Contribution of APOBEC3G/F activity to the development of low-abundance drug-resistant human immunodeficiency virus type 1 variants

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Abstract

Plasma drug-resistant minority human immunodeficiency virus type 1 variants (DRMVs) increase the risk of virological failure to first-line non-nucleoside reverse transcriptase inhibitor antiretroviral therapy (ART). The origin of DRMVs in ART-naive patients, however, remains unclear. In a large pan-European case–control study investigating the clinical relevance of pre-existing DRMVs using 454 pyrosequencing, the six most prevalent plasma DRMVs detected corresponded to G-to-A nucleotide mutations (V90I, V106I, V108I, E138K, M184I and M230I). Here, we evaluated if such DRMVs could have emerged from apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3G/F (APOBEC3G/F) activity. Out of 236 ART-naive subjects evaluated, APOBEC3G/F hypermutation signatures were detected in plasma viruses of 14 (5.9%) individuals. Samples with minority E138K, M184I, and M230I mutations, but not those with V90I, V106I or V108I, were significantly associated with APOBEC3G/F activity (Fisher's $P < 0.005$), defined as the presence of > 0.5% of sample sequences with an APOBEC3G/F signature. Mutations E138K, M184I and M230I co-occurred in the same sequence as APOBEC3G/F signatures in 3/9 (33%), 5/11 (45%) and 4/8 (50%) of samples, respectively; such linkage was not found for V90I, V106I or V108I. In-frame STOP codons were observed in 1.5% of all clonal sequences; 14.8% of them co-occurred with APOBEC3G/F signatures. APOBEC3G/F-associated E138K, M184I and M230I appeared within clonal sequences containing in-frame STOP codons in 2/3 (66%), 5/5 (100%) and 4/4 (100%) of the samples. In a re-analysis of the parent case control study, the presence of APOBEC3G/F signatures was not associated with virological failure. In conclusion, the contribution of APOBEC3G/F editing to the development of DRMVs is very limited and does not affect the efficacy of non-nucleoside reverse transcriptase inhibitor ART.

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Introduction

Cumulative evidence indicates that minority (or low-abundance) drug-resistant human immunodeficiency virus type 1 (HIV-1) variants (DRMVs) impair virological responses to first-line antiretroviral therapy (ART) containing non-nucleoside reverse transcriptase inhibitors (NNRTIs) [1–3]. The origin of such variants remains unclear: whereas drug-resistant variants can be transmitted [4], they could also be generated spontaneously through a variety of mechanisms [5]. In a previous European case–control study [3], we showed that pre-existing DRMVs harbouring resistance to reverse transcriptase inhibitors more than doubled the risk for virological failure in ART-naive patients starting efavirenz- or nevirapine-containing regimens. Interestingly, the six most prevalent mutations detected in the study corresponded to G-to-A nucleotide mutations, which resulted in valine to isoleucine (V90I, V106I and V108I), methionine to isoleucine (M184I and M230I) or glutamate to lysine (E138K) mutations. This observation prompted us to investigate whether such DRMVs could have been generated through APOBEC3G/F editing.

APOBEC3G/F (apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3G/F) proteins are cellular proteins within the APOBEC family that inhibit the replication of HIV-1 and other lentiviruses [6] through cytidine deamination of their genomes [7]. Although all APOBEC proteins can induce G-to-A mutations, APOBEC3G/F (and H) are highly expressed in $CD4^+$ T cells [8]. APOBEC3G/F molecules are packaged into nascent virions and mutate the cDNA produced by the viral reverse transcriptase. Cytidines in the newly synthesized cDNA strand are deaminated and turned into uracils, so leading to G-to-A mutations in the viral plus DNA strand. The preferred genetic context of APOBEC3G and APOBEC3F is 5′-<u>G</u>G-3′ and 5′-<u>G</u>A-3′, respectively, where the 5′ nucleotide (underlined) is mutated [9,10]. Furthermore, sequence context specificity can be influenced by the secondary structure of single-stranded DNA [10]. Of note, 5′-T<u>G</u>GG-3′ is an important hotspot for APOBEC3G activity, whereas 5′-T<u>G</u>AA-3′ is most often used by APOBEC3F [9]. The antiviral effect of the APOBEC3G/F proteins derives from their ability to hypermutate the genome of HIV-1, leading to DNA degradation, and from generation of replication-incompetent viruses through the development of in-frame STOP codons.

The effect of APOBEC3G/F is counteracted by the HIV-1 virus infectivity factor (*Vif*) protein, which binds to APOBEC3G/F and recruits a ubiquitine ligase protein complex that targets APOBEC3G/F for degradation [6,11]. As the strength of this specific interaction and subsequent APOBEC3G/F degradation varies on a small set of *Vif* and APOBEC3G/F mutations [12–15], *Vif*-mediated inhibition of APOBEC3G/F activity may be total or partial; the latter can generate sublethal levels of viral genetic diversity [16–18]. Therefore, virus–host co-evolutionary dynamics through APOBEC3G/F–*Vif* interactions provide HIV-1 with additional paths for adaptation to selective pressure and can lead to the generation of new resistance mutations. Particularly, previous studies using different modified cell lines have shown that mutations generated by APOBEC3G/F can be used by HIV-1 to generate diversity and, in some cases, mutations in reverse transcriptase (RT) that provide resistance to several antiretroviral drugs [12,17,19]. Furthermore, impaired processivity of the HIV-1 RT, for instance, due to the presence of the M184I mutation, can increase the APOBEC3G/F G-to-A mutation rate [20].

Here, we sought to elucidate the contribution of APOBEC3G/F editing in the development of minority drug-resistant HIV-1 variants in ART-naive patients.

Methods

Study design and 454 pyrosequencing

This was a post-hoc sub-analysis of a previously published European case–control study aimed at determining the effect of pre-existing DRMVs on the virological outcomes of first-line ART including NNRTIs [3]. The most prevalent DRMVs detected in the parent study were V90I, V106I, V108I, E138K, M184I and M230I. Here, we analysed HIV-1 reverse transcriptase 454 sequences (amplicons B, C and D (see Supplementary material, Fig. S1)) from the parent study participants. Briefly, cryopreserved plasma samples with HIV-1 RNA levels ≥10 000 copies/mL were collected from six different European cohorts. Samples were sequenced in a 454 FLX Genome Sequencer using the Titanium chemistry and analysed centrally, as previously described [3]. The DRMVs were defined as those found at between 0.5% and 25% of the viruses comprising the overall viral population.

Data analysis

Generation of sequence alignments. (see Supplementary material, Fig. S2) Error-corrected and collapsed unique sequences were obtained using AVA software (Amplicon Variant Analyzer v2.7.0; Roche, Basel, Switzerland) for all samples. The number of raw reads supporting each unique sequence was used to

calculate the abundance both of mutations and of APOBEC3G/F hypermutation signatures as defined below. Sequence alignments were produced by MOSAIK ALIGNER Software (v.2.1.73) [21]. PERL SCRIPTS, R [22] and SAMTOOLS [23] software libraries were used for sequence data processing and statistical analysis. For each sample, a reference-guided pairwise alignment using the HIV-1 reference strain HXB2 (GenBank ID: K03455.1) was generated. This HXB2-guided alignment was used for two purposes: first, to obtain amplicon-based haplotypes with amino acid substitutions for each sequence read, and second, to obtain a patient-specific consensus sequence, which was used to define APOBEC3G/F target and control sites. Sequence reads for each sample were then re-aligned to the corresponding patient-specific consensus sequence.

APOBEC3G/F status definition. Target and control tri-nucleotide sequences were defined as GRD and GYN|GRC, respectively, using IUPAC notation [24]. These were located in each sequence read. Using an approach mimicking the HYPERMUT2 tool for Sanger sequences (http://www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html), reads were considered mutated or not by comparing aligned sequence reads with patient-specific consensus. The number of mutated versus non-mutated nucleotides in target and control sites was evaluated in each sequence read. An 'APOBEC3G/F-edited' status flag was defined for each sequence read if a Fisher's test provided a p value < 0.05, indicating that APOBEC3G/F mutations were preferentially occurring in expected target sites. Presence of overall 'APOBEC3G/F activity' in individual samples was defined as the existence of >0.5% of sample sequences with an 'APOBEC3G/F-edited' status flag (see Supplementary material, Fig. S2).

Association between DRMV and APOBEC3G/F status. The association between DRMVs and APOBEC3G/F activity was evaluated at clonal levels (i.e. co-ocurrence of DRMVs and APOBEC3G/F signatures in the same clonal sequence) and as well as at sample levels (i.e. presence of APOBEC3G/F activity in samples with and without DRMVs) using 2 × 2 contingency tables and Fisher's exact tests to evaluate the statistical significance of the observed associations.

Clinical relevance of APOBEC3G/F activity. To assess the influence of APOBEC3G/F activity on virological outcomes, we reanalysed the multivariate models of risk of virological failure from the parent study [3], incorporating the variable 'APOBEC3G/F activity' as one of the exposure variables of interest.

Results

The 454 pyrosequencing data were available from 326 plasma samples (see Supplementary material, Fig. S1). For this substudy we only analysed 236 samples that had at least 400 sequences for amplicon E and for either amplicon C or D to ensure sufficient sequencing depth to detect both DRMVs and APOBEC3G/F hypermutation signatures in individual sequences (Table 1). Overall, the most frequent nucleotide substitutions detected in our data set were A-to-G (23.3%) and G-to-A (20.9%) transitions, followed by T-to-C (15.7%) and C-to-T (13.6%) transitions. Similar distribution was observed when amplicons were analysed separately, ruling out amplicon bias (see Supplementary material, Fig. S3).

Associations at sample level. Fourteen/236 (5.9%) samples contained APOBEC3G/F editing signatures in at least 0.5% of sequences, being flagged as 'hypermutated' samples. The percentage of APOBEC3G/F-edited sequences within hypermutated samples ranged from 0.6% to 7.2% (Table 2). Different two-nucleotide downstream sequence context preferences were found for G-to-A mutations in APOBEC3G/F edited and non-edited samples. GA sequence context was preferred for APOBEC3G/F edited samples, whereas the AA sequence context was more frequent in non-edited samples. These differences were not observed for A-to-G mutations (see Supplementary material, Fig. S4A).

For samples harbouring the DRMVs of interest, the sequence context at M184I, M230I and E138K sites corresponded to well-defined G-to-A APOBEC3G and APOBEC3F mutation hotspots 5′-TGGR-3' (M184I, M230I) and 5′-TGAR-3' (E138K), respectively. Mutations V90I, V106I and V108I, conversely, occurred in a sequence context not previously defined as a hotspot for APOBEC3G/F G-to-A mutations. Additionally, samples harbouring M184I and M230I, but not E138K, V90I, V106 or V108I, showed a significantly higher proportion of G-to-A mutations occurring within any of APOBEC3G/F target sites than those obtained from the global data set (see Supplementary material, Fig. S4B).

TABLE 1. Summary of sequencing results per amplicon

	Amplicon		
	C	D	E
Samples with >400 sequence reads	252	236	284
Median (MAD) number of sequence reads per sample	1620 (940)	1634 (893)	1650 (1051)
Amplicon length (base pairs)	316	309	337
Median (MAD) number of APOBEC3G/F target sites	37 (3)	37 (3)	38 (3)
Global % of G-to-A mutated target site positions	1.4	1.0	1.5
Median number of APOBEC3G/F control sites	23	24	25
Global % of G-to-A mutated control site positions	1.2	1.7	1.7
Global % of APOBEC3G/F-edited sequences	0.31%	0.04%	0.04%

Abbreviations: APOBEC3G/F, apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3G/F; MAD, median absolute deviation.

TABLE 2. Frequency of APOBEC3G/F hypermutation and IAS-USA 2013 mutations in individual samples

Sample ID	Total no. of sequences	Hypermutated sequences (%)	M184I (%)	M230I (%)	E138K (%)	V108I (%)	V106I (%)	V90I (%)
CHAIN033	5061	0.55	–	–	–	–	–	–
CHAIN061	2327	2.12	–	–	–	1.86	–	–
CHAIN083	1390	3.24	4.53	–	0.32	–	–	–
CHAIN138	3466	0.71	1.07	2.11	–	–	–	1.53
CHAIN140	6994	0.62	0.38	–	0.63	–	–	–
CHAIN144	4143	0.72	–	–	–	–	54.9	–
CHAIN145	6150	0.99	–	–	1.48	–	–	–
CHAIN146	6048	1.45	–	–	–	–	–	–
CHAIN151	5702	2.09	1.77	2.60	–	–	3.27	–
CHAIN198	5608	0.95	–	–	–	–	–	–
CHAIN210	5432	6.20	13.28	17.87	–	–	–	–
CHAIN247	8522	7.24	–	8.68	–	–	–	–
CHAIN338	4088	1.49	0.61	1.00	–	–	–	–
CHAIN361	28956	0.76	–	–	0.76	–	–	–

Abbreviations: APOBEC3G/F, apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3G/F; IAS-USA: International Antiviral Society-USA HIV-1 Drug Resistance Mutation List (March 2013 update); –, not detectable.
Mutation proportions are calculated using total number of sequences covering each specific position.

At a sample level, M184I, M230I and E138K were at least eight-fold more likely to be detected in samples with evidence of APOBEC3G/F hypermutation than in samples without evident APOBEC3G/F activity. Conversely, V90I, V106I and V108I did not preferentially occur in samples with evidence of APOBEC3G/F hypermutation (Table 3).

Associations at clonal level. At the clonal level, mutations E138K, M184I and M230I occurred in the same sequence as APOBEC3G/F signatures in 3/9 (33%), 5/11 (45%) and 4/8 (50%) samples, respectively (Table 4). Conversely, none of the samples harbouring minority V90I, V106I and V108I showed co-occurrence of any of such mutations with APOBEC3G/F signatures within the same sequence read.

Interestingly, all clonal sequences with both an APOBEC3G/F footprint and either M184I or M230I also harboured STOP codons, whereas one sample harboured E138K at 0.76% within APOBEC3G/F hypermutated sequence reads without an in-frame STOP codon within the sequenced region of the HIV-1 genome. In addition to STOP codons, many other polymorphisms, all of them corresponding to G-to-A mutations, were linked to APOBEC3G/F. Of note, 16% of APOBEC3G/F-edited sequence reads in the whole data set did not contain any in-frame STOP codon.

TABLE 3. Chances of detecting drug-resistant HIV-1 minority variants in samples with evidence of APOBEC3G/F edition

Mutation	No. of samples with mutation	No. of hypermutated samples with mutation	Relative risk (95% CI) of mutation occurring in an hypermutated sample	p-value[a]
M184I	11	5	13.2 (3.8–41.8)	<0.001
M230I	8	5	26.4 (6.0–130.3)	<0.001
E138K	9	3	8.0 (1.6–31.2)	<0.005
V90I	18	1	0.9 (0.1–5.4)	1
V106I	13	1	1.3 (0.1–8.1)	1
V108I	8	1	2.3 (0.1–16.0)	0.97

Abbreviations: APOBEC3G/F, apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3G/F; HIV-1, human immunodeficiency virus type 1.
[a]Fisher's exact test, Yates-corrected, p-value 14/263 samples evaluated were considered as hypermutated.

Impact of ABOBEC3G/F editing on virological outcomes. Detection of APOBEC3G/F activity was not associated with virological failure to first-line ART including NNRTIs (OR 1.13, 95% CI 0.42–3.02, p 0.807) (Table 5). Conversely, factors already identified in the parent case–control study (3) remained significantly and independently associated with an increased risk of virological failure to first-line NNRTI ART even after incorporating the variable APOBEC3G/F activity in the model, i.e. detection of at least one IAS-USA 2013 MV (OR 2.66, 95% CI 1.29–5.47, p 0.008); time from sample to NNRTI initiation (OR 1.06, 95% CI 1.02–1.11, per month longer, p 0.007), and black ethnicity (OR 13.17, 95% CI 2.20–78.97, p 0.005).

Discussion

The NNRTI-based regimens combine high antiviral potency with affordability, being the recommended first-line ART in low- and middle-income countries. Understanding the origin of DRMVs is relevant to devise strategies to prolong the efficacy of these regimens as much as possible. In a previous study [3] we observed that the most prevalent DRMVs in ART naive subjects were G-to-A mutations. This suggested a role for APOBEC3G/F editing in the spontaneous development of plasma DRMVs. In the present thorough analysis, however, APOBEC3G/F editing contributed minimally to the development of DRMVs and had no evident impact on subsequent virological outcomes.

Evidence of APOBEC3G/F activity was only observed in 14/236 (5.9%) individuals tested using sensitive sequencing technology (≥0.5%). Mutations V90I, V106I, V108I were not associated with APOBEC3G/F activity in any of the analyses performed at either sample or clonal sequence level. Moreover,

TABLE 4. Linkage between resistance mutations, APOBEC3G/F edits and STOP codons at sequence level

Sample ID	HM status[a]	Mutation	Amplicon	Mutation frequency (%)	Mutation + STOP frequency (%)	Linkage of mutation and APOBEC3G/F edits (p-value)[b]
CHAIN264	–	E138K	C & E	0.61	0.00	1
CHAIN179	–	E138K	C & E	0.65	0.00	1
CHAIN178	–	E138K	C & E	0.90	0.00	1
CHAIN078	–	E138K	C & E	1.80	0.00	1
CHAIN279	–	E138K	C & E	2.10	0.00	1
CHAIN224	–	E138K	C & E	2.69	0.00	1
CHAIN140	+	E138K	C & E	0.63	0.63	<0.001
CHAIN361	+	E138K	C & E	0.76	0.00	<0.001
CHAIN145	+	E138K	C & E	1.48	1.48	<0.001
CHAIN092	–	M184I	D & E	0.53	0.00	1
CHAIN264	–	M184I	D & E	0.61	0.61	1
CHAIN162	–	M184I	D & E	0.76	0.00	1
CHAIN135	–	M184I	D & E	1.38	0.00	1
CHAIN011	–	M184I	D & E	1.47	1.30	1
CHAIN216	–	M184I	D & E	1.61	0.00	1
CHAIN338	+	M184I	D & E	0.61	0.61	<0.001
CHAIN138	+	M184I	D & E	1.07	1.07	<0.001
CHAIN151	+	M184I	D & E	1.77	1.77	<0.001
CHAIN083	+	M184I	D & E	4.53	4.53	<0.001
CHAIN210	+	M184I	D & E	13.28	13.28	<0.001
CHAIN231	–	M230I	D	0.64	0.00	1
CHAIN311	–	M230I	D	2.90	0.00	1
CHAIN247	–	M230I	D	8.68	0.00	1
CHAIN338	+	M230I	D	1.00	1.00	<0.001
CHAIN229	+	M230I	D	1.88	1.88	<0.001
CHAIN138	+	M230I	D	2.11	2.11	<0.001
CHAIN151	+	M230I	D	2.60	2.60	<0.001
CHAIN210	–	M230I	D	17.87	17.87	0.32

Abbreviations: APOBEC3G/F, apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3G/F; HM, hypermutation; IAS-USA: International Antiviral Society-USA HIV-1 Drug Resistance Mutation List (March 2013 update).
[a]HM: APOBEC3G/F hypermutation status, defined as presence of at least 0.5% of sequence reads with an APOBEC3G/F signature.
[b]Fisher's p-value defining the strength of the statistical linkage between the IAS-USA mutation of interest and the presence of APOBEC3G/F signatures in the same sequence. Mutations V90I, V106I and V108I are not included in this table because none of them was statistically linked with APOBEC3G/F signatures in the same sequence (all Fisher's test p 1.0).

TABLE 5. Odds of virological failure in the parent CHAIN minority variant case-control study [3] including APOBEC3G/F hypermutation status information in the regression model

	Standard logistic regression Crude and adjusted odds ratios of viral load >200 copies/mL					
	Cases (n = 76)	Controls (n = 184)	Crude OR (95% CI)	p-value	Adjusted[a] OR (95% CI)	p-value
Calendar year of starting NNRTI						
per more recent	2003 (2001–2005)	2004 (2002–2006)	0.88 (0.79–0.97)	0.010	0.85 (0.71–1.01)	0.060
Time from sample to NNRTI initiation						
per month longer	2.97 (1.21–5.02)	1.61 (0.00–3.31)	1.04 (1.00–1.08)	0.038	1.06 (1.02–1.11)	0.007
Viral load at NNRTI initiation						
per log10 copies/mL higher	4.85 (4.54–5.30)	4.91 (4.59–5.36)	0.94 (0.58–1.54)	0.817	1.22 (0.65–2.27)	0.533
NNRTI started, *n* (%)						
Nevirapine	21 (27.6%)	21 (11.4%)	1.00		1.00	
Efavirenz	55 (72.4%)	163 (88.6%)	0.34 (0.17–0.66)	0.002	0.47 (0.20–1.10)	0.083
NRTI pair started, *n* (%)						
Currently recommended ABA/3TC, TEN/FTC	17 (22.4%)	64 (34.8%)	1.00		1.00	
Currently alternative ZDV/3TC, TEN/3TC	36 (47.4%)	96 (52.2%)	1.41 (0.73–2.73)	0.304	0.80 (0.29–2.24)	0.673
Currently not recommended	23 (30.3%)	24 (13.0%)	3.61 (1.65–7.89)	0.001	2.16 (0.66–7.06)	0.201
Detection of ≥1 IAS-USA 2013 minority variants, *n* (%)						
No	52 (68.4%)	153 (83.2%)	1.00		1.00	
Yes	24 (31.6%)	31 (16.8%)	2.28 (1.23–4.23)	0.009	2.66 (1.29–5.47)	0.008
Ethnicity, *n* (%)						
Non-Black	69 (90.8%)	182 (98.9%)	1.00		1.00	
Black	7 (9.2%)	2 (1.1%)	9.23 (1.87–45.53)	0.006	13.17 (2.20–78.97)	0.005
Subtype, *n* (%)						
B	52 (68.4%)	139 (75.5%)	1.00		1.00	
Non-B	24 (31.6%)	45 (24.5%)	1.43 (0.79–2.57)	0.238	1.32 (0.65–2.72)	0.444
Gender, *n* (%)						
Male	54 (71.1%)	153 (83.2%)	1.00		1.00	
Female	22 (28.9%)	31 (16.8%)	2.01 (1.07–3.77)	0.029	1.57 (0.71–3.48)	0.270
APOBEC3G/F signal, *n* (%)						
No	66 (88.0%)	164 (91.1%)	1.00		1.00	
Yes	9 (12.0%)	16 (8.9%)	1.40 (0.59–3.32)	0.448	1.13 (0.42–3.02)	0.807

Abbreviations: APOBEC3G/F, apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3G/F; ART, antiretroviral treatment; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; IAS-USA: International Antiviral Society-USA HIV-1 Drug Resistance Mutation List (March 2013 update); MV, minority variant; ABC, abacavir; TDF, tenofovir disiproxyl fumarate; 3TC, lamivudine; FTC, emtricitabine.
[a]Adjusted for calendar year of starting NNRTI, time from sample, viral load, NRTI pair started, NNRTI started ethnicity, HIV-1 subtype, gender, and cohort study.

such V-to-I mutations were found in 5′-AGTN-3′, a non-target sequence context for APOBEC3G/F. All this suggests that mutations V90I, V106I, V108I had other origins, which might include patient-to-patient transmission or non-APOBEC3G/F-mediated spontaneous generation due to RT G-to-A polymerization bias [25].

Conversely, mutations E138K, M184I and M230I were associated with APOBEC3G/F hypermutation activity resulting from G-to-A transitions in well-defined APOBEC3G/F sequence context targets. In particular, M184I and M230I target sites were 5′-TGGR-3′, which is preferred by APOBEC3G [7,9]. The E138K nucleotide change occurred in a 5′-TGAG-3′ target site, preferred by APOBEC3F [7]. However, a statistically significant clonal linkage between APOBEC3G/F edits and mutations E138K, M184I and M230I was only observed, at most, in half of the samples containing such DRMVs, leaving the origin of the remainder unexplained.

To our knowledge, this is the first evaluation of APOBEC3G/F activity in plasma viruses using next-generation sequencing. Previous studies attributed the origin of M184I, M230I and E138K and other mutations to the activity of APOBEC3 family proteins on proviral DNA from peripheral blood mononuclear cells, viral reservoirs and tissues. Similar results were obtained *in vitro*, using *vif* defective viral constructs on non-permissive cell lines, using standard and ultra deep sequencing [12,17,19,20,26–29]. Fourati et al. [19] described that *in vitro* M184I and E138K mutations developed through exclusive edition by APOBEC3G/F proteins in peripheral blood mononuclear cells from patients on successful highly active ART, concluding that such mutations could pre-exist in the viral reservoir as a result of APOBEC3G/F activity. Additionally, Mulder et al. [17] detected an increased abundance of hypermutated, non-viable, M184I-harbouring viral genomes in $CD4^+$ T-cell-derived proviruses and suggested that these viruses could serve as a pool of resistance mutations to non-edited wild-type viruses, through recombination mechanisms.

The observation of APOBEC3G/F-edited sequences in plasma might suggest different possibilities.

(a) Our findings reflect sub-lethal APOBEC3G/F activity, which would have enabled the active replication of viable viruses and contributed to their diversification *in vivo*. This possibility would be in agreement with previous observations. Recently, Armitage et al. [30] found E138K linked to APOBEC3G/F activity in HIV-1 RNA bulk sequences from a large data set including acutely and chronically HIV-1-infected subjects. Although in our study, all M230I and M184I variants linked to APOBEC3G/F sequence edits were also linked to STOP codons, E138K was found with and without STOP codons.

(b) We might have sequenced non-viable intracellular or circulating free viral RNA and/or virions. This could be the case in sequences containing in-frame STOP codons. In fact, due to the short amplicon length and our targeted sequencing design, we might have largely underestimated the true burden of non-viable RNA sequences in our data set. This would have fewer clinical implications because sequences with STOP codons are unlikely to affect virological outcomes, even in the presence of DRMVs, except in the event of recombination.

(c) The APOBECG3G/F-edited sequences actually correspond to contaminating proviral DNA instead of free viral RNA. Such proviral DNA might not necessarily be expressed and therefore might not affect virological outcomes. We cannot fully rule out this possibility, which would be consistent with the low frequency of observations. However, the frequency of cells in plasma is low and in previous PCR controls without reverse transcriptase we did not observe DNA amplification (not shown).

In summary, our findings support a role for APOBEC3G/F proteins in the generation of DRMVs. However, this role seems to be limited to a small subset of mutations and does not explain most of the DRMVs evaluated. More importantly, APOBECG/F activity also created neighbouring in-frame STOP codons and had no significant effect on virological outcomes of ART. Other factors, including onward HIV-1 transmission, G-to-A RT bias, and possibly host genetics, viral genomic background or *vif* polymorphisms affecting APOBEC3G/F degradation efficacy, might also be relevant in the generation of DRMVs and merit further study.

Transparency Declaration

Alessandro Cozzi-Lepri declares no conflict of interest although he received consulting fees from ViiV Healthcare, Gilead Sciences and Roche for other projects. Martin Däumer has received travel grants from Abbott and MSD Sharp & Dohme, has received a research grant from MSD Sharp & Dohme, and has been an adviser for Roche, MSD Sharp & Dohme, Gilead Sciences and Janssen & Cilag. Francesca Ceccherini-Silberstein has received funds for research grants, attending symposia, speaking and organizing educational activities from Abbott, Merck Sharp & Dohme, Gilead, Janssen, ViiV Healthcare, Roche and Virco, Bristol-Myers Squibb. Antonella D'Arminio Monforte has received grants from BMS, Abbvie, ViiV, MSD, Janssen and Gilead. Erika Castro has received travel grants, honoraria and grants for other projects from Gilead Sciences, Janssen

Cilag-AG and Merck Sharp & Dohme. Hansjakob Furrer declares no conflict of interest although the institution of HF has received payments for participation in advisory boards and/or unrestricted educational or scientific grants and/or travel grants from Abbott, BMS, ViiV Healthcare, Roche, Gilead, MSD, Boehringer Ingelheim and Tibotec-Janssen. Huldrych Günthard has been an adviser and/or consultant for the following companies: GlaxoSmithKline, Abbott, Gilead, Novartis, Boehringer Ingelheim, Roche, Tibotec, Pfizer and Bristol-Myers Squibb, and has received unrestricted research and educational grants from Roche, Abbott, Bristol-Myers Squibb, Gilead, Astra-Zeneca, GlaxoSmithKline and Merck Sharp & Dohme (all money went to institution). Roger Paredes has received consulting fees from Pfizer, ViiV Healthcare, Merck Sharpe & Dohme, Bristol-Myers Squibb and grant support from Pfizer, ViiV Healthcare, Roche Diagnostics, Siemens, Merck Sharpe & Dohme and Boehringer-Ingelheim. Karin J. Metzner has received travel grants and honoraria from Gilead Sciences, Roche Diagnostics, Tibotec, Bristol-Myers Squibb, Merck Sharp & Dohme and Abbott; the University of Zurich has received research grants from Gilead Sciences, Roche Diagnostics and Merck Sharp & Dohme for studies that KJM serves as principal investigator and advisory board honoraria from Gilead Sciences and ViiV. All other authors declare no conflict of interest. Roger Kouyos has been supported by the Swiss National Science Foundation (PZ00P3_142411 and BSSGI0_155851). RDK received travel grants from Gilead. Huldryck Gunthard has been supported by Swiss National Science Foundation No. 159868 and 141067.

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Appendix A. Supporting information

Additional Supporting Information may be found at http://dx.doi.org/10.1016/j.cmi.2015.10.004.

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