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1 **Anti-gp41 antibody levels reflect HIV viral suppression and cellular reservoir in**
2 **long-term antiretroviral treated participants**

3

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39

40 **Synopsis**

41 **Background**

42 A major challenge to HIV cure strategies is the quantification of persistent reactivation-
43 prone virus in people living with HIV.

44 **Objectives**

45 Our aim was to determine whether anti-gp41 antibody levels correlate with viral
46 suppression and HIV-1 DNA levels on antiretroviral therapy.

47 **Patients and methods**

48 Participants with plasma HIV-1 RNA below 50 copies/ml for >12 months were included
49 from 3 ANRS cohorts (COPANA, MONOI and APROCO). Antibody levels to gp41 were
50 measured by a low-sensitivity enzyme-linked immunoassay. Correlations with individual
51 and viral characteristics, plasma HIV-1 RNA (standard and ultrasensitive) and cell-
52 associated HIV-1 DNA were assessed.

53 **Results**

54 Seventy seven percent of the 683 participants were men. Median age was 41, median
55 CD4+ T-cell count was 582/ μ L and median viral suppression duration was 6.6 years
56 (IQR, 2.0-9.5). The overall median anti-gp41 antibody titer was 1.3 (0.6-1.9); median
57 (IQR) HIV-1 DNA level was 2.6 (2.1-3.0) \log_{10} copies/ 10^6 cells; ultrasensitive HIV-1 RNA
58 tested below 1 copy/mL in 56% of samples. A lower titer of anti-gp41 antibodies was
59 correlated to male gender, longer viral suppression and lower HIV-1 DNA burden.
60 Consistent HIV-1 RNA <1copy/mL was associated with lower gp41 levels (median: 1.1
61 (0.5-1.6) vs 1.4 (0.7-1.9), $p=0.009$).

62 **Conclusions**

63 Anti-gp41 levels decreased with the duration of antiviral suppression on ART. Lower
64 titers were associated with lower HIV-1 DNA levels and maximal viral suppression,
65 reflecting minimal antigen stimulation. Anti-gp41 antibody titration may be a useful
66 biomarker reflecting long-term HIV-1 suppression on ART.

67

68 **Introduction**

69 Combination antiretroviral therapy (ART) suppresses human immunodeficiency virus
70 type 1 (HIV-1) replication but does not cure the infection, due to the persistence of long-
71 lived latent HIV-1 reservoirs.¹ Reactivation-prone HIV-1 DNA persists in infected CD4+
72 T cells and macrophages, thus preventing viral elimination in spite of long-term ART.

73 ART leads to the suppression of plasma HIV-1 RNA viral load below the limit of
74 quantification of standard clinical assays (20-50 copies/mL). Using an ultrasensitive
75 “single copy” assay with a limit of quantification of 1 copy/mL, Palmer *et al*
76 demonstrated that low-level viremia persists for at least 7 years in many patients on
77 suppressive antiretroviral therapy.²

78 The origin of this residual viremia remains elusive. Low-level replication may persist in
79 tissue sanctuaries due to limited drug penetration, and/or the production of HIV-1 RNA
80 may arise from the sporadic reactivation of infected T cells. In addition, the majority of
81 integrated HIV-1 DNA sequences are defective and a fraction only of the 10% viral
82 genomes preserved from deletions and hypermutation will produce infectious viral
83 particles following *in vitro* activation of infected CD4+ T cells.³ Nonetheless, defective
84 proviruses are able to produce protein-encoding RNA species in patients on ART.⁴
85 Regardless of the origin of the residual viremia, current tools based on PCR
86 amplification to detect plasma HIV-1 RNA fall short of an assay that would detect the
87 production of actual infectious particles.

88 A major challenge to HIV cure strategies is the quantification of persistent replication-
89 competent HIV. Current methods to quantify the HIV reservoir in blood and tissues in
90 individuals on ART are continually challenged. The quantification of total cell-associated
91 HIV-1 DNA overestimates the actual reservoir size, while the viral outgrowth assay

92 measuring the production of infectious virus from resting CD4+ T cells underestimates
93 the number of reactivation-prone cells and requires large volumes of blood,
94 emphasizing the need for new approaches.⁵ HIV-1 infection elicits a strong adaptive
95 immune response involving both cellular and humoral immunity. Measuring the specific
96 immune responses to HIV may be an alternate way to assess HIV persistence and
97 replication in blood and tissue.

98 Interestingly, immunoassays for recent HIV infection that were developed to estimate
99 HIV incidence are based on the maturation of HIV-specific antibody responses. They
100 mostly measure antibody levels or antibody avidity towards major antigenic epitopes of
101 HIV-1^{6,7}. Previous studies demonstrated the impact of ART on the proportion of HIV-
102 specific IgG, and that patients who were falsely classified as recently infected (low titers
103 or/and low avidity) had been treated for a longer period and with longer viral
104 suppression than those correctly classified^{8,9}. The prevailing hypothesis is that the
105 suppression of viral replication is likely to lessen antigen presentation to immune cells,
106 leading to a decrease in anti-HIV antibody production. Several groups have since
107 studied anti-HIV antibodies as potential surrogate markers of HIV reservoir. These
108 studies included various types of participants (untreated, ART-treated at the early or
109 the chronic stage, elite controllers) in limited numbers^{10,11} and described declining
110 antibody levels during ART. Our aim was to determine whether anti-gp41 antibody
111 levels are correlated with HIV-1 reservoir size and residual viremia and could be a
112 valuable marker of this reservoir in a large homogenous group of successfully ART-
113 treated participants from three ANRS cohorts, who initiated ART at the chronic stage
114 and had prolonged viral suppression.

115

116

117 **Patients and methods**

118 **Participants**

119 We analyzed a large number of participants in a cross-sectional design, accounting for a
120 wide range of ART durations. Adult participants with ART-treated chronic HIV infection
121 suppressed below 50 copies/mL were included from three French ANRS cohorts.
122 Participant characteristics, clinical and biological data were obtained from the ANRS
123 cohort databases.

124 We obtained samples from the final evaluation of ANRS-EP11-APROCO (Anti-PROtease
125 COhort) treatment-controlled participants followed for a median 11 years (IQR 10-
126 12)^{12,13}. We included on-ART samples from participants with sustained viral
127 suppression below 50 copies/mL for at least 12 months in the ANRS-C09-COPANA
128 prospective cohort of HIV-1-infected adults initiating therapy.¹⁴ Baseline pre-
129 intervention samples from the ANRS-136 MONOI trial (NCT00412551),¹⁵ which
130 included ART-treated participants with plasma HIV-1 RNA suppressed below 400
131 copies/mL for at least 18 months and below 50 copies/mL at the screening visit were
132 also included.

133 Prospectively stored (-80°C) serum and whole blood samples were obtained from the
134 ANRS Biobank.

135 **Ethics**

136 All participants to the 3 studies provided written informed consent. Research protocols
137 were conducted in accordance with the Declaration of Helsinki and with national and
138 institutional standards and were approved by the relevant local interventional review
139 boards (Cochin-Tarnier, Paris-Cochin). Personal information and samples were de-
140 identified and analyzed anonymously.

141 **Detection of antibodies to the immunodominant epitope of the gp41 viral protein:**

142 **EIA-RI**

143 The level of antibody to the immunodominant epitope (IDE) of gp41 was estimated
144 following a procedure already described^{6,8,18}. We used an equimolar mixture of two 30
145 amino-acids oligopeptides, representing the consensus sequences of the IDE of HIV-1
146 group M and subtype D, respectively. A low concentration of this mixture (0.05 µg/mL
147 each) allows the binding of antibodies that are present at sufficient level or sufficient
148 avidity rendering feasible the semi-quantitative detection by spectrophotometry. The
149 antibody levels were expressed as absorbance values (OD values).

150 **Cell-associated HIV-1 DNA quantification**

151 HIV-1 DNA data from the ANRS-MONOI trial were described elsewhere.¹⁶ For COPANA
152 samples, total cellular DNA was extracted from blood using the DSP DNA Mini Kit with a
153 QiaSymphony instrument (Qiagen, Courtaboeuf, France). The number of extracted
154 genomes in DNA samples was quantified by a qPCR of the human albumin gene. Total
155 HIV-1 DNA load was assayed using the qPCR Generic HIV-1 DNA Cell® kit (Biocentric,
156 Bandol, France) and normalized by the albumin quantification.

157 **Ultrasensitive HIV-1 RNA viral load (usVL)**

158 As previously described¹⁶ MONOI participants were tested twice for usVL: at the
159 screening visit (week -10) and at inclusion (week 0: sample included in our study).
160 Participants with undetectable usVL (<1copy/mL) at both sampling times were
161 considered negative and participants with one or both positive results were considered
162 positive for usVL. Plasma samples from COPANA participants were assayed using the
163 Cobas Ampliprep/Cobas Taqman HIV-1 Test, version 2.0 (Roche Diagnostics). The
164 published limit of detection for this system is 20 copies/mL of plasma. The actual copy

165 number of <20 HIV RNA copies/mL was determined by extrapolating Ct values as
166 described by Chun *et al*¹⁷. UsVL data was not available for APROCO.

167 **Statistical analysis**

168 Patient characteristics are presented as medians and interquartile ranges for
169 continuous variables and percentages for categorical variables. In the univariate
170 analysis, the association between anti-gp41 levels and the following variables was
171 tested using Pearson correlation coefficients or the Kruskal-Wallis test: age, sex, time
172 since HIV diagnosis (years), ART duration (years), viral suppression duration (years),
173 CD4 and CD8 counts (cell/ μ L), nadir CD4 (cell/ μ L), CD4/CD8 ratio, total cell-associated
174 HIV-1 DNA (\log_{10} copies/ 10^6 cells), usVL (positive or negative as described above) and
175 anti-gp41 levels (OD). A series of univariate models were fitted to the data and all
176 variables providing $p < 0.05$ were retained into the final multivariate models. Each
177 multivariate model included the retained variables and either the duration of viral
178 suppression or HIV-1 DNA quantification. Final models were selected using a stepwise
179 procedure selecting variables with a $p < 0.05$. Statistical analyses were performed using
180 SAS software.

181

182 **Results**

183 We quantified anti-gp41 antibody levels in 683 chronically HIV-1-infected participants
184 from ANRS-APROCO (n=354), COPANA (n=119) and MONOI (n=210) studies. Individual
185 and immuno-virological characteristics at the time of anti-gp41 testing are detailed in
186 **Table 1**. Anti-gp41 antibody levels (OD) ranged from 0.0 to 3.5, the median value was
187 1.3 (IQR 0.6-1.9). 77.5% of the participants were male, with a median age of 41 years.
188 The median time from HIV-1 diagnosis was 13 years. All participants were treated with
189 ART, spanning a wide range of treatment durations from 1 to 18 years (median: 10.7
190 years) and virally suppressed below 50 copies/mL. The overall duration of viral
191 suppression was a median 6.6 years (IQR, 2-9.5). usVL data was available for 328
192 participants, amongst whom 56% (n=184) tested negative (<1 copy/ml). The overall
193 median cell-associated HIV-1 DNA level was 2.6 (IQR, 2.1-3.0) log₁₀copies/10⁶ cells.
194 HIV-1 DNA level decreased with the number of years on ART (p<0.0001).
195 Associations between anti-gp41 antibody levels and participants' clinical, virological
196 and immunological characteristics were assessed in a univariate analysis (**Table 2**).
197 Anti-gp41 level did not correlate with the overall duration since HIV-1 diagnosis.
198 Alternatively, it decreased with the duration of ART (Pearson r=-0.26, p<0.0001) and
199 most markedly with the duration of effective viral suppression <50 copies/mL (r=-0.46,
200 p<0.0001) (**Figure 1**). When taking into account the 328 available results for usVL,
201 there was no association with anti-gp41 antibodies (negative usVL, 1.4 OD (0.7-2.1) vs
202 positive usVL, 1.5 OD (0.8-1.9), Kruskal-Wallis p=0.77). However, in the MONOI
203 participants, who were tested for usVL both at week -10 and at inclusion, anti-gp41
204 levels were significantly lower when usVL tested consistently negative (negative usVL,
205 1.1 OD (0.5-1.6) vs 1 or 2 positive usVL, 1.4 OD (0.7-1.9), p=0.01) (**Figure 2**). Anti-gp41

206 levels were positively correlated to cell-associated HIV-1 DNA quantification ($r= 0.23$,
207 $p<0.0001$). In addition, a lower titer of anti-gp41 antibodies was observed in
208 participants with non-detectable HIV DNA compared to those with quantified HIV DNA
209 (0.9 (0.5-1.6) vs 1.5 (0.8-2.1), $p=0.02$). Finally, antibody levels were higher in women
210 than in men (1.5 (0.8-2.1) vs 1.2 (0.6-1.8), $p=0.0002$).

211 In multivariate models, anti-gp41 levels remained significantly correlated with the
212 duration of ART ($p=0.03$), the duration of viral suppression (<0.0001) and the levels of
213 cell-associated HIV-1 DNA ($p=0.0002$), as well as with gender ($p=0.008$).

214

215

216 **Discussion**

217 We studied the anti-gp41 antibody levels in long-term ART participants with
218 suppressed HIV-1 viremia below the clinical threshold of 50 copies/mL. Participants
219 initiated ART at the chronic phase of HIV-1 infection and were treated for a median of
220 10.7 years (1 to 18 years). Our results confirm the hypothesis that a decrease in anti-
221 gp41 antibodies mirrors the loss of virus production and antigenic stimulation. Anti-
222 gp41 levels were inversely correlated with the duration of viral suppression below 50
223 copies/mL, and were positively correlated with HIV-1 DNA quantification, residual
224 viremia and female gender. Thus, there was an association between specific HIV
225 antibody levels and both the size of the total cell-associated HIV-1 DNA burden and the
226 presence of a residual HIV-1 RNA viremia.

227 These results confirm previous studies in smaller cohorts which described a decrease in
228 anti-HIV antibodies along time on ART, in both chronically infected individuals and
229 primary HIV-1 infection participants, as well as in perinatally infected children^{8,10,11,18,19}.
230 Similarly to our results, Chaillon *et al.* using the same assay also reported higher
231 antibody levels in ART-treated women compared to men⁸. Gender differences in HIV
232 pathophysiology remain debated, with conflicted reports of stronger immune activation
233 and antiviral responses in HIV-infected women²⁰.

234 Previous reports however vary regarding the correlation between anti-gp41 levels and
235 the viral reservoir size. Brice and colleagues did not find an association between both
236 variables in a cross-sectional study of children who initiated ART at a median age of 3
237 years.¹⁸ Anti-gp41 levels, although estimated through the same immunoassay as we
238 used in the current study, were markedly lower than the numbers we observed. The use
239 of Dried Serum Spot samples may explain this discrepancy, along with the differences in

240 immune responses between childhood and adult age. Lee *et al.* used a luciferase
241 immunoprecipitation assay to quantify anti-HIV-1 antibodies^{10,19} and did not observe an
242 association with integrated HIV-1 DNA levels. Technical variations between that study
243 and ours may explain the differences in results.

244 Alternatively, our results back up the study from Keating and colleagues in recently
245 infected participants, reporting a correlation between anti-gp41 levels by the gp41
246 limiting antigen avidity assay (LAG, described elsewhere⁷) and PBMC-associated HIV-1
247 DNA for up to six years of viral suppression.¹¹ We observed a similar correlation
248 between anti-gp41 EIA-RI and CD4+ T-cell-associated HIV-1 DNA for 12 years ($r=0.25$).
249 These results suggest the maintenance of a higher production of anti-gp41 antibodies
250 by the immune system when HIV-1 DNA burden is high. Total cell-associated HIV-1 DNA
251 has the advantage of taking virtually all viral species into account, given that full-length
252 intact viral DNA is not mandatory to expose viral antigens to the immune system. On the
253 whole, the general reduction in anti-gp41 antibodies we observed on sustained and
254 prolonged viral suppression on ART may arise concurrently from the reduction of the
255 overall viral reservoir over time and/or from its enrichment in defective proviruses,
256 leading to an impaired production of viral particles and/or viral antigens.^{3,21}

257 In addition, we provide new data on usVL clinical significance. We observed an
258 association between lower anti-gp41 levels and a repeatedly negative residual viremia,
259 suggesting that systemic exposure to low-level viremia contributes to the maintenance
260 of anti-gp41 humoral responses and that the time dimension of antigenic exposure is
261 critical. Interestingly, such a time component is the basis of the “viremia copy-years”
262 biomarker, a time-updated measure of cumulative HIV-1 exposure akin to cigarette
263 pack-years in smoking exposure. Cumulative viremia copy-years is a calculation of the
264 area under the plasma viral load curve along follow-up and was described to associate

265 with clinical and immunological outcomes of HIV infection²²⁻²⁴. To our knowledge, such
266 a marker has not been described regarding cumulative single-copy viremia, and would
267 be tremendously demanding technically. Interestingly, our results point at the potential
268 of anti-gp41 antibody activity as a surrogate marker of residual viral exposure on long-
269 term ART, warranting further longitudinal studies.

270 As expected and in accordance with the previously published data, we observed
271 heterogeneous anti-gp41 levels in the participants to our study. Individual humoral
272 responses are driven by a variety of individual and viral factors and not all participants
273 initiate ART with the same baseline antibody level^{11,19}. At least by current laboratory
274 assays, defining a “significance threshold” seems elusive. Accordingly, the potential of
275 anti-gp41 titration as a biomarker for the reactivation-prone viral reservoir may be
276 further evaluated timewise for individual follow-up, or at the population level.

277 Our study has several limitations. The retrospective and cross-sectional design did not
278 allow a longitudinal follow-up of paired values of viremia and anti-gp41 antibodies,
279 although the cross-sectional design allowed the inclusion of a wide range of ART
280 durations. As the 3 different ANRS cohorts used for this study were run independently,
281 some retrospective data were missing and sufficient sample material was not always
282 available to complete HIV-1 DNA or usVL testing.

283 A major challenge to HIV cure strategies is the quantification of persistent replication-
284 competent HIV. A variety of laboratory assays coexist and none is fully satisfactory. In
285 addition, transient low-level viremia or viral blips cannot be always detected because of
286 sparse viral load testing performed as part of the standard care for HIV-1 infection.
287 Further longitudinal studies will be necessary to closely explore the kinetics of anti-
288 gp41 levels and their variations in relation to low-level viremia and individual factors. A
289 biomarker for the persistence of replication-competent HIV would be invaluable to the

290 evaluation of long-term adherence and efficacy of ART, to the development of new
291 antiretroviral strategies as well as to HIV cure studies.

292 In conclusion, assessing HIV specific immune response through the measurement of
293 anti-gp41 antibody levels could reflect the size of the viral reservoir and residual
294 replication during prolonged viral suppression on long-term ART. Lower titers of anti-
295 gp41 might reflect low antigen stimulation and better control of chronic HIV infection.

296

297

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306 **Transparency declarations**

307 All authors: none to declare.

308

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373

374

375 **Tables**376 **Table 1: Viral and immunological features at the time of anti-gp41 assay**

Participants: n=683	ANRS COPANA (n=119)		ANRS MONOI (n=210)		ANRS APROCO (n=354)	
	n	median [IQR]	n	median [IQR]	n	median [IQR]
Individual characteristics						
Age (years)	119	39.5 [33.8-48.7]	210	45.7 [40.3-53]	354	37.9 [33.3-43.7]
Gender: Male	119	71.4%	210	75.2%	354	80.8%
Time from HIV-1 diagnosis (years)	79	4.6 [3.6-6.2]	209	10.7 [4.9-15.6]	354	16.1 [12.3-19.8]
Time on ART (years)	119	2.0 [1.6-2.4]	207	8.4 [3.8-11.3]	352	11.3 [10.8-11.9]
HIV-1 RNA <50cp/mL (years)	119	1.6 [1.3-1.9]	NA	...	351	8.3 [5.6-10.2]
Immunology						
Nadir CD4+ T cells(cells/ μ L)	119	264 [205-325]	210	220 [147-298]	354	207 [104-320]
Current CD4+ T cells (cells/ μ L)	119	497 [367-655]	189	583 [445-772]	350	618 [442-785]
Current CD4/CD8 ratio	119	0.9 [0.6-1]	181	0.8 [0.6-1.1]	350	0.8 [0.6-1.1]
Biomarker measures						
usVL HIV-1 RNA <1cp/mL (%)	119	72%	210	46%	NA	...
HIV-1 DNA (\log_{10} cp/ 10^6 cells)	119	2.9 [2.3-3.3]	186	2.4 [2-2.7]	NA	...
Anti-gp41 antibody (OD value)	119	1.7 [1-2.3]	210	1.3 [0.7-1.8]	354	1.1 [0.6-1.7]

n: number with data; IQR: interquartile range; cp: copy ; NA : not available

377 **Table 2: Association between anti-gp41 levels and quantitative characteristics**
 378 **(univariate analysis)**

Variables	Participants (n)	Pearson coefficient	Significance (2-tailed)
Age	683	-0.03	0.41
HIV-1 infection (years)	642	0.02	0.55
ART (years)	678	-0.26	<0.0001
Viral suppression (years)	470	-0.46	<0.0001
Nadir CD4+ T-cell count	683	-0.01	0.88
Current CD4+ T-cell count	658	-0.08	0.02
CD4+/CD8+ ratio	650	-0.10	0.04
Cell-associated HIV-1 DNA	305	0.23	<0.0001

Variables	Participants (n)	Kruskall-Wallis test	Significance
Sex	683	Male vs Female	0.0002
usVL HIV-1 RNA	328	Negative vs Positive	0.77
Cell-associated HIV-1 DNA below limit of detection	305	Negative vs Positive	0.02

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381 **Figure Legends**

382 **Figure 1: Anti-gp41 titers and the duration of viral suppression**

383 Association between anti-gp41 antibody titers (OD) and duration of viral suppression
384 below 50 copies/mL (cross-sectional analysis of 470 participants).

385

386 **Figure 2: Anti-gp41 titers according to residual viremia**

387 Anti-gp41 antibody levels according to the usVL status in MONOI participants.

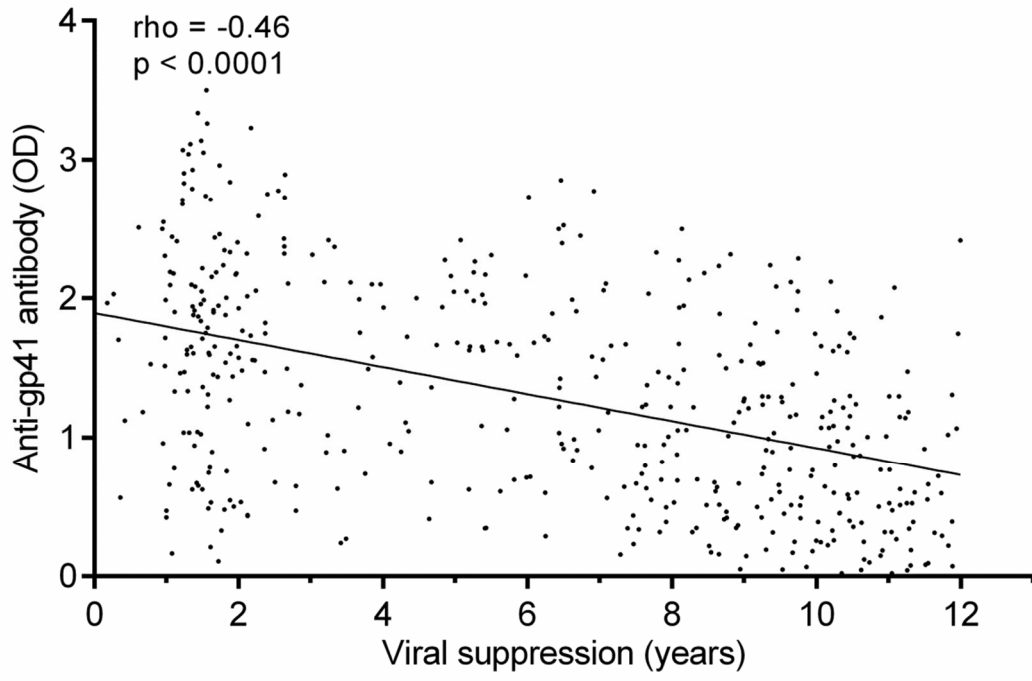
388 Undetectable usVL: negative assay at both week -10 and the inclusion visit. Positive

389 usVL: ≥ 1 copy/mL usVL at one or both visits.

390

391 **Figures**

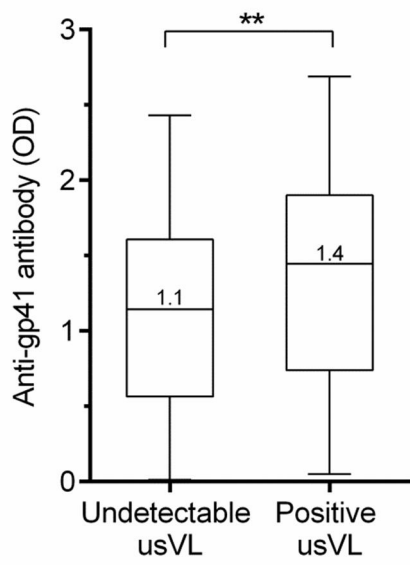
392 **Figure 1**



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395 **Figure 2**



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