

Subtype Analysis and Mutations to Antiviral Drugs in HIV-1-Infected Patients From Mozambique Before Initiation of Antiretroviral Therapy: Results From the DREAM Programme

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Phylogenetic analysis and evaluation of drug-resistance were carried out upon 59 plasma samples from 58 treatment-naïve HIV-1 infected patients from Mozambique, enrolled in a free antiviral-therapy protocol in the frame of Drug-Resource-Enhancement against AIDS and Malnutrition (DREAM) programme. Sequencing of the first 1,300 bases of the *pol*-gene shows that all virus strains cluster within clade C, with the exception of a single patient carrying a G-subtype virus. Relevant mutations in the reverse transcriptase (RT) are rare: 118A/I/L/G (four patients), 179E/D/I (three patients), 333E/D (two patients), 101R, and 210F (one patient each). In Protease (PR), V82I (10.3%) is the only relevant mutation, while natural polymorphisms/secondary mutations are found, some at very high frequency: 20R (25.9%), 36I (91.4%), 36L (8.6%), 60E (31.0%), 63P (29.3%), and 93L (96.6%). Among them, mutations with a frequency >25% were further investigated to assess their covariation pattern with PI resistance associated mutations. The pattern of covariation observed for K20R and D60E (but not L63P and M36I) was different between C and B subtype isolates from PR-inhibitor-treated patients. The sequences were also analyzed to calculate the ratio of non-synonymous to synonymous substitution. The ratio for PR and RT was 0.116 and 0.093, respectively, suggesting a greater conservation in RT than PR in both subtypes B and C HIV strains. Taken together, the results demonstrate a consistent clade-homogeneity of viral strains circulating in Mozambique, and the very limited presence, in drug-naïve patients, of mutations associated with

resistance to RT-inhibitors. The high frequency of secondary mutations/polymorphisms in HIV-PR deserves further studies to evaluate its relevance in clinical settings. **J. Med. Virol. 76:452–458, 2005.** © 2005 Wiley-Liss, Inc.

KEY WORDS: *pol* gene; phylogenetic analysis; drug resistance; drug-naïve patients; Africa; Mozambique

INTRODUCTION

Three distinct groups of HIV, isolated from diverse geographic origins, are currently circulating in humans, named M (main), O (outlier), and N (non-M and non-O) [Perrin et al., 2003]. Group M is responsible for the global HIV-1 pandemic, whereas the other groups are essentially restricted to the West Africa. Subtypes and circulating recombinant forms (CRFs) of group M [Robertson et al., 2000], are all present in central Africa. The predominance of one or few subtypes is observed in other African countries [Thomson et al., 2002].

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Grant sponsor: Italian Ministry of Health (AIDS project and Ricerca Finalizzata and Corrente); Grant sponsor: European Union; Grant sponsor: Unicredit Bank-Unidea Foundation (an unrestricted educational).

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Accepted 17 April 2005

DOI 10.1002/jmv.20382

Published online in Wiley InterScience
(www.interscience.wiley.com)

The high prevalence of subtype-B in western countries may explain why anti HIV-1 drug development, susceptibility tests and characterization of drug resistance mutations are confined to subtype B isolates. But the rapidly increasing prevalence of non-B subtypes in developed countries, (perhaps resulting from the high level of population migration) especially in countries with ties to Africa and in some Eastern European areas [Liitsola et al., 1998], and the increasing availability of antiviral therapy in poor resource settings make it important to study the geographical distribution of clades, and spread of mutations associated with resistance in non-B subtypes.

HIV-subtype C represents around 56% of all circulating group M strains, present in large areas such as India, China, south Asia, South Africa, and the Horn of Africa [Esparza and Bhamarapravati, 2000]. The monophyletic HIV-1 subtype C contrasts with the heterogeneity of the various subtypes and CRFs reported in other sub-Saharan Africa regions, related to internal migration of rural inhabitants, attracted by the urban centers for social and political reasons. Such studies, however, are based mainly upon analysis of the *env* region [Bredell et al., 1998; Engelbrecht et al., 1998; Downing et al., 2000], while the recent availability of antiviral drugs in Africa makes it necessary to define the *pol* sequence of subtype C, and to document the presence of other subtypes. This is particularly true for Mozambique, a developing country where a large programme of free antiviral therapy has been started recently in the Drug-Resource Enhancement against AIDS and Malnutrition (DREAM) project of the Community of Sant'Egidio. This program is run in collaboration with the Ministry of Health of Mozambique [Emberti Gialloreti et al., 2003]. To date, there is no available information regarding either HIV subtypes in Mozambique, or the circulation of resistant strains.

For these reasons, we studied the HIV *pol* regions of patient samples from Mozambique. The inter- and intra-population variability, both nucleotide and amino acid sequences, and the presence of mutations associated to drug resistance were investigated.

METHODS

Study Population

Sixty-one plasma samples from drug-naïve patients were collected in the first months of 2003 from patients referring to Day Hospitals of Matola and Machava (two main centers located near Maputo, the capital of Mozambique), and enrolled in the antiviral programme of DREAM.

RNA Extraction, RT-PCR, and Sequencing

RNA was extracted from 500 µl of each plasma sample using the QiaAmp Viral RNA (Qiagen), according to the manufacturer protocol. RNA was recovered in 50 µl of sterile nuclease-free water and stored at -80°C for later analysis. cDNA was synthesized from 10 µl of extracted

RNA by RT-PCR kit (Viroseq 2, Abbott), according to the manufacturer protocol. All samples were successfully amplified, but, due to the quality of the signal of two samples, sequencing of the first 340 amino acids of reverse transcriptase (RT) and of all 99 amino acids of the protease (PR) was completed for 59 samples from 58 patients. For one patient two plasma samples, obtained at different time, are available. HIV Sequencing Module RUO (ViroSeq2, Abbott), was performed according to the manufacturer protocol. Sequencing reactions were run in the capillary automated DNA sequencer (ABI model 3100 Applied). Sequences were analyzed by the software program HIV Analysis, and the obtained reports were submitted into the Stanford web site for Drug Resistance Algorithm (http://hivdb2.stanford.edu/asi/deployed/hiv_central.pl?program=hivdb, Beta Test). The reference mutation list used to evaluate resistance was that reported in Stanford HIV Drug Resistance Database [<http://hivdb.Stanford.edu>]. Sequence obtained in this study are in the process of submission to GenBank.

Phylogenetic Analysis

The processed sequences were compared to reference sequences for both the HIV-1 subtypes (subtype A, 328902 and 2570232 ID; subtype B, 1906382, 1465777, 328440, and 3285651 ID; subtype C 4324725, 16751238, 22596251, 15281459, 3569317, 3252946, 6016887, 4324715, 1353860, 13569307, 2194183, 15281449, 13569327, 13569227, 13569247, and 32261454 ID; subtype D, 328154, 329377, and 326675 ID; subtype F, 3114544 ID; subtype H, 3114562 ID; subtype J, 4336328 and 4336329 ID; subtype N, 3288388 ID; subtype O, 463057 ID; subtype A/E, 1537050 ID; subtype G, 3403225 and 4262336 ID; NCBI database, <http://www.ncbi.nlm.nih.gov/retroviruses/subtype/refseqHIV1.html>) and the Circulating Recombinant Forms (CRFs) isolates (CRF02_AG, CRF05_DF, CRF07_BC, CRF08_BC; CRF10_CD, CRF12_BF; Los Alamos HIV Sequence Database: <http://hiv-web.lanl.gov/content/hiv-db/Help/Docs/subtypes.html>). The subtype O was considered as outgroup subtype. The reference sequences for C subtype were selected among those available from different worldwide countries. The entire sequence set (59 processed and 34 subtype sequences plus 19 CRFs isolates, 1300 characters) was aligned by the CLUSTAL X 1.80 program and subjected to manual adjustment by using the BioEdit software (BioEdit version 5.0.9). The nucleotide pairwise evolutionary distance was computed by DNADist under the Kimura-2 parameter method and the transition/transversion parameter ratio of 2.77 (PHYLIP software 3.6 Version). This parameter ratio was estimated from our data set by using the substitution model HKY, running under the TREE-PUZZLE 5.0. The tree was constructed by the Neighbor-Joining method. The bootstrap analysis was elaborated on the basis of 1,000 replicates and a final consensus tree was produced. Trees were drawn by using the TREE-VIEW version 1.4 program. Additional trees were

calculated by using the Mega3 program (version 2.1) with N-J and Maximum Parsimony methods with the 1,000 replicate numbers for the bootstrap calculation.

Non-Synonymous/Synonymous Analysis

Fifty-eight nucleic acid sequences from HIV-1 C subtype infected patients from Mozambique and 60 sequences from HIV-1 B subtype infected patients from an anonymous database were also analyzed using the method of Nei and Gojobori, incorporating a statistic developed in Ota and Nei in SNAP (Synonymous/Non-synonymous Analysis Program; <http://www.hiv.lanl.gov/>).

Covariation Analysis

The binomial correlation coefficient (ϕ) was calculated for all the possible pair-wise combinations between K20R, D60E, L63P, M36I (all present in our cohort of HIV-1 C subtype naïve patients with a frequency >25%), and the other known PI-resistance associated mutations.

Covariation analysis was performed separately in 96 HIV-1 C subtype and 699 HIV-1 B subtype infected patients who were failing an antiretroviral regimen containing at least one PI.

In the case of 96 PI-treated HIV-1 C subtype infected patients, 60.4% experienced treatment with nelfinavir, 32.2% with indinavir, 29.2% with saquinavir, 13.5% with ritonavir, 6.2% with lopinavir/ritonavir, 2.1% with amprenavir. At the time of genotypic analysis, 45.8% of patients were under treatment with nelfinavir, 25% with indinavir, 18.8% with saquinavir, 16.1% with ritonavir, 5.2% with lopinavir/ritonavir, 2.1% with amprenavir.

In the case of 699 PI-treated patients carrying HIV-1 B subtypes, 63% of patients experienced indinavir, 61.6% nelfinavir, 41.3% saquinavir, 25% ritonavir, 10.5% lopinavir/ritonavir, 2.2% amprenavir, 0.1% tipranavir. At the time of genotypic analysis, 51.4% of patients were under treatment with nelfinavir, 28.4% with indinavir, 13% with ritonavir, 10.5% with saquinavir, 8.7% with lopinavir/ritonavir, 1.4% with amprenavir, 0.1% with tipranavir.

All 699 B subtype and 9 C subtype sequences from PI-treated patients were stored in a specifically designed anonymous database that included also demographic, immunologic, virologic and therapeutic parameters. 87 C-subtype sequences were taken from HIV Stanford Drug Resistance Database (<http://hivdb.stanford.edu>).

Statistically significant pair-wise correlations were those with a P -value < 0.05. The Benjamini-Hochberg method (false discovery rate of 0.05) was used to assess pairwise combinations that were significant in the presence of multiple-hypothesis testing.

RESULTS

The amplification, sequencing, and phylogenetic analysis of the 1.3 Kb region of HIV-1 *pol* gene (containing all

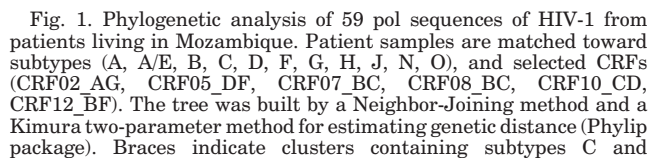
known mutations associated with resistance to antiretrovirals) were completed successfully for 59 plasma samples from 58 patients. Median values of CD4- and CD8-lymphocytes were 361/ μ l (IQR 154–549) and 798/ μ l (IQR 432–1,304), respectively; median viral load was 90,000 HIV-RNA copies/ml (IQR 18,400–250,000). Phylogenetic analysis shows that only one sample clusters within subtype G and CRF02_AG under a branch node (95% bootstrap); all the other 58 samples cluster into subtype C and CRF08_BC clade (Fig. 1). In particular, a clear relationship with clade C was shown in all these samples, even though they maintain a remarkable degree of each other genetic diversity (few nodes appeared to be characterized by bootstrap values >95%). No phylogenetic correlation was observed with a particular subtype C isolated from an adjacent country.

Two monophyletic groups were identified, represented by a mother with her son (MC003 and MC060, bootstrap value of 99%), and by a single patient whose HIV sequences were obtained from blood samples drawn at 2 months interval (MT000370_1 and MT000370_2 bootstrap value of 100%). These phylogenetic relationships were confirmed in the trees inferred by Maximum Parsimony and Neighbor-Joining program from Mega software package (data not shown).

Mutations associated with resistance to antiviral drugs and present in drug-naïve patients are described in Table I. Regarding RT, natural polymorphisms somewhat associated with resistance to RT inhibitors are poorly represented in this cohort of drug-naïve patients. Indeed, only four patients carried mutations at codon 118, one at 98, one at 44, three at 179, one at 101, and finally two at 333. None of these mutations are associated with high degree of resistance to RT inhibitors. Interestingly, one patient carries an atypical mutation F (instead of the classical W) at codon 210 of RT. The relevance of this mutation in term of resistance to nucleoside analogs has yet to be determined.

In PR the most frequent secondary mutations/polymorphisms are at position 20R (25.9% patients), 36I/L (91.4/8.6%–100% of the cases), 60E (31.0%), 63P (29.3%), and 93L (96.6%); other polymorphisms present at lower frequency are reported in Table I. No major primary mutations in PR were found. Furthermore, in six patients (10.3%) the mutation V82I, a natural polymorphism in C subtype recently reported to be correlated with a three-fold decreased susceptibility to Amprenavir [Holguin et al., 2004], was observed; this mutation may be intermediate between the wild type Valine and the double-base mutation for amino acids (such as Alanine, Serine, Threonine, Phenylalanine) which confers major resistance to most protease inhibitors.

PR mutations, detected in drug-naïve patients with frequency >25%, were further investigated to assess their co-variation pattern with PI-resistance mutations in both HIV-1 C and B subtype isolates from PI-treated patients. In particular, to identify pair-wise correlations, the binomial correlation coefficient (ϕ) and the



G+CRF02_AG. Subtype reference strain C, selected among those available from different worldwide countries, (<http://www.ncbi.nlm.nih.gov/retroviruses/subtype/refseqHIV1.html>) (○) and samples coming from Maputo and Machava hospital (●) are shown. CRF02_AG (<http://hiv-web.lanl.gov/content/hiv-db/HelpDocs/subtypes.html>) and subtype reference strain G are shown, respectively (□). Number next to the nodes of the tree represents bootstrap values (1,000 replicates).

multi-drug resistance mutation, thus showing a pattern of covariation superimposable to that observed in B subtype ($\phi = 0.27$, $P = 2.2e^{-12}$). None of these four mutations correlated with either V82I, the most common amino acid substitution at position 82 in C subtype, or with V82A.

TABLE I. Mutations/Polymorphisms Detected in HIV-Protease and RT of 58 Drug-Naive Patients From Mozambique

Protease			Reverse transcriptase		
Mutations	Number	Percent of mutations (%)	Mutations	Number	Percent of mutations (%)
10I	1	(1.7)	44K	1	(1.7)
20R	15	(25.9)	98S	1	(1.7)
20I	1	(1.7)	101R	1	(1.7)
20M	1	(1.7)	118A	1	(1.7)
32G	1	(1.7)	118L	1	(1.7)
36I ^a	53	(91.4)	118G	1	(1.7)
36L ^a	5	(8.6)	118I	1	(1.7)
36T ^a	1	(1.7)	179E	1	(1.7)
50L	1	(1.7)	179D	1	(1.7)
60E	18	(31.0)	179I	1	(1.7)
63P ^b	17	(29.3)	210F	1	(1.7)
63V	5	(8.6)	333E	1	(1.7)
63S ^b	5	(8.6)	333D	1	(1.7)
63T	3	(5.2)			
63E	1	(1.7)			
63A ^b	1	(1.7)			
63I	1	(1.7)			
63H	1	(1.7)			
77I	1	(1.7)			
82I	6	(10.3)			
93L	56	(96.6)			

^aAt aminoacid 36 of HIV-protease one patient showed a quasispecies I/T and an other I/L.

^bAt aminoacid 63 of HIV-protease one patient showed a quasispecies A/S and an other P/S.

Interestingly, K20R in B subtype was strongly correlated with V82A ($\phi = 0.24$, $P = 2.0 \times 10^{-10}$). But in C subtype had a totally different pattern of covariation, represented by M46L ($\phi = 0.31$, $P = 0.002$). M36I, occurring in 90% of HIV-1 C subtype PI-treated patients, which was not correlated with any PI-resistance associated mutations, while in B subtype was correlated with I54V ($\phi = 0.15$, $P = 7.4 \times 10^{-5}$). In both subtypes, M36I showed a very strong negative correlation with V77I ($\phi = -0.40$, $P = 6.4 \times 10^{-26}$; $\phi = -0.45$, $P = 9.0 \times 10^{-6}$ in B and C subtype, respectively).

Lastly, D60E showed a very different pattern of covariation between C and B subtype represented by L33F ($\phi = 0.31$, $P = 0.002$) and F53L ($\phi = 0.13$, $P = 0.0006$), respectively.

Fifty-eight nucleic acid sequences of HIV-1 C subtype patients from Mozambique were also analyzed using the method of Nei and Gojobori, incorporating a statistic developed in Ota and Nei in SNAP (Synonymous/Non-synonymous Analysis Program) to calculate the ratio of

non-synonymous to synonymous amino acid substitution (dn/ds) as a measure of natural selection at protein level (Table II). In our analysis the values of dn were substantially lower compared to ds both in PR (0.02 dn vs. 0.17 ds) and in RT (0.02 dn vs. 0.18 ds). The values obtained for C subtype have been compared to those of the B subtype. The same trend was observed (PR 0.03 dn vs. 0.09 ds and RT 0.02 dn vs. 0.11 ds). Both in C and in B subtypes the PR has a dn/ds higher than RT (0.116 vs. 0.093 in C subtype and 0.234 vs. 0.185 in B subtype) thus suggesting a greater conservation in the RT versus PR. The values of dn for PR and RT in both C and B subtypes are similar, (0.02–0.03 range), while those of ds show a different trend in C subtype (0.17 in PR and 0.18 in RT) and in B subtype (0.09 in PR and 0.11 in RT).

DISCUSSION

To assess the characteristics of HIV strains circulating in Mozambique, a strategy was used which provides both phylogenetic and genotypic drug-susceptibility

TABLE II. Ratio of Non-Synonymous to Synonymous Substitution (dn/ds)

Naïve	ds	Average ds	Standard deviation(ds)	dn	Average dn	Standard deviation(dn)	dn/ds
Subtype C							
PR	0.17	0.164	0.024	0.02	0.024	0.007	0.116
RT	0.18	0.179	0.014	0.02	0.019	0.003	0.093
Subtype B							
PR	0.09	0.080	0.014	0.03	0.026	0.006	0.234
RT	0.11	0.109	0.009	0.02	0.017	0.003	0.185

Nucleic acid sequences of 57 patients from Mozambique (Naïve, subtype C) and of 60 patients stored in a specifically designed anonymous database (Naïve, subtype B) were analyzed using the method of Nei and Gojobori, incorporating a statistic developed in Ota and Nei in SNAP (Synonymous/Non-synonymous Analysis Program).

analysis by sequencing the HIV-*pol* frame encoding for protease and a major 3' part of RT. This strategy for phylogenetic analysis has been validated by other groups of investigators, either from African countries and from Europe, and achieved reliable and reproducible results [Balotta et al., 2001; Yahi et al., 2001; Njouom et al., 2003].

The achievement of PCR signal in 100% of samples tested suggests that the method used is able to detect subtype C with high sensitivity, and excludes the possibility of a bias due to the negative selection of outlier strains genetically different, and therefore not recognized by primers chosen and designed for known HIV-sequences. Overall, this suggests the reliability of this method for C-subtypes (highly prevalent in Southern Africa).

To our knowledge, this is the first set of data presenting the characteristics of HIV-1 *pol* sequences circulating in Mozambique, and the natural prevalence of mutations associated with resistance to antiviral drugs in that area (only limited *env* sequences are available for HIV circulating in this country) [Bredell et al., 1998; Engelbrecht et al., 1998; Downing et al., 2000]. Our data suggest the presence, in the suburban area analyzed, of virus strains all belonging to subclade C (with a single exception of one clade G), not carrying mutations associated to resistance to antiretrovirals. This, at least for RT, may exclude the possibility of a "founder" effect linked to a virus naturally resistant to antivirals.

The interpretation of the results of PR sequences is more complex, in view of the presence, in many of these patients, of natural polymorphisms/secondary mutations usually detected in patients treated with protease inhibitors. Interestingly, a recently characterized cohort of subtype-B patients showed that the most common polymorphisms present in these virus strains were 63P (present in 44.2% of patients), followed by 77I (18.9%), 36I (17.2%), and 10I (5.7%) [Perno et al., 2002]. Perno's pattern of polymorphisms is different than in our subtype C viruses. We found the most common polymorphisms were 36I (91.5% of patients), 93L (96.6%), 60E (32.2%), 63P (28.8%), and 20R (25.4%). These are all mutations known to be involved in PI-resistance in B subtype virus. Whether this different pattern correlates with different levels of natural resistance to protease inhibitors still needs to be elucidated. Interestingly, in our study it was found that the naturally occurring protease polymorphisms in the C subtype virus, not only increase the catalytic efficiency of protease enzyme [Apetrei et al., 1998; Descamps et al., 1998; Velazquez-Campoy et al., 2001], but also decrease the binding affinities of existing clinical inhibitors by factor between 2 and 7.5 [Velazquez-Campoy et al., 2002], thus conferring a baseline resistance to PIs.

In addition, under selective pressure from PIs, many of these mutations (K20R, M36I, D60E) were associated with PI-resistance mutations different than those observed in B subtype. This suggests that in different virus subtypes the resistance to PIs may be regulated through different mutational pathways. Two other

studies suggest similar results with a Nelfinavir containing first line regimen. In fact, D30N was the common pathway for subtype B viruses, but L90M occurred in subtypes A, C, or G [Gomes et al., 2002; Kantor and Katzenstein, 2003]. Our covariation analysis shows that M36I is not correlated with specific PI-resistance mutations, thus suggesting that this mutation, already present at baseline, may presumably contribute to PI cross-resistance. Interestingly, two recent papers have shown, in a mainly B-clade infected population, that the presence of mutation M36I before antiviral therapy is associated with a greater risk of virological failure [Perno et al., 2001], and with the appearance of primary mutation L90M [Perno et al., 2004]. Whether this mutation decreases the genetic barrier to PI-resistance, not only in B subtype but also in C subtype warrants further investigation.

We have also analyzed the non-synonymous or non-silent (dn) and synonymous or silent (ds) substitution rates occurring in PR and RT enzymes in C subtype virus. In the absence of positive selection, substitutions that are silent (synonymous mutations) would be expected to exceed substitutions that lead to amino acid changes (non-synonymous mutations), since most structural changes in a protein are deleterious. This is confirmed in our analysis where the values of dn are substantially lower compared to those of ds for both PR and RT. The values obtained for C subtype virus have been compared to those of the B subtype virus where the same trend was observed. In the absence of treatment pressure our analysis confirmed that both in C and in B subtypes the PR has a ratio dn/ds higher than RT. In fact, at the amino acid level a higher frequency of non-silent mutations was observed in the PR enzyme with respect to RT. At nucleotide level, silent mutations are different between C and B subtypes, while non-silent mutations are almost equal. In addition, both PR and RT from C subtype showed a dn/ds two-fold lower than PR and RT from B subtype, thus suggesting a greater conservation of the *pol* gene in C subtype with respect to B subtype. This is mainly determined by the differences in ds values, as dn values are similar, thus suggesting that in the absence of selective pressure the rate of amino acid substitutions among subtypes is similar, while at nucleotide silent substitutions level the subtypes are different.

Overall the data suggest the key importance of performing large studies in patients carrying non-B strains of HIV treated with protease inhibitors, particularly in light of a potential long-term relevance of secondary mutations, whose effect may not be easy to detect in studies characterized by short-term endpoints.

The results described in this paper have a high level of clinical relevance, in view of the widespread DREAM programme in Mozambique, which brings free-of-charge, triple-drug antiretroviral therapy (mostly two nucleoside analogs plus one non-nucleoside inhibitor) to patients requiring treatment for their advanced disease, and/or to pregnant women in the framework of a

mother-to-child prevention programme. Similar programmes are ongoing in other African countries, and access to therapy will be implemented in the next years in southern Africa. Overall, the absence of relevant mutations in the RT of these subtype C patients suggests that NRTI plus NNRTI based antiretroviral therapy is likely to be as successful as developed countries, pending the accomplishment of other parameters such as the adherence to long-term therapy. Large and long-term studies are required to verify whether this holds true in clinical practice.

ACKNOWLEDGMENTS

We are gratefully to Ada Bertoli, Fabio Continenza, Sara Giannella for their technical assistance.

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