The failure of raltegravir (RAL) is generally associated with the selection of mutations at integrase position Y143, Q148, or N155. However, a relatively high proportion of failures occurs in the absence of these changes. Here, we report the phenotypic susceptibilities to RAL and elvitegravir (EVG) for a large group of HIV-infected patients failing on RAL-containing regimens. Plasma from HIV-infected individuals failing on RAL-containing regimens underwent genotypic and phenotypic resistance testing (Antivirogram v2.5.01; Virco). A control group of patients failing on other regimens was similarly tested. Sixty-one samples were analyzed, 40 of which belonged to patients failing on RAL-containing regimens. Full RAL susceptibility was found in 20/21 controls, while susceptibility to EVG was diminished in 8 subjects, with a median fold change (FC) of 2.5 (interquartile range [IQR], 2.1 to 3.1). Fourteen samples from patients with RAL failures showed diminished RAL susceptibility, with a median FC of 38.5 (IQR, 10.8 to 103.2). Primary integrase resistance mutations were found in 11 of these samples, displaying a median FC of 68.5 (IQR, 23.5 to 134.3). The remaining 3 samples showed a median FC of 2.5 (IQR, 2 to 2.7). EVG susceptibility was diminished in 19/40 samples from patients with RAL failures (median FC, 7.71 [IQR, 2.48 to 99.93]). Cross-resistance between RAL and EVG was high ($R^2 = 0.8; P < 0.001$), with drug susceptibility being more frequently reduced for EVG than for RAL (44.3% versus 24.6%; $P = 0.035$). Susceptibility to RAL and EVG is rarely affected in the absence of primary integrase resistance mutations. There is broad cross-resistance between RAL and EVG, which should preclude their sequential use. Resistance to EVG seems to be more frequent and might be more influenced by integrase variability.

**MATERIALS AND METHODS**

**Study population.** HIV-1-infected individuals treated at several clinics in Spain who experienced virological failure on a RAL-containing antiretroviral regimen were identified during 2009. Virological failure was defined as the first sample with plasma HIV RNA levels of ≥50 copies/ml confirmed with a second specimen collected within a month. Plasma specimens collected at the time of the first RAL failure were then sent to Hos-
pital Carlos III along with information on viral load, CD4 counts, and antiretroviral combinations. In addition, samples from INI-naïve patients were also sent to be used as controls. Most participating centers belonged to RIS (Red de Investigación en SIDA), the government-funded Spanish AIDS research network, which involves around 25 HIV clinics across the country and whose main characteristics were described elsewhere previously (17). All specimens that were examined in the current study were part of the repository collected for the SINRES study, a previous survey that characterized RAL failures virologically in a total of 106 patients (6). Signed informed consent was obtained from each individual.

### Genotypic resistance analysis
The analysis of the HIV integrase gene was performed by bulk sequencing using an in-house PCR protocol with primers and conditions previously described (7). Primary integrase resistance mutations were defined as those changes that caused a significant loss of drug susceptibility by themselves. This was the case for the Y143RHC, Q148HRK, and N155H mutations.

### Phenotypic resistance analysis
Viral RNA was isolated from all plasma samples by using NucliSENS EasyMAG (bioMérieux). One amplification per sample containing the reverse transcriptase-integrase (RT-IN) regions was sequenced and cloned into an HXB2 backbone. Recombinant viruses were titrated and subjected to an antiviral experiment with MT4-LTR-eGFP cells, as previously described (20). Briefly, MT4-LTR-eGFP cells were inoculated with a titrated amount of virus in the presence of 3-fold dilutions of the compound tested. After 3 days of incubation at 37°C, infection was quantified by means of fluorescence microscopy measuring HIV Tat-induced enhanced green fluorescent protein (eGFP) expression. Using HIV-1 wild-type strain IIIB as a reference, fold change (FC) values were calculated by dividing the mean 50% effective concentration (EC50) for a recombinant virus stock by that for the reference strain. A biological cutoff (BCO) for RAL and EVG was calculated with a clonal database (991 clones from 153 clinical isolates) as the 97.5th percentile of the log FC phenotypes of patient-derived clonal viruses. The main characteristics of these patients are reported in Table 3, including information on RAL plasma levels as an indirect marker of drug adherence.

### Statistical analysis
Results are expressed as medians (interquartile ranges [IQRs]) and percentages. Comparisons were performed with nonparametric tests using Mann-Whitney or chi-square tests. FC values were log transformed for analyses of phenotypic resistance correlations using a bivariate analysis. All statistical analyses were carried out by using SPSS v15 software (SPSS Inc., Chicago, IL).

### Nucleotide sequence accession numbers
All sequences examined in this study have been submitted to the GenBank database under accession numbers JQ716857 to JQ716918.

### RESULTS

#### Study population
A total of 61 samples were examined: 21 belonging to INI-naïve patients and 40 obtained from patients experiencing early virological failure on RAL-containing regimens. All INI-naïve individuals were antiretroviral experienced. The main characteristics of this population are summarized in Table 1.

### Resistance testing for INI-naïve patients
Phenotypic results for specimens collected from the 21 INI-naïve individuals showed that all but 1 (95.2%) were susceptible to RAL. The single sample displaying an FC of 2.4 for RAL did not harbor any primary or secondary known integrase resistance mutation. However, compared to reference sequences, the following changes in the integrase gene were present: E111D, S24G, D25E, S39C, M501M, I72V, L101I, V201I, and S283G. In contrast, up to 8/21 (38%) INI-naïve samples displayed an FC above 1.9 for EVG. Neither primary nor secondary resistance changes were found in these specimens, as shown in Table 2.

### Resistance testing for RAL failures
Overall, 11 out of 40 (27.5%) samples collected from patients experiencing early RAL failure harbored primary INI resistance mutations, including the Q148H/R (n = 5), N155H (n = 3), and Y143R/H11005 n (n = 2) mutations and a complex pattern including the Y143HY and N155H mutations (n = 1).

Fourteen (35%) samples displayed phenotypic FCs above 2 for RAL, including the 11 specimens with primary INI resistance mutations. Three samples, which did not contain any primary INI resistance change, displayed low FC values for RAL (2, 2.5, and 2.7, respectively). As a comparison, the median FC was 58.9 (IQR, 26.5 to 125.3) for the 11 patients with INI resistance mutations. The main characteristics of these patients are reported in Table 3, including information on RAL plasma levels as an indirect marker of drug adherence.

When EVG sensitivity testing was carried out, 19 (47.5%) RAL failures displayed impaired EVG sensitivity. Overall, the median FC was 7.71 (IQR, 2.48 to 99.93), but it was much higher in the 11 samples harboring primary INI resistance mutations than in the 8 samples without major integrase changes (FC of 99.93 [IQR, 46.92 to 99.93] versus 2.45 [IQR, 2.36 to 2.83]; P < 0.001). Table 3 reports the genotypic and phenotypic data for EVG in these patients.

Primary INI resistance mutations led to high-level resistance to both RAL and EVG although with slight differences. The Y143R mutation conferred a higher level of resistance to RAL than to EVG, with the FCs ranging from 26.5 to 58.9 and from 3.92 to 7.68, respectively. However, it is noteworthy that this change, which is not considered a primary resistance mutation for EVG, significantly compromised EVG susceptibility in the presence of secondary resistance mutations (T97A plus V151I for one specimen and L74M for another).

### Impact of minor integrase changes on RAL and EVG susceptibility
Comparisons of RAL and EVG FCs in the presence or absence of changes at single integrase positions were performed by using nonparametric tests. Only samples without primary INI resistance mutations were considered for this purpose, and there-
Elvitegravir and Raltegravir Cross-Resistance

The variability at the viral integrase has been shown to be

A few samples in our study displayed small, but potentially clinically significant, reductions in susceptibility to integrase inhibitors, based upon the established biological cutoffs of FC values of 2.0 and 1.9 for RAL and EVG, respectively. The reduced sensitivity was more prominent for EVG in the absence of primary INI resistance mutations. One specimen from a subject failing on a RAL-containing regimen did not show any known INI resistance mutation but displayed FC values of 2.5 for RAL and 7.71 for EVG. This sample harbored only the R263K mutation as being potentially responsible for this effect on INI susceptibility. Interestingly, the R263K mutation was previously shown to be selected in vitro by EVG, conferring an FC of 5.2 to the drug (10). In the present study, this mutation also led to a slight but significant decrease in RAL susceptibility. Another patient displayed an FC of 2.7 for RAL despite lacking primary INI resistance mutations. However, the L74I mutation had been selected in this patient failing on a RAL-containing regimen. Given that the L74M mutation is a secondary resistance mutation generally selected along with the N155H mutation in patients failing on RAL-containing regimens (8, 15), we suggest that the L74I mutation might equally compromise RAL susceptibility.

Overall, the testing of samples collected from patients with RAL failures in the absence of primary INI resistance mutations allowed the recognition of certain minor changes that might impact INI susceptibility in a clinically relevant manner. This was the case for the S24G/N, D25E, L74I, K215N, and Q221H/S changes for RAL and the L45Q/V, V72I, and R263K changes for EVG. Additional resistance studies are being performed by Virco to clear up the phenotypic effect of each of these changes. However, their clinical impact should be further investigated with larger groups of patients experiencing INI failures.

Most patients displaying resistance to RAL in our study also exhibited a reduced EVG susceptibility. The extent of cross-resistance was high for all different resistance patterns and may have important clinical implications regarding the consideration of the sequential use of RAL and EVG. Moreover, it was intriguing that EVG showed increased FC values more often than RAL, suggesting that integrase changes had a greater influence on EVG. Hypothetically, our findings could be explained by the faster rate of dissociation from the viral integrase for EVG than for RAL, with half-lives of 2.4 and 9 h, respectively (9).

### TABLE 2 Genotypic and phenotypic results for patients naïve to HIV integrase inhibitorsa

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>ARV regimen</th>
<th>Subtype</th>
<th>FC for RAL</th>
<th>FC for EVG</th>
<th>Minor mutation</th>
<th>Other changes</th>
</tr>
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<tbody>
<tr>
<td>N_1</td>
<td>ABC, AZT, TDF, DRV/r</td>
<td>B</td>
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<td>2.08</td>
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<td>Q44K, I72V, T112IT, I113V, S119P, T122IT, E152G, T206S, T218S</td>
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<td>N_5</td>
<td>TDF, AZT, 3TC, ABC, fAPV/r</td>
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<td>0.80</td>
<td>2.56</td>
<td>S230NS</td>
<td>D6E, E11D, L28I, K34R, V37L, V201I, L234L, D253DE, A265AV</td>
</tr>
</tbody>
</table>

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a ART, antiretroviral therapy; FC, fold change; RAL, raltegravir; EVG, elvitegravir; ABC, abacavir; 3TC, lamivudine; FTC, emtricitabine; TDF, tenofovir; fAPV, fosamprenavir; ATV, atazanavir; LPV, atazanavir/ritonavir. Values in boldface indicate fold change above cutoff.

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<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Subtype</th>
<th>ARV regimen</th>
<th>Time on RAL (mo)</th>
<th>Detectable RAL plasma levels</th>
<th>FC for RAL</th>
<th>FC for EVG</th>
<th>Major mutation(s)</th>
<th>Minor mutation(s)</th>
<th>Other changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>F_1</td>
<td>B</td>
<td>ETR, DRV/r, RAL</td>
<td>8.9</td>
<td>−</td>
<td>2</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>F_2</td>
<td>B</td>
<td>AZT, 3TC, ATV, RAL</td>
<td>7.6</td>
<td>−</td>
<td>2.7</td>
<td>1.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F_3</td>
<td>B</td>
<td>MVC, ATV, RAL</td>
<td>NA</td>
<td>+</td>
<td>2.5</td>
<td>7.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F_4</td>
<td>B</td>
<td>3TC, MVC, RAL</td>
<td>9.6</td>
<td>+</td>
<td>78</td>
<td>99.9</td>
<td>Q148HQ</td>
<td></td>
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</tr>
<tr>
<td>F_5</td>
<td>B</td>
<td>FTC, TDF, ETR, DRV/r, RAL</td>
<td>5.3</td>
<td>+</td>
<td>161.2</td>
<td>99.9</td>
<td>Q148H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F_6</td>
<td>B</td>
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<td>7</td>
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<td>125.3</td>
<td>99.9</td>
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<td>Q148H</td>
<td></td>
<td></td>
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<tr>
<td>F_8</td>
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<td>NA</td>
<td>+</td>
<td>95.9</td>
<td>99.9</td>
<td>Q148R</td>
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<td></td>
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<tr>
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<td>NA</td>
<td>NA</td>
<td>+</td>
<td>13.5</td>
<td>48.0</td>
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<tr>
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<td>6.7</td>
<td>+</td>
<td>14.6</td>
<td>99.9</td>
<td>N155H</td>
<td></td>
<td>L74LM</td>
</tr>
<tr>
<td>F_11</td>
<td>B</td>
<td>AZT, 3TC, ETR, DRV/r, T20, RAL</td>
<td>5.2</td>
<td>+</td>
<td>27.6</td>
<td>46.9</td>
<td>N155H</td>
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<tr>
<td>F_12</td>
<td>B</td>
<td>d4T, TDF, ETR, DRV/r, RAL</td>
<td>21.7</td>
<td>+</td>
<td>26.5</td>
<td>3.9</td>
<td>Y143R</td>
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<tr>
<td>F_13</td>
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<td>FTC, TDF, RAL</td>
<td>12.8</td>
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<td>58.9</td>
<td>7.6</td>
<td>Y143R</td>
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<tr>
<td>F_14</td>
<td>B</td>
<td>FTC, TDF, RAL</td>
<td>10.4</td>
<td>+</td>
<td>49.3</td>
<td>99.9</td>
<td>Y143HY, N155H</td>
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</tr>
<tr>
<td>F_15</td>
<td>B</td>
<td>FTC, TDF, ETR, RAL</td>
<td>11.8</td>
<td>−</td>
<td>1.3</td>
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<td>F_16</td>
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<td>AZT, 3TC, T20, DRV/r, RAL</td>
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<td>F_17</td>
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<td>FTC, TDF, DRV/r, RAL</td>
<td>31.7</td>
<td>+</td>
<td>1.1</td>
<td>2.4</td>
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<tr>
<td>F_18</td>
<td>B</td>
<td>DRV/r, RAL</td>
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<td>−</td>
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<td></td>
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<tr>
<td>F_19</td>
<td>B</td>
<td>FTC, TDF, ETR, RAL</td>
<td>16.5</td>
<td>−</td>
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<td>2.5</td>
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<td>F_20</td>
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<td>3TC, LPV/r, RAL</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>F_21</td>
<td>B</td>
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<td>18</td>
<td>+</td>
<td>0.6</td>
<td>2.8</td>
<td>S230N</td>
<td></td>
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</tbody>
</table>

aFC, fold change; RAL, raltegravir; EVG, elvitegravir; −, undetectable RAL plasma levels; +, detectable RAL plasma levels; DRV, darunavir; ETV, etravirine; MVC, maraviroc; ABC, abacavir; 3TC, lamivudine; FTC, emtricitabine; TDF, tenofovir; LPV, fosamprenavir; ATV, atazanavir; LPV, lopinavir; d4T, stavudine; T20, enfuvirtide; NA, not applicable. Values in boldface indicate fold change above cutoff.
greater in antiretroviral-experienced than in drug-naïve patients 
(1, 7, 21). All our patients, regardless of RAL exposure, were anti- 
retroviral experienced, which might have led to a higher rate of 
polymorphisms, providing an explanation of the relatively high 
proportion of subjects with slight reductions in RAL and espe- 
cially EVG susceptibilities in INI-naïve patients.

In summary, our results for the testing of phenotypic and ge- 
genotypic samples from patients with RAL failures and controls 
support that susceptibility to RAL and EVG is impaired mainly 
by the Q148H/R, N155H, and/or Y143R resistance mutation. 
Slight reductions in susceptibility to any of these drugs can 
occur in the presence of other integrase changes. The wide 
extent of cross-resistance between RAL and EVG should pre- 
clude their subsequent use.

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