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Presence of HIV-1 G-to-A mutations linked to APOBEC editing is more prevalent in non-B HIV-1 subtypes and is associated with lower HIV-1 reservoir

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1 Presence of HIV-1 G-to-A mutations linked to APOBEC editing is more prevalent in non-B
2 HIV-1 subtypes and is associated with lower HIV-1 reservoir
3 Running head: Association of APOBEC3 footprints in HIV-1 DNA with non-B HIV-1 and
4 low HIV-1 reservoir

5

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29 **Abstract**

30 **Objectives**

31 APOBEC3 editing activity contributes to sequences variation and viral diversification. We
32 aimed to characterize virological and clinical factors associated with G-to-A mutations and
33 stop codons in the HIV-1 reservoir, markers of APOBEC3 footprints, in order to better
34 understand HIV-1 diversity among virologically suppressed HIV-1 infected patients.

35 **Methods**

36 Immuno-virological and clinical factors were compared between 92 patients harboring G-to-A
37 mutations and stop codons (APOBEC+) in the reverse transcriptase gene and 92 patients
38 without G-to-A mutations (APOBEC-) nor stop codons in their DNA genotypes.

39 **Results**

40 Patients were predominantly men (74.5%) and were mostly infected by B-subtype (69.0%),
41 with 44.1% and 55.9% in APOBEC+ and APOBEC- groups, respectively. At time of HIV
42 DNA genotypes, the total cell associated HIV-1 DNA load was 2.34 log₁₀ copies/10⁶ cells in
43 median (IQR 1.85-2.67) and 33.2% of them had a detectable ultra-sensitive plasma viral load.
44 Hypermutated sequences were identified in 28.2% in APOBEC+ group. The median of total
45 cell-associated HIV-1 DNA level was significantly lower in APOBEC+ than APOBEC-
46 group: 2.13 log₁₀ copies/10⁶ cells (IQR 1.60-2.60) *versus* 2.52 log₁₀ copies/10⁶ cells (IQR
47 2.19-2.71), ($p<0.001$). Presence of G-to-A mutations and stop codon was independently
48 associated with HIV-1 subtype non B ($p=0.017$).

49 **Conclusion**

50 These results show an independent association between the presence of G-to-A mutations and
51 stop codons with HIV-1 subtype non-B and low proviral DNA that could be explained by the
52 APOBEC3 footprints and restriction of DNA synthesis and integration. However, further
53 investigations are needed to study the contribution of Vif amino acid variability among HIV-1
54 subtypes.

55 **Introduction**

56 APOBEC3 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) is an
57 important innate immune family of cytidine deaminase proteins. These cellular proteins block
58 actively retroviral infection by hitchhiking newly produced viral particles.¹ Different
59 APOBEC3 family members were distinguished but mainly two enzymes APOBEC3F and
60 APOBEC3G that have slightly different substrate specificities, produce G-to-A transitions and
61 inhibit profoundly the replication of HIV-1.^{1,2} The antiviral activity of APOBEC3 is
62 counteracted by the HIV viral infectivity factor (Vif) protein by mediating its degradation by
63 the proteasome. When Vif is defective, the result is hypermutation, an inordinate number of
64 identical transitions G to A.^{1,3,4}

65 Several studies have shown that APOBEC3 contributes to sequences variation and viral
66 diversification because of naturally occurring defective HIV variants.^{3,5}

67 One study demonstrates also that defective genomes were systematically detected in 5
68 patients on long-term ART and this high level of defective genomes was correlated with a
69 small size of HIV proviral DNA.⁶

70 A better understanding of HIV-1 diversity and the persistence of the HIV-1 reservoir is
71 necessary to characterize the viruses in order to develop new therapeutic approaches and to
72 better target HIV-1 patients susceptible to receive optimize eradication strategies. Thus, we
73 aim here to compare patients with or without HIV-1 APOBEC mutations and stop codons in
74 the HIV-1 reservoir in order to determine which virological or clinical factors could be
75 associated with APOBEC editing activity.

76

77 **Materials and Methods**

78 **Study design and patients**

79 One hundred eighty-four HIV-1 patients were retrospectively studied at the Pitié-Salpêtrière
80 hospital, Paris, France. The study was focused on the RT gene, the most region targeted by
81 APOBEC editing activity according of the Stanford University list of signature APOBEC
82 mutations (<https://hivdb.stanford.edu/page/apobec/>). The only 5 G-to-A transitions identified
83 in this list as RT DRAMs (D67N, E138K, M184I, E190G/S and M230) were used to select
84 patients. Then, we included all HIV-1 patients between January 2011 and June 2019 with a
85 Plasma Viral Load (pVL) ≤ 20 copies/mL under ART having at least one of these G-to-A drug
86 resistance mutations (DRAMs) and stop codons in the reverse transcriptase gene (RT) in their
87 HIV-1 DNA genotypic drug resistance test performed by Sanger sequencing according to the
88 ANRS HIV Drug Resistance procedures (APOBEC+) (n=92). A second group of patients,
89 with a pVL ≤ 20 copies/mL and with nor G-to-A DRAMs nor stop codons in their DNA
90 genotypes were included adjusting on the genotype's time period (APOBEC-) (n=92).

91 Cumulative HIV-1 RNA and DNA DRAMs were interpreted using the latest ANRS resistance
92 algorithm (<http://www.hivfrenchresistance.org/>).

93 This work was a retrospective non-interventional study with no addition to standard care
94 procedures. The study was carried out in accordance with the Declaration of Helsinki.

95

96 **Total HIV-DNA quantification**

97 Cell-associated HIV-1 DNA was quantified by ultrasensitive real-time PCR (Generic HIV-
98 DNA assay, Biocentric, Bandol, France) as previously described.⁷

99

100 **Ultra-sensitive viral load**

101 The pVL was quantified using the Cobas AmpliPrep/CobasTaqMan HIV-1 assay (Roche
102 Diagnostics, Switzerland; lower detection limit of 20 copies/ml). Ultra-sensitive viral load
103 (USpVL) in the range of 1–20 copies/ml was indicated qualitatively (presence or absence of
104 detectable signal).⁸

105

106 **Identification of hypermutated G-to-A sequences**

107 Hypermutation was analyzed using the Hypermut Analysis and Detection of APOBEC-
108 induced Hypermutation program
109 (<https://www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html>) in DNA sequences.
110 A sequence was considered hypermutated if it registered a P value less than 0.05 on the
111 Fisher's exact test that compared the number of G-to-A changes due to APOBEC3 versus
112 HxB2 reference (K03455).

113

114 **Statistical analysis**

115 All reported values are medians with IQR for continuous variables and frequencies and
116 percentages for categorical variables. We used the Mann–Whitney test and the Chi2 test for
117 comparison between patients' groups APOBEC+ and APOBEC-. Univariable and
118 multivariable models were used to identify factors associated with G-to-A mutations: age, sex,
119 ethnicity, time from HIV diagnosis, duration of ART, time to undetectable pVL, zenith of
120 pVL, Nadir of CD4 cells count, baseline CD4 cells count, baseline CD4/CD8 ratio, HIV-1
121 subtype and at time of HIV-1 DNA genotypic drug resistance test: HIV-1 DNA viral load,
122 USpVL, CD4 cells count, CD4/CD8 ratio and hypermutation. Variable with an univariable p

123 value <0.2 were retained in the multivariable analysis. All reported p values are two-tailed,
124 with significance set at 0.05. Analyses were performed using Statview for Windows (SAS
125 Institute Inc., version 5.0.1, Cary, NC, USA).

126

127 **Results**

128

129 **Patients' characteristics**

130 One hundred eighty-four patients were included in the study. They were predominantly men
131 (74.5%) and Caucasian (71.7%). They were HIV-1 diagnosed in median 22.8 years ago (15.5-
132 27.2), ART treated for 19.1 years (13.7-22.2) and they had undetectable pVL for 6.9 years in
133 median (3.1-11.0) (table 1). 183/184 (99.45%) of patients had received RT inhibitors in the
134 past. The patients were mostly infected by B-subtype (69.0%), with 44.1% and 55.9% in
135 APOBEC+ and APOBEC- ($p=0.017$), respectively. Details of frequency of HIV-1 subtypes
136 are presented in table S1 in supplementary data. At time of HIV DNA genotype test, the total
137 cell associated HIV-1 DNA load was 2.34 \log_{10} copies/ 10^6 cells in median (IQR 1.85-2.67)
138 and 33.2% of all patients had a detectable USpVL. The median of total cell-associated HIV-1
139 DNA level was significantly lower in APOBEC+ than APOBEC-: 2.13 (IQR 1.60-2.60)
140 versus 2.52 \log_{10} copies/ 10^6 cells (IQR 2.19-2.71), ($p<0.001$).

141

142 **HIV-1 sequences characteristics**

143 The distribution of HIV-1 G-to-A DRAMs and stop codons in the study participant's
144 sequences of the APOBEC+ (n=92) was: 15.2% (14/92) D67N, 2.2% (2/92) E138K, 33.7%
145 (31/92) M184I, 1.1% (1/92) E190G/S, 41.3% (38/92) M230I and 100% (92/92) stop codon.
146 Stop codons were mostly found at the following amino acid positions: W71, W88, W153,
147 W212, W229, W239 and W266.

148 The distribution of HIV-1 DRAMs not APOBEC related is shown in table S2 in
149 supplementary data. Globally, there is no a significant difference between the two groups
150 (APOBEC+ and APOBEC-) according to the DRAMs prevalence.

151 Hypermutated sequences were identified in 28.2% (26/92) in APOBEC+. As expected, no
152 patients in APOBEC- had hypermuted HIV-1 sequences.

153

154 **Risk factors associated with APOBEC mutations and stop codons**

155 Univariate and multivariate logistic regression analyses were performed to assess independent
156 associations between immune-virological and clinical data and APOBEC mutation's presence
157 (table 2). Three factors were retained for the multivariable analysis: ethnicity ($p=0.018$), total
158 cell-associated HIV-1 DNA level ($p=0.016$) and HIV-1 subtype ($p=0.016$). The multivariable
159 analysis showed that G-to-A DRAMs and stop codons presence was independently associated
160 with HIV-1 subtype and level of total cell associated HIV-1 DNA. The presence of G-to-A
161 DRAMs and stop codons was 2.8 times higher (95% CI 1.25-6.35) for patients infected by
162 HIV-1 non-B subtypes and inversely related to the level of total cell associated HIV-1 DNA
163 (OR 0.34, 95% CI 0.18-0.63).

164

165 **Discussion**

166 In the context of viral diversity and the development of new therapeutic approaches for HIV-1
167 cure, careful consideration must be given to the identification and characterization of HIV-1
168 reservoir. The ability of APOBEC editing activity to introduce mutations into viral DNA may
169 contribute to viral diversity. Here, we find in a large number of virologically well controlled
170 HIV-1 patients that G-to-A mutations DRAMs and stop codons presence in RT sequences
171 were associated with a smaller size of HIV-1 reservoir and HIV-1 non-B subtypes.

172 Our results are in line with other studies showing an inverse association between APOBEC3
173 protein activity and level of HIV proviral DNA. Fourati *et al.*, studied the relationship
174 between the size of the reservoir and the frequency of defective genomes in 5 patients on
175 successful ART and 5 untreated patients and concluded that a high level of defective genomes
176 was correlated with a small size of HIV proviral DNA.⁶ Other researchers quantified provirus
177 and APOBEC3G levels in resting CD4+ T lymphocytes in HIV controllers and ART-
178 suppressed non-controllers and concluded that the highest levels of APOBEC3G protein in
179 resting memory CD4+ T cells were significantly associated with the lowest levels of DNA
180 provirus.⁹ These results could be explained by the innate APOBEC3 footprints on the viral
181 genome. Indeed, APOBEC3 family is the host restriction factor that inhibits HIV-1
182 replication, DNA synthesis and integration by blocking viral plus-strand DNA transfer and
183 inhibiting provirus establishment in the host genome.¹⁰

184 We showed also that the presence of HIV-1 G-to-A DRAMs linked to APOBEC editing were
185 more prevalent in non-B HIV-1 subtypes. This finding could be explained by the genetic
186 variability of Vif that counteracts the antiviral activity of APOBEC3 by proteasome
187 degradation and polymorphism in APOBEC3 gene family members depending on the HIV-1
188 viral subtype. Previous works have focused on the contribution of Vif amino acid variability
189 among HIV-1 subtypes and they all reported that Vif proteins derived from HIV-1 clinical
190 and viral isolates of different subtypes varied in their activities against APOBEC3 but there
191 were some discrepancies between results.¹¹⁻¹³ Mawuena *et al.* have found that several non-B
192 subtypes Vif alleles have an efficient anti-APOBEC3 activity similar to the commonly used
193 subtype B Vifs.¹¹ Other researchers showed that Vif derived from a subtype C molecular
194 clone was less effective at overcoming APOBEC3-mediated inhibition than Vif derived from
195 either subtype B or CRF02_AG molecular clones.¹² However, Yukie *et al.* found that Vif
196 protein derived from subtype C strains harbored the most robust anti-APOBEC3 activity.¹³

197 Limitation of all these studies is the sample sizes which are not sufficiently representative of
198 all the HIV-1 subtypes' diversity. In our study, we were not able to identify a specific non-B
199 subtype that was associated with the presence of G-to-A DRAMs and stop codons. Thus,
200 further investigations should be conducted focusing on particular non-B subtypes (i.e. C or
201 CRF02_AG).

202 Our study had some limitations as the sample size, although all HIV-1 patients with APOBEC
203 DRAMs and stop codons were selected on the studied period time. Multivariate models with
204 relative small sample sizes tend to be unstable, thus the future study could benefit more from
205 a larger sample size. Furthermore, the use of the classical Sanger sequencing technology
206 could underestimate resistance and stop codons. The sensitivity of Sanger sequencing in
207 detecting resistant variants within quasi-species of WT viruses is ~20%, whereas the
208 sensitivity of the ultra-deep sequencing (UDS) assay allows the use of a sensitivity threshold
209 as low as 1%.¹⁴ Rodriguez and colleagues showed that UDS have better sensitivity in
210 detecting mutations. They also found more stop codons by UDS 1% than bulk sequencing
211 (44% versus 26%).¹⁴ However, Sanger sequencing is a widely used technic in standard follow
212 up of HIV-patients.

213 The ability of APOBEC3 to introduce G-to-A DRAMs and stop codons into HIV-1 viral
214 DNA, and its contribution to viral diversity and viral pathogenesis is now clearly established.
215 However, some factors could influence this contribution and this study showed that there is an
216 independent association between the presence of G-to-A DRAMs and stop codons with HIV-
217 1 subtype non-B and low proviral DNA. The biological mechanisms underlying these
218 associations should be further investigated in order to better characterize HIV-1 reservoir and
219 to help in developing new therapeutic approaches for HIV-1 cure.

220

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222

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226

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	All patients n=184	APOBEC- group n=92	APOBEC+ group n=92	P value
Sex (Female versus Male), n (%)				
Female	47 (25.5)	23 (48.9)	24 (51.1)	0.866
Male	137 (74.5)	69 (50.4)	68 (49.6)	
Age (years), median (IQR)	58 (53-65)	59 (54-65)	56 (51-65)	0.063
Ethnicity (Caucasian versus Non Caucasian), n (%)				
Caucasian	132 (71.7)	66 (50)	66 (50)	1.000
Non Caucasian	52 (28.3)	26 (50)	26 (50)	
Time from HIV diagnosis (years), median (IQR)	22.8 (15.5-27.2)	24.6 (17.6-27.6)	21.7 (14.3-26.5)	0.072
Duration of ART (years), median (IQR)	19.1 (13.7-22.2)	19.78 (15.4-22.5)	18.7 (12.4-22.0)	0.187
Time to undetectable pVL (years), median (IQR)	6.95 (3.1-11.0)	6.77 (3.5-10.9)	7.0 (2.1-12.3)	0.845
Zenith of pVL (log ₁₀ copies/mL), median (IQR)	4.96 (4.25-5.44)	4.99 (4.25-5.38)	4.90 (4.25-5.47)	0.743
Nadir of CD4 (cells count /mm ³), median (IQR)	176 (78-289)	176 (61-281)	173 (80-297)	0.998
CD4 at baseline (cells count/mm ³), median (IQR)	315 (176-434)	333 (202-504)	312 (161-419)	0.401
CD4/CD8 ratio at baseline, median (IQR)	0.36 (0.20-0.60)	0.37 (0.20-0.60)	0.34 (0.20-0.60)	0.753
HIV-1 subtype, n (%)				
B	127 (69.0)	71 (55.9)	56 (44.1)	0.017
Non-B	57 (31.0)	21 (36.8)	36 (63.2)	
Total cell associated HIV-1 DNA (log ₁₀ / 10 ⁶ cells), median (IQR)	2.34 (1.85-2.67)	2.52 (2.19-2.71)	2.13 (1.60-2.60)	<0.001
Ultra-sensitive pVL, n (%)	61 (33.2)	32 (52.5)	29 (47.5)	0.638

CD4 (cells count/mm ³), median (IQR)	617 (446-836)	669 (462-852)	602 (402-789)	0.092
CD4/CD8 ratio, median (IQR)	0.82 (0.58-1.26)	0.83 (0.60-1.41)	0.81 (0.54-1.18)	0.469

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289 **Table 1: Patient's characteristics.** All reported values are medians with IQR for continuous
290 variables and frequencies and percentages for categorical variables. Mann–Whitney and Chi2 tests
291 are used for comparison between patients APOBEC+ and APOBEC-. Statistical significance set at
292 0.05 is indicated in bold. pVL= plasma Viral Load.

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	Univariate analysis		Multivariate analysis	
	OR (95% IC)	<i>p</i>	OR (95% IC)	<i>p</i>
Sex (Female versus Male)				
Female	1			
Male	2.38 (0.62-9.20)	0.206		
Age (years)	0.98 (0.94-1.02)	0.464		
Ethnicity (Non Caucasian versus Caucasian)				
Non-Caucasian	1		1	
Caucasian	4.87 (1.31-18.08)	0.018	1.76 (0.76-4.05)	0.180
Time from HIV diagnosis (years)	1.00 (1.00-1.00)	0.825		
Duration of ART (years)	1.00 (1.00-1.00)	0.650		
Time to undetectable pVL (years)	1.00 (1.00-1.00)	0.928		
Zenith of pVL (log ₁₀ copies/mL)	0.94 (0.56-1.57)	0.819		
Nadir of CD4 (cells count /mm ³)	1.00 (0.99-1.00)	0.830		
CD4 (cells count/mm ³)	1.00 (0.99-1.00)	0.833		
CD4/CD8 ratio	1.87 (0.20-17.23)	0.578		
HIV sub-type (B versus Non_B)				
B	1		1	
Non_B	5.08 (1.36-18.98)	0.016	2.81 (1.25-6.35)	0.013
HIV parameters at time of HIV-1 DNA genotypic drug resistance				
Total cell associated HIV-1 DNA (log ₁₀ /10 ⁶ cells)	0.30 (0.11-0.80)	0.016	0.34 (0.18-0.63)	0.001
Ultra-sensitive pVL	1.51 (0.55-4.17)	0.421		
CD4 (cells count/mm ³)	1.00 (0.99-1.00)	0.677		
CD4/CD8 ratio	0.77 (0.21-2.78)	0.695		

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311 **Table 2: Univariate and multivariate analysis to identify factors associated with**

312 **APOBEC editing activity.** Univariable P value <0.2 were retained in the multivariable

313 analysis. Statistical significance set at 0.05 are indicated in bold. MSM= Men who have Sex

314 with Men; pVL= plasma Viral Load

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Supplementary materials

	All patients n=184	APOBEC- group n=92	APOBEC+ group n=92
B	127 (69)	71 (55.9)	56 (44.1)
CRF02_AG	25 (13.6)	11 (44)	14 (56)
A	3 (1.63)	1 (33.3)	2 (66.7)
D	3 (1.6)	1 (33.3)	2 (66.7)
F	1 (0.5)	1 (100)	0 (0)
G	4 (2.2)	1 (25)	3 (75)
H	1 (0.5)	0 (0)	1 (100)
CRF06_cpx	4 (2.2)	1 (25)	3 (75)
CRF13_cpx	2 (1.1)	1 (50)	1 (50)
CRF45_cpx	1 (0.5)	0 (0)	1 (100)
Non determined	13 (7.1)	4 (30.8)	9 (0)

Table S1: **Frequency of HIV-1 subtype.** All reported values are frequencies and percentages for categorical variables

	All patients n=184	APOBEC- group n=92	APOBEC+ group n=92	P value
HIV-1 drug resistance associated mutations				
M41L	46 (25)	27 (58.7)	19 (41.3)	0.173
E44D	8 (4.3)	5 (62.5)	3 (37.5)	0.470
K65R	3 (1.6)	1 (33.3)	2 (66.7)	0.560
T69D	7 (3.8)	3 (42.9)	4 (57.1)	0.700
K70R	15 (8.2)	7 (46.7)	8 (53.3)	0.788
L74V	12 (6.5)	5 (41.7)	7 (58.3)	0.550
K101E	4 (2.2)	2 (50)	2 (50)	1.000
K103N	15 (8.2)	5 (33.3)	10 (66.7)	0.178
Y115F	2 (1.1)	1 (50)	1 (50)	1.000
E138A	11 (6)	4 (36.4)	7 (63.6)	0.351
M184V	55 (29.9)	30 (54.5)	25 (45.5)	0.421
Y181C	10 (5.4)	5 (50)	5 (50)	1.000
Y188L	2 (1.1)	1 (50)	1 (50)	1.000
G190A	5 (2.7)	2 (40)	3 (60)	0.650
L210W	30 (16.3)	15 (50)	15 (50)	1.000
T215Y	44 (23.9)	24 (54.5)	20 (45.5)	0.489
T215D	6 (3.3)	2 (33.3)	4 (66.7)	0.406
T215E	2 (1.1)	1 (50)	1 (50)	1.000
T215H	3 (1.6)	1 (33.3)	2 (66.7)	0.560
T215S	19 (10.3)	6 (31.6)	13 (68.4)	0.090
K219Q	6 (3.3)	3 (50)	3 (50)	1.000
P225H	3 (1.6)	0 (0)	3 (100)	0.081

Table S2: Cumulative HIV-1 drug resistance associated mutations not APOBEC related

in reverse transcriptase gene. All reported values are frequencies and percentages for categorical variables. Chi2 test is used for comparison between patients APOBEC+ and APOBEC-. Statistical significance set at 0.05 is indicated in bold.