical information refer to a period of
395 several months around the date of sampling. When indicated, patients were categorized in

396 controlled viremia group (patients presenting HIV RNA copies/ml plasma always below LOD for at
397 least 1 year prior sampling) or non-controlled viremia group (patients presenting $>1.60 \log_{10}$ copies
398 of HIV RNA/ml plasma at date of sampling or displaying less than 12 months of continuous
399 suppression). Regarding immunological status, patients are categorized as immunologically
400 competent when $CD4^+$ T-cells count was >350 cells/ μ l for two or more consecutive measurements;
401 or in immunological failure when $CD4^+$ T-cells per μ l was constantly <350 cells/ μ l for all blood
402 sample collected for at least 1 year including the date of sampling for HIV in platelets. We
403 obtained, for each selected patient, a mean $CD4^+$ T cell number for counts registered 18 months
404 before sampling (retrospective count) and for 18 months after sampling (prospective count). Figure
405 4B shows the mean retrospective count, the mean count at sampling date and the mean prospective
406 count of patients which $CD4^+$ T cell count was surveyed.

407

408 ***Statistical analysis***

409 In all HIV detection assays performed, patient samples are stratified in two groups (negative
410 or positive) according to the presence of HIV in platelets. Median numbers of HIV-containing
411 platelets were calculated exclusively from samples of the positive group, to precisely determine
412 how many platelets do contain HIV in the specific group of patients displaying HIV in platelets.
413 The results are expressed as median, followed by the 95% confidence interval (CI).

414 All statistical analyses were performed using IBM SPSS Statistics software (IBM). Pairwise
415 comparisons of normal distributions were performed by Student's t-test. Pairwise and multiple
416 comparisons of non-normal distributions were performed using the non-parametric tests Mann-
417 Whitney U and Kruskal-Wallis, respectively. Patients' clinical parameters at date of sampling were
418 examined altogether by multivariate analysis using Categorical Principal Component Analysis
419 (PCA), a data reduction technique that simultaneously quantifies categorical variables while
420 reducing the dimensionality of the data. Results are presented as PCA biplot, a graphical display
421 that illustrates the relationships among the components, original variables and observations (49).

422 Patients' clinical data at date of sampling were normalized to remove any undue influence on the
423 analysis. In the biplot patients' clinical parameters are shown as variable vectors (component
424 loadings). Samples (objects in biplot) are labeled according to experimental detection of HIV in
425 platelets (positive or negative).

426 Repetition of parameter values from patient history due to multiple samplings (namely
427 nadir) do not interfere in the PCA performed, as when randomly selecting one sampling per patient
428 before running the PCA, results did not change. Multinomial Logistic Regression was performed
429 using detection of HIV in platelets as dependent variable and patients' clinical parameters at date of
430 sampling as covariates (excluding CD4⁺ T-cell historical nadir due to colinearity with CD4⁺ T-cell
431 count [Pearson's coefficient= 0.635, p<0.001]). Fisher's exact test was used for odds ratio (OR) and
432 risk estimates. *P values* <0.05 were considered significant.

433

434 Additional materials and methods are available in Supplementary Material.

435

436 **LIST OF SUPPLEMENTARY MATERIAL**

437

438 **SUPPLEMENTARY FIGURES**

439 Fig. S1: Flow cytometry gating strategy for the detection of HIV in platelets from HIV⁺
440 cART-treated patients

441 Fig. S2: Platelet-rich plasma (PRP) preparations from HIV-infected patients are not
442 contaminated by HIV-infected CD4⁺ T-cells

443 Fig. S3: HIV-containing platelets detected by electron and confocal microscopy

444 Fig. S4: Infection of macrophages by HIV contained in platelets is due to direct interaction
445 between macrophages and platelets and not to residual cell-free viruses present in PRP

446 Fig. S5: Detection of HIV-containing platelets do not correlate with other clinical parameters

447 Fig. S6: HIV-1 integrated proviral DNA in megakaryocytes (MK) from cART-suppressed
448 patients

449 Fig. S7: Deep sequencing and phylogenetic analysis of HIV *env* V3 before and after
450 virological suppression in different compartments of the same cART-suppressed patient

451

452 SUPPLEMENTARY TABLES

453 Table S1: Global clinical information on HIV-infected cART-treated patients sampled for
454 HIV in platelets

455 Table S2: Clinical information of each HIV-infected cART-treated patient sampled for HIV in
456 platelets

457 Table S3: Available viral load measurements of patients positive for HIV in platelets with
458 viral load below limit of detection (LOD) at the date of sampling

459 Table S4: CD4⁺ T-cell counts and viral load measurements of patients positive for HIV in
460 platelets with controlled viremia

461 Table S5: cART treatment regimen of patients classified as positive or negative for the
462 presence of HIV in platelets

463 Table S6: List of gag mRNA probe sequences used for FISH-flow

464 Table S7: Clinical information of each HIV-infected cART-treated patient sampled for HIV in
465 megakaryocytes

466 Movie S1: Laser scanning confocal image of an HIV-containing platelet engulfed by a
467 macrophage

468

469 SUPPLEMENTARY REFERENCES (50-69)

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700

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710 **AUTHORSHIP CONTRIBUTIONS**

711 FR, ECB, MB: conceived the experiments; CC, SG, ER, ECB: collected the patient samples
712 and provided clinical information; FR, CC, MB: performed flow cytometry and confocal
713 microscopy experiments; FR, GS, JZ: performed indicator cell assays; FR, AS, AZ, LX: performed
714 qPCR experiments; FR, CC, JMM, MB: performed electron microscopy experiments; FR, RA,
715 MLG, MB: set up fluorescence *in situ* hybridization coupled to flow cytometry; FR, MB, EE, MD:
716 designed and performed experiments on *in situ* hybridization of megakaryocytes and platelets; MC,
717 PD, JI: performed deep sequencing and phylogenetic analyses of HIV *env* V3; FR, SG, ER, ECB,
718 MB: performed retrospective and prospective analysis of patient clinical status; FR, ECB, MB:
719 wrote the manuscript.

720 **COMPETING INTERESTS**

721 The authors declare no competing financial interests in conducting this study.

722 **DATA AND MATERIALS AVAILABILITY**

723 All data associated with this study are in the paper or supplementary materials. HIV *env* V3
724 sequences can be found as GenBank accession numbers MN453286, MN453287, MN453286

725 MN453288, MN453289, MN453290, MN453291, MN453292, MN453293, MN453294,
726 MN453295.

727

728 **FIGURE AND TABLE CAPTIONS**

729

730 **Figure 1: HIV is detected in platelets from cART-treated patients.**

731 (A) Number of HIV LTR RNA copies per million platelets obtained from platelet-rich plasma
732 (PRP) of cART-treated patients displaying viral loads detected or below limit of detection (<LOD)
733 at date of sampling. Healthy donors PRP were used as negative controls. In orange, patient platelet
734 samples in which HIV was detected; in grey, samples negative for detection. n=number of different
735 individuals tested; N=7 independent experiments (B) HIV RNA⁺/Gag⁺ double-positive platelets
736 analyzed by flow cytometry. HIV-containing platelets were detected in patients with both <LOD
737 and detected viral load at date of sampling. Left boxplot: percentage of HIV-containing platelets in
738 PRP samples from healthy donors or HIV-infected cART-treated patients as assessed by the gating
739 strategy. The quantification threshold (red dotted line) was established based on healthy donor
740 samples, grouping the HIV-infected patient samples according to the absence or the presence of
741 HIV in platelets (grey and orange boxplots, respectively). Normalized percentage values are
742 indicated in italic on the right axis. The amount of detected HIV-containing platelets is significantly
743 different from the background detected events retrieved in healthy donors or in patients considered
744 negative for the presence of HIV in platelets ($p<0.001$). Right boxplot: percentages converted into
745 HIV RNA⁺/Gag⁺ platelets per million platelets after data normalization, subtracting the maximum
746 value obtained from healthy donor controls. n=number of different individuals tested; N=5
747 independent experiments. (C) Images: Platelets from HIV-infected cART-treated patient
748 immunostained for CD41 (green) and p24-Gag (red), observed by confocal microscopy (left
749 images) and reconstructed in three-dimensions (right images). Arrowheads indicate HIV contained
750 within platelets. Representative of n=11 different individuals. Bar=1 μ m. Graph: p24-Gag

751 fluorescence intensities recorded from healthy donors or HIV-infected cART-treated patients'
752 platelets as detected by flow cytometry. A threshold (red dotted line) was established based on
753 healthy donor samples, separating HIV-infected patient samples in negative and positive groups
754 concerning the presence of HIV in platelets (grey and orange boxplots, respectively). The
755 fluorescence intensities of p24 immunostaining retrieved in positive groups are significantly
756 different from the background fluorescence intensities detected either in healthy donors or in
757 patients from the negative groups ($p < 0.001$). n=number of different individuals tested; N=7
758 independent experiments. (D) Transmission electron microscopy of HIV-containing platelets: a
759 viral particle in a small vacuolar compartment is indicated by green dotted line boxes, and shown at
760 higher magnification in image inset. Representative of n=5 different individuals. 10nm
761 Immunogold p24-Gag labeling of cryosections was performed prior to electron microscopy
762 observation to confirm the presence of HIV within platelet compartments (arrowheads).
763 Representative of n=2 different individuals and 10 different images. α G: alpha-granule; V:
764 Vacuole. Bar= 0.5 μ m or 100 nm. (E) Image: Infected GFP⁺ indicator cells after interaction with
765 PRP from an HIV-infected patient. Nuclei were labeled with DAPI (blue) and fluorescence signal
766 merged with phase contrast is shown (arrowheads). Bar= 5 μ m. Graph: Quantification of infectious
767 units per million platelets found in platelet samples of HIV-infected cART-treated patients
768 displaying <LOD or detected viral load at date of sampling (grey= samples without infectious virus;
769 orange= samples with infectious virus). n=number of different individuals tested; N=8 independent
770 experiments.

771

772 **Figure 2: HIV-containing platelets propagate infection to macrophages *in vitro*.**

773 (A) Assay to quantify the propagation of infection from HIV-containing platelets to macrophages
774 (M Φ). (B-C) Confocal microscopy images of HIV-containing platelets interacting with
775 macrophages *in vitro*, after immunostaining for CD41 (green) and p24-Gag (red) merged with
776 phase contrast image. Arrowheads: virus labeling contained within platelets. Insets: three-

777 dimensional reconstruction or projections in xy, yz and xz dimensions of HIV-containing platelet
778 images. Representative of n=5 different individuals. In main and inset figures, Bar=5 μ m. (D)
779 Number of integrated copies of HIV DNA per 10^6 macrophages 7 days after interaction with cART-
780 treated virally controlled patient platelets treated or not with Abciximab (orange dot and orange
781 circle, respectively) or cell-free HIV-1 (positive control, green dot). Healthy donor platelets were
782 used as negative controls (grey dot). n=5 different individuals, N=3 independent experiments.
783 Mann-Whitney U test applied for pairwise experimental data obtained from HIV⁺ cART patients
784 platelets treated or not with abciximab. (E) Infectious units produced per 10^6 macrophages after 7
785 days of co-culture with cART-treated virally suppressed patient platelets, treated or not with
786 Abciximab (orange dot and orange circle respectively) or cell-free HIV-1 (positive control, green
787 dot). Healthy donor platelets were used as negative controls (grey dot). n=5 different individuals,
788 N=3 independent experiments. Mann-Whitney U test applied for pairwise experimental data
789 obtained from HIV⁺ cART patients platelets treated or not with abciximab.

790

791 **Figure 3: Presence and infectiousness of HIV in platelets is associated with low CD4⁺ T-cell**
792 **counts (<350/ μ l).**

793 (A) Comparison of CD4⁺T-cell counts at date of sampling of patients whose platelets contain
794 (orange boxplot, positive) or not (grey boxplot, negative) HIV as assessed by HIV LTR qPCR,
795 FISH-flow (HIV RNA/Gag), p24-Gag flow cytometry and indicator cell assays. Patients with HIV-
796 containing platelets consistently present lower CD4⁺T-cell count (<350/ μ l) compared to patients
797 which platelets do not contain virus. Mann-Whitney U test comparing negative and positive groups,
798 for each technique. (B) Principal Component Analysis (PCA) of patient samples categorized in
799 positive (orange dots) or negative (grey dots) according to presence of HIV in platelets and patients'
800 clinical parameters at date of sampling represented as variable vectors (arrows).

801

802 **Figure 4: Presence of HIV in platelets is correlated with poor immunological recovery.**

803 (A) CD4⁺ T-cell historical nadir from HIV-infected cART-treated patients whose platelets
804 contained or not HIV (orange and grey bars, respectively). Patients with HIV-containing platelets
805 have lower mean CD4⁺ T-cell nadir (<200 cells/ μ l) compared with the negative group. Mann-
806 Whitney U test. (B) CD4⁺ T-cell mean count of patients positive (orange line) or negative (grey
807 line) for the presence HIV in platelets, in a period of 18 months before and after the date of
808 sampling. Analysis was first performed taking n=35 selected patients (see methods) together
809 irrespective of viremia (first graph), then further divided regarding patients with controlled viremia
810 (>1 year with viral load always below LOD prior sampling, second graph, n=25) and patients with
811 non-controlled viremia (detectable viral load at date of sampling or displaying less than 12 months
812 of continuous suppression, third graph, n=10). CD4⁺ T-cell count mean of patients with HIV-
813 containing platelets remained persistently below a 350 cells/ μ l threshold (red dotted line).
814 n=number of different individuals selected. (C) Percentage of patients in immunological failure
815 (<350 CD4⁺T-cell count constantly for >1 year of follow-up including at date of sampling) among
816 the HIV-infected cART-treated patients grouped according to the presence of HIV in platelets
817 (negative: grey bar; positive: orange bar). Taking the selected 35 patients together (left bar graphs),
818 there is a >20-fold higher chance of immunological recovery failure compared to patients without
819 HIV in platelets, with patients in immunological failure corresponding to 80% of the patients
820 displaying HIV in platelets (OR:22.6, [CI:3.8-132.1], p=0.0005). When analysis was partitioned
821 relative to patient viremia status, patients in immunological failure correspond to 88% and 66% of
822 the patients displaying HIV in platelets when viremia is controlled (middle bar graphs) or non-
823 controlled (right bar graphs), respectively. The chance of a patient displaying HIV in platelets to fail
824 immunological recovery is higher and statistically significant in the controlled viremia group,
825 compared with the non-controlled viremia group (OR:56, [CI:4.3-719.2], p=0.002 and OR:6
826 [CI:0.3-101.5], p=0.21, respectively). n=number of different individuals selected. OR=odds ratio.

827

828

847 **Table 1: List of patients selected for retrospective and prospective analysis of immunological**
848 **failure and virological control shown in Figure 4.** Patients were classified as positive or negative
849 for HIV in platelets according to confirmatory results in at least two different techniques. Only
850 patients which clinical data is available on 18 months around the date of sampling were included in
851 the analysis. The period of virological suppression in months prior date of sampling is indicated for
852 each patient tested for HIV in platelets.
853

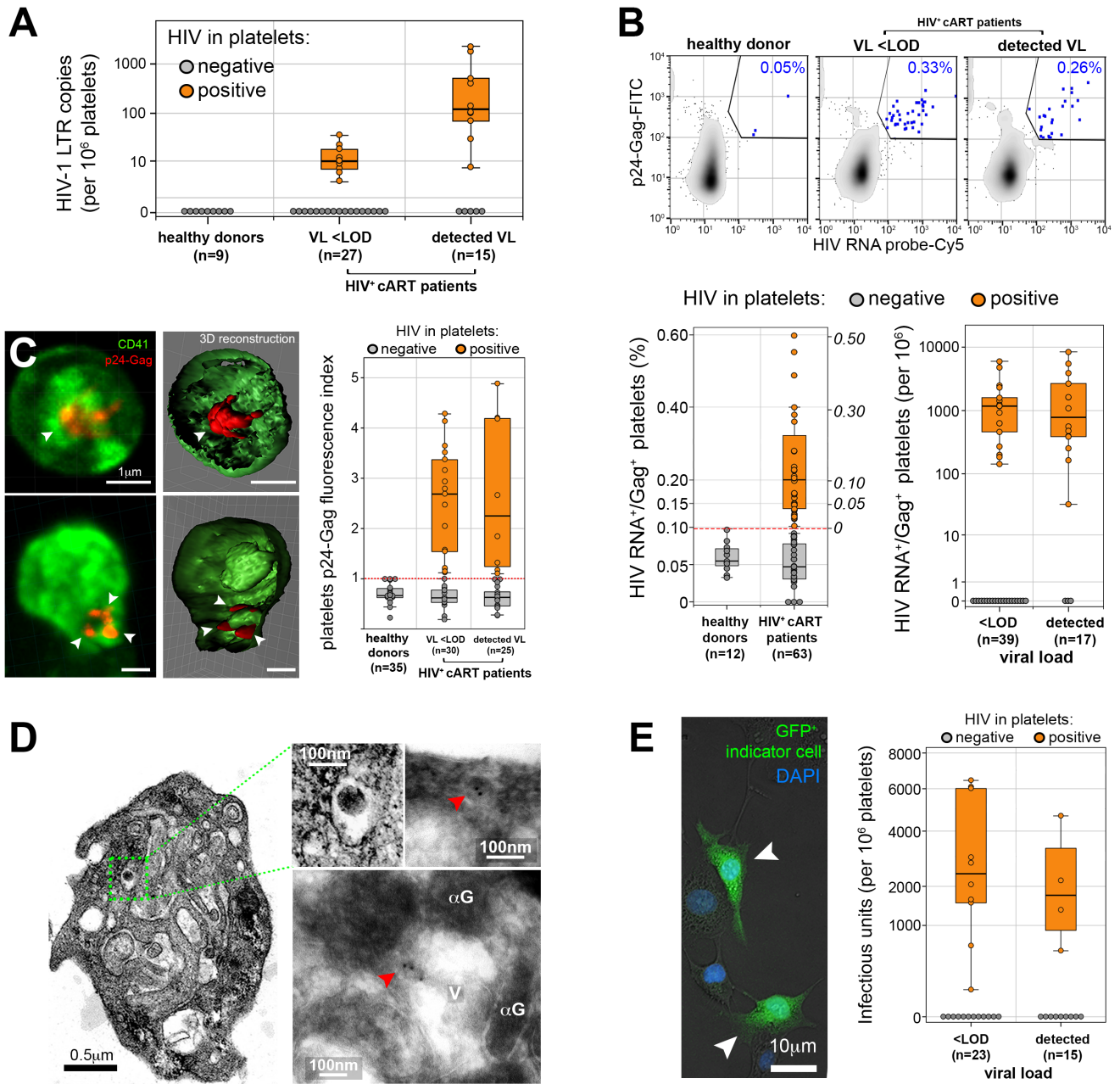


Figure 1

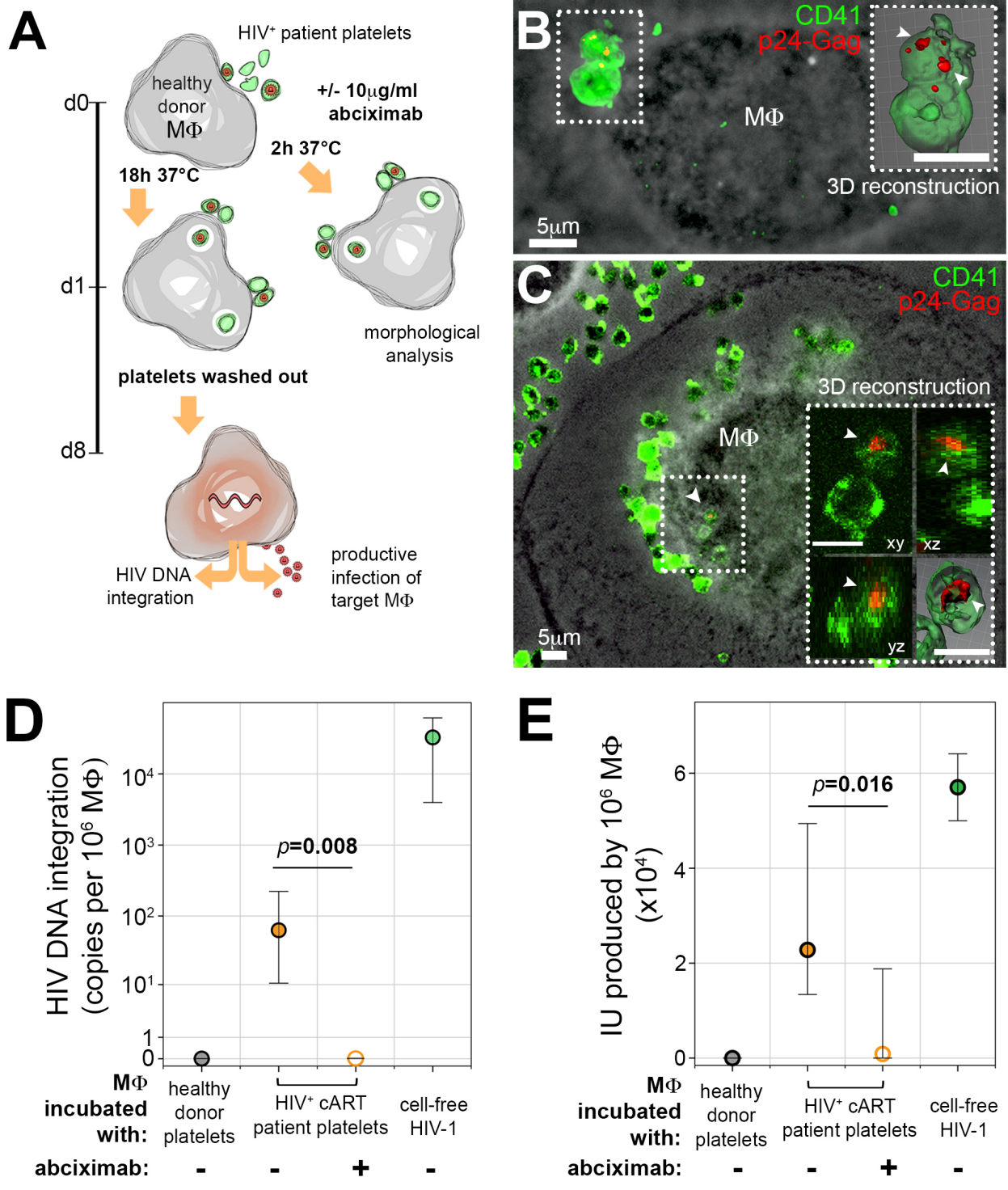


Figure 2

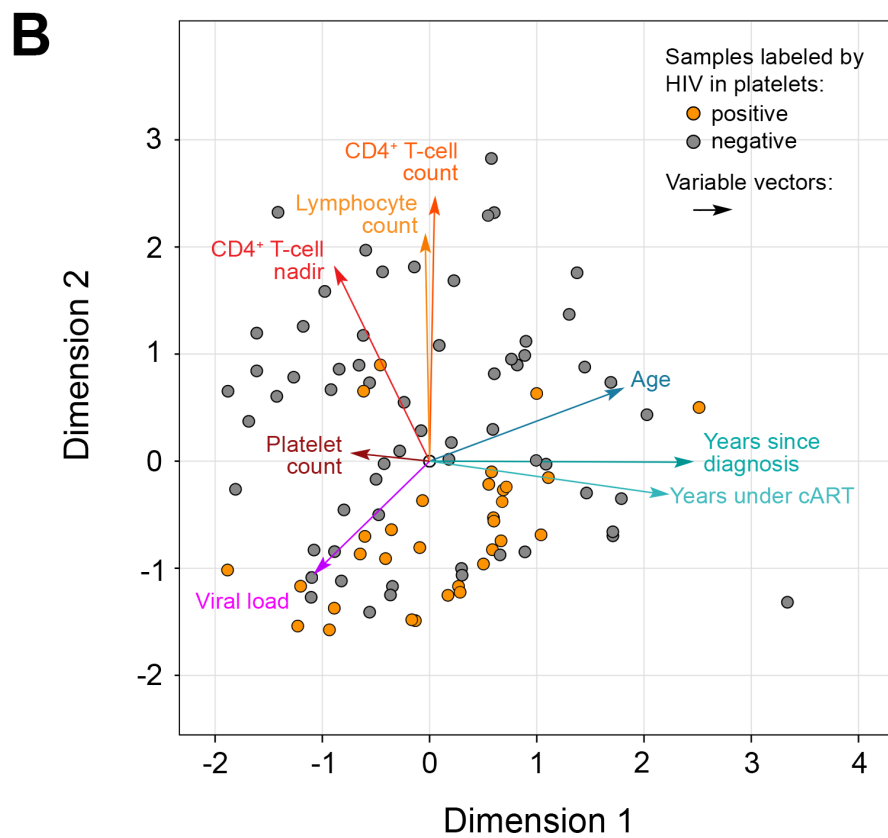
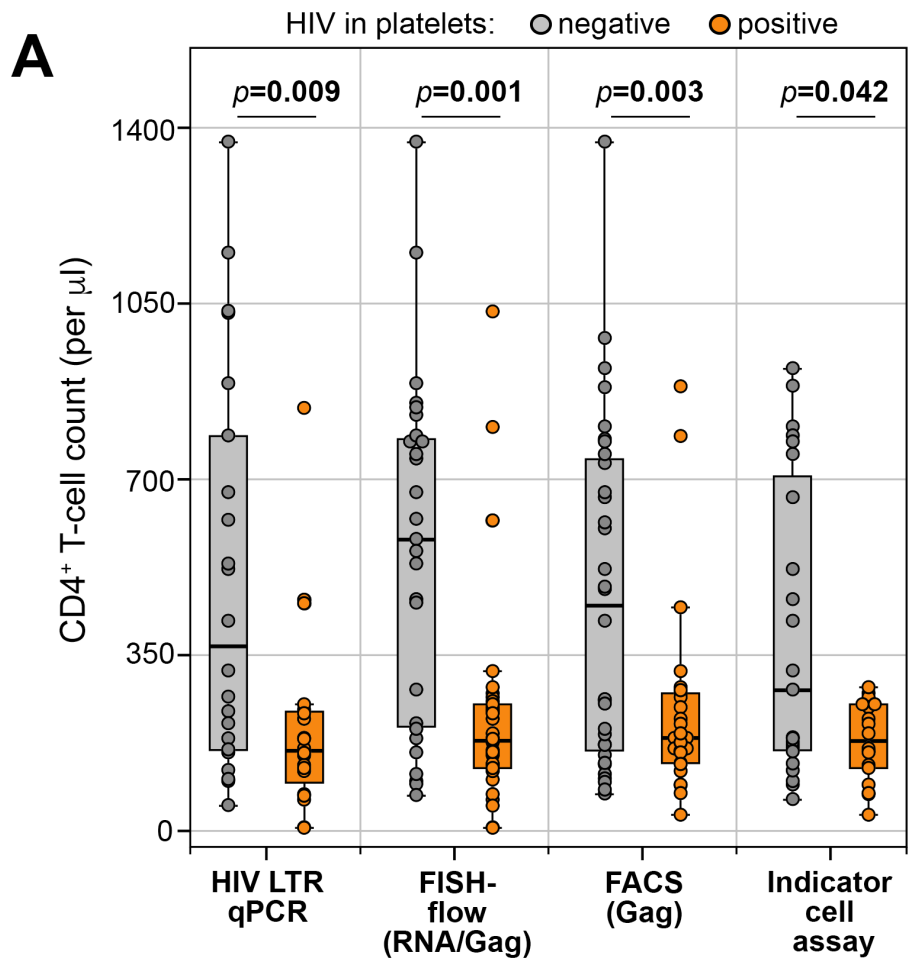


Figure 3

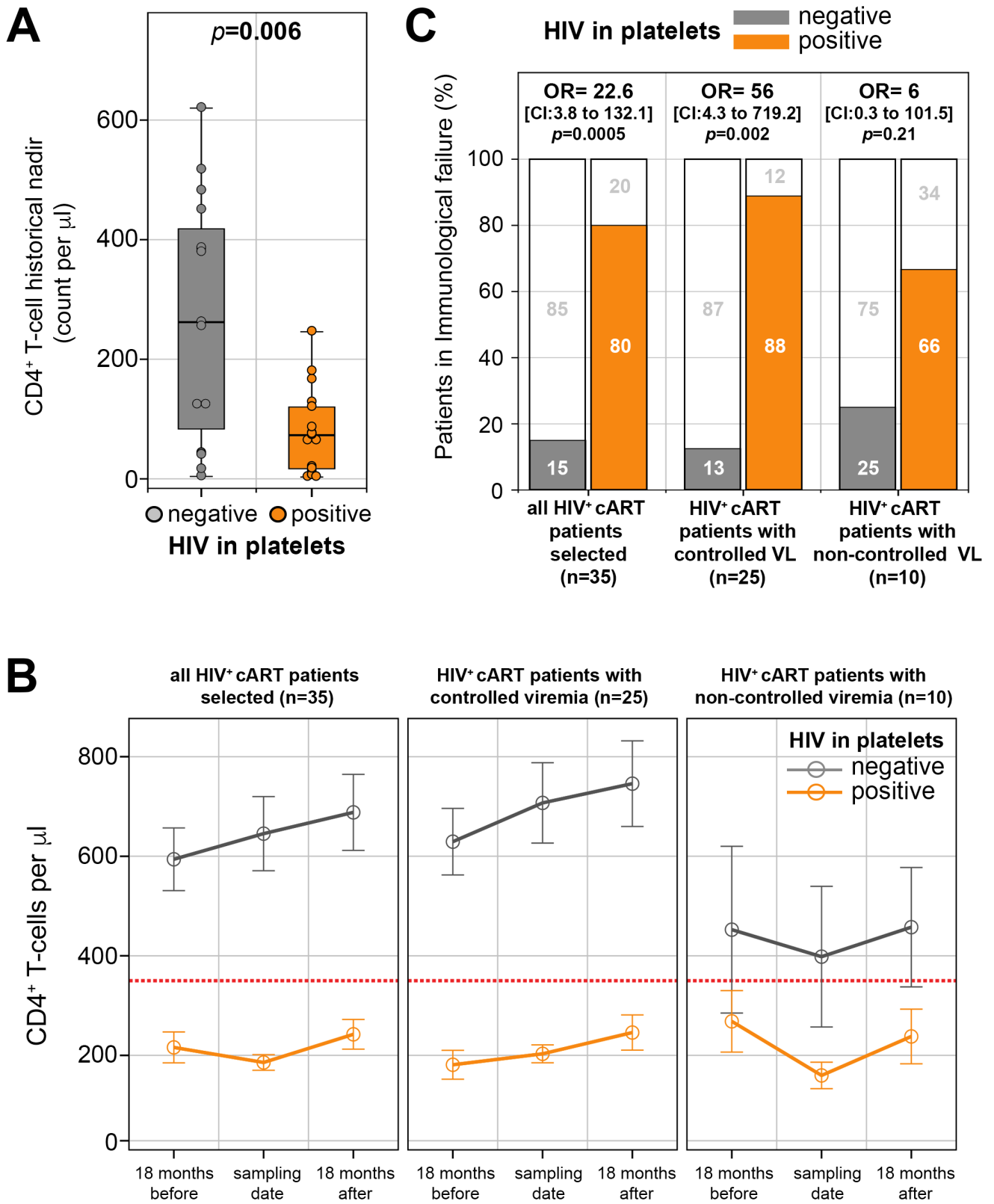


Figure 4

Table 1. List of patients selected for retrospective and prospective analysis of immunological failure and virological control shown in Figure 4

| | patient number | Immunological status ² | viremia ³ | months suppressed prior sampling | |
|--|---|-----------------------------------|----------------------|----------------------------------|-------------------|
| Patients tested negative for HIV in platelets ¹ | 39 | competent | controlled | 17 | |
| | 32 | competent | controlled | 18 | |
| | 75 | competent | controlled | 31 | |
| | 54 | competent | controlled | 18 | |
| | 52 | competent | controlled | 12 | |
| | 11 | competent | controlled | 18 | |
| | 51 | competent | controlled | 18 | |
| | 16 | competent | controlled | 12 | |
| | 15 | competent | controlled | 13 | |
| | 61 | competent | controlled | 13 | |
| | 66 | competent | controlled | 47 | |
| | 67 | competent | controlled | 31 | |
| | 4 | competent | controlled | 78 | |
| | 56 | competent | controlled | 62 | |
| | 53 | in failure | controlled | 22 | |
| | 59 | in failure | controlled | 18 | |
| | median months under controlled viremia [confidence interval] | | | | 18 [15-31] |
| | 7 | in failure | non-controlled | <12 | |
| | 60 | competent | non-controlled | <12 | |
| | 24 | competent | non-controlled | <12 | |
| | 46 | competent | non-controlled | <12 | |
| Patients tested positive for HIV in platelets ¹ | 9 | in failure | controlled | 24 | |
| | 27 | in failure | controlled | 38 | |
| | 48 | in failure | controlled | 19 | |
| | 6 | in failure | controlled | 20 | |
| | 49 | in failure | controlled | 50 | |
| | 64 | in failure | controlled | 52 | |
| | 79 | in failure | controlled | 14 | |
| | 80 | in failure | controlled | 62 | |
| | 37 | competent | controlled | 13 | |
| | median months under controlled viremia [confidence interval] | | | | 24 [14-52] |
| | | 74 | competent | non-controlled | <12 |
| | | 25 | competent | non-controlled | <12 |
| | | 18 | in failure | non-controlled | <12 |
| | | 78 | in failure | non-controlled | <12 |
| | 40 | in failure | non-controlled | <12 | |
| | 41 | in failure | non-controlled | <12 | |

¹confirmed by at least two different techniques

²**competent immunological status:** >350 CD4⁺T-cells per µl for two or more consecutive measurements in blood samplings during at least 1 year including date of sampling for HIV in platelets;

immunological status in failure: <350 CD4⁺T-cells per µl in all blood samplings during at least 1 year including date of sampling for HIV in platelets

³**controlled viremia:** HIV RNA log₁₀ copies/ml plasma always tested <1.60 log₁₀ copies/ml during at least 1 year prior sampling for HIV in platelets;

non-controlled viremia: HIV RNA log₁₀ copies/ml plasma tested >1.60 log₁₀ copies/ml at date of sampling or displaying less than 12 months of continuous suppression

Table 1