Human Immunodeficiency Virus Type 1 Protease Genotype Predicts Immune and Viral Responses to Combination Therapy with Protease Inhibitors (PIs) in PI-Naive Patients

Elena E. Perez,1,a Stephanie L. Rose,1 Brian Peyser,1 Susanna L. Lamers,2 Brant Burkhardt,2 Ben M. Dunn,3 Alan D. Hutson,4 John W. Sleasman,2 Susanna L. Lamers,5 Brant Burkhardt,1 Ben M. Dunn,3 Alan D. Hutson,4 John W. Sleasman,2 and Maureen M. Goodenow1

Protease genotype, as a variable in outcome to combination therapy for human immunodeficiency virus (HIV) type 1 infection, was evaluated among protease inhibitor–naive children and adolescents who had received extensive treatment with reverse-transcriptase inhibitors. After 24 weeks of combination therapy, 35% had viral and immune success (VSIS patients), 19% had viral and immune failure (VFIF patients), and 46% had viral failure but marked improvement in CD4 T cells (VFIS patients). Disease stage was the only pretherapy clinical variable associated with outcome (P = .02). Although reverse-transcriptase genotype was unrelated to outcome, pretherapy protease genotype was related significantly to therapy response (P = .005). Odds for immune or viral failure were 17.7 to 1 and 2.5 to 1, respectively, for protease genotype as a single variable. Protease genotype combined with disease stage and CD4 cell percentage predicted correct therapy response for 81% of patients (100% of VFIF, 78% of VSIS, and 75% of VFIS patients). Naturally occurring amino acid polymorphisms in protease provide sensitive biomarkers for treatment response among inhibitor-naive patients with advanced HIV disease.

Antiretroviral therapy using protease inhibitors (PIs) in combination with reverse-transcriptase inhibitors (RTIs) has produced a dramatic impact on the natural history of human immunodeficiency virus (HIV) type 1 disease by delaying progression to AIDS through the control of viral replication and by reversing or preventing immunodeficiency [1, 2]. Plasma virus levels and CD4 T cell counts are intermediate markers for disease progression that are generally applied as prognostic measures of therapy outcome and that provide the basis of current guidelines for the use of antiretroviral agents in HIV-infected adults and children [3–9]. Unfortunately, a number of HIV-infected patients who receive PI combination therapy fail to achieve or sustain undetectable plasma virus levels, despite prognostic markers that would predict successful therapy response [6, 7, 10–14]. In addition, both adults and children can reconstitute and sustain improved CD4 T cell counts when treated with combination therapy, even though virus levels rebound [14–17].

Designing effective combination therapy from the multiple choices of PIs and RTIs that are available necessitates a more diverse repertoire of prognostic markers for successful therapy outcome. Genetic characteristics of protease (PR) and reverse transcriptase (RT), which are targets in the viral genome for drug action, provide an additional level of prognostic value in predicting treatment outcomes [18–20]. Switching antiretroviral drugs on the basis of RT and PR genotype increases the likelihood of successful response for treatment-experienced patients [21, 22]. In contrast, few data to assess the value of RT and PR genotyping for treatment of therapy-naive patients are available [23].

Theoretical calculations based on rate of virus mutation, amount of replication, and levels of virus in vivo estimate that HIV-1 quasi species with 1, or even 2, amino acid substitutions, which could reduce drug efficacy, exist in treatment-naive individuals [24, 25]. Assessments of therapy-naive patient populations indicate the occurrence of natural polymorphisms in either RT or PR that could reduce drug sensitivity when therapy


Patients and/or their legal guardians provided informed consent. Guidelines for research involving human subjects of the US Department of Health and Human Services and the Institutional Review Board of the University of Florida were followed.

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1Department of Pathology, Immunology, and Laboratory Medicine, 2Division of Immunology and Infectious Diseases, Department of Pediatrics, and 3Department of Biochemistry and Molecular Biology, College of Medicine, and 4Department of Statistics, Division of Biostatistics, College of Liberal Arts and Sciences, University of Florida, Gainesville, 5Gene Genic, Thibodaux, Louisiana

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is initiated [26–28]. Consequences of low levels of resistance may be difficult to detect in in vitro assays that measure biochemical activity of PR [29]. Natural polymorphisms in PR might reduce sensitivity to drugs and affect viral responses to combination therapy, setting the stage for rapid emergence of viruses with multiple drug-resistance mutations [30]. Consequently, pretherapy resistance may provide an additional factor to account for failure to sustain suppression of virus in patients who appear to be compliant with medications [31].

We designed a prospective study to evaluate whether genotypic determinants in PR or RT would improve the prognostic value of pretherapy viral and immune variables in predicting therapy outcome. The study focused on a cohort of HIV-infected children and adolescents, who were naive to PI treatment but were at great risk for combination therapy failure, because of advanced disease and high virus burden.

Patients and Methods

Study patients. The study cohort comprised HIV-infected children and adolescents who were enrolled prospectively between January 1996 and October 1999 in a treatment protocol that involved combination therapy with 1 PI and 1 or 2 RTIs [9]. Eligible patients were 1–18 years of age, naive to PI therapy, and immune compromised (CDC immune stage 2 or 3 [1993 definition]), with plasma virus levels >4.0 log₁₀ copies/mL, as measured by the Amplicor assay, with 400 copies/mL as the lower limit of detection (Roche Molecular Systems). Prior therapy with RTIs was permitted if patients were naive to ≥1 RTI in their new combination therapy protocol. Among a population of <100 infected children, 26 patients fulfilled the inclusion criteria and also demonstrated compliance with combination therapy. Seventeen patients (65%) received combination therapy with ritonavir, 3 (12%) with nelfinavir, and 6 (23%) with indinavir. Selection of PI was based on the availability of pediatric formulations, as well as the ability of the patient to swallow capsules or tolerate liquid formulations and to adhere to the treatment regimen. Optimal drug dosing was based on pharmacokinetic studies of PIs in children [32–34]. Adherence was carefully monitored at each study visit by measurement of returned medications and interviews with the patient and/or the family. Adherence to therapy was defined as evidence that a patient received correctly ≥90% of the prescribed doses of medications [35, 36].

The cohort had a median age of 8 years (range, 1–17 years) and included 8 adolescents. Thirteen patients were girls and 13 were boys. Thirteen children (50%) were African American, 12 (46%) were white, and 1 (4%) was Asian American. A majority of the cohort (24 [92%] of 26) had been infected perinatally, either through maternal transmission (20/24) or through HIV-contaminated blood products (4/24). Two adolescents were infected by sexual transmission ≥2 years before initiation of therapy. All patients were infected before the introduction of PI therapy into the adult population. Most patients displayed symptoms of HIV infection (CDC clinical stage B or C; 20 [77%] of 26) and immune suppression (CDC immune stage 2 or 3; 25 [96%] of 26) [37]. Pretherapy median CD4 T cell counts were 147 cells/µL (25th–75th quartile range, 41–309 cells/µL), the median CD4:CD8 ratio was 0.15, and median HIV plasma RNA levels were 5.15 log₁₀ copies/mL (25th–75th quartile range, 4.5–5.7 log₁₀ copies/mL).

Clinical monitoring, sample collection, and processing. Within 8 weeks before initiation of antiretroviral therapy, ≥2 blood samples and a complete clinical evaluation were obtained from each patient. Additional clinical examinations were performed and additional blood samples were collected at 4, 12, 16, 24, 32, 44, and 48 weeks after initiation of therapy. Plasma HIV levels, complete blood cell counts, and T cell subsets were evaluated at each time point. T cell subsets were determined by flow cytometry analysis. Plasma was separated and was stored at −80°C within 2 h of collection. Peripheral blood mononuclear cells were obtained by Lymphoprep (Nycomed) density centrifugation, were cryopreserved by using a liquid nitrogen step freezer, and were stored in liquid nitrogen [38]. All samples were processed and stored in a biological safety level-2 facility that was free from HIV-1 cultures and amplified or plasmid HIV-1 DNA.

Response to therapy. Study end points were defined as any new AIDS-defining illness or failure to improve CD4 T cell counts by 24 weeks of therapy [39]. Response to therapy was classified by plasma virus levels and CD4 T cell numbers. Each patient had either viral success (VS) or viral failure, on the basis of the extent and duration of viral suppression after initiation of therapy. VS consisted of a decline in plasma viral RNA by >1.5 log₁₀/mL during the first 4 weeks of therapy, with sustained suppression at ≤400 copies/mL for ≥16 weeks. Plasma virus levels below the limits of detection for the assay were calculated as 399 copies/mL. Patients whose plasma viral RNA levels declined <1 log₁₀/mL or had rebounded to detectable levels by 16 weeks of therapy were defined as having viral failure.

Immune success (IS) was defined as an increase in the CD4 T cell count by ≥1 CDC stage by 24 weeks of therapy. Children who were immunosuppressed and showed no improvement in CD4 T cell counts were classified as having immune failure. Five patients who had immune and viral failure were discontinued from the study at 24 weeks, and their drug regimen was changed to a new antiretroviral therapy. Children who experienced increased CD4 T cell counts but no clinical disease progression, despite high virus levels, were continued on study for an additional 24 weeks.

Sequence analysis. Plasma samples collected before the initiation of combination therapy were used to evaluate RT and PR genotypes. Viral RNA was extracted with the QIaAmp Viral RNA kit (QIAGEN), which included controls to rule out carryover. Population sequencing of RT and PR was performed with the TruGene HIV-1 Genotyping Assay (Visible Genetics), as described by the manufacturer. In brief, amplification of cDNA by RT–polymerase chain reaction (PCR) was followed by combination PCR/sequencing reactions, using forward and reverse primers labeled with 2 different dyes. Reaction products were analyzed using the OpenGene automated DNA sequencing system (Visible Genetics).

The sensitivity of population sequencing allowed detection of polymorphisms in RT or PR that occurred with a frequency >20%. To verify the population sequence of PR, allele-specific PR sequences were generated, using reverse primer p2 (5'-CTTTTG-GGCCATCATTTCCGTC-3') from nucleotides 2170–2193 in the HIVLAI genome) [40] and Superscript II RNaSE H–RT (Gibco) to synthesize cDNA. First-round amplification was performed with forward primer p1 (5'-CAGAGCCAACAGCCCCACCAG-3'; nu-
cleotides 1724–1740) and reverse primer p2, followed by second-round amplification with nested primers p3 (5′-ACTGTACCTT- TAACTCC-3′, nucleotides 1817–1836) and p4 (5′-AGGTC- CAATAGGACTAATGGG-3′, nucleotides 2132–2152), to yield a 335-bp product that included the entire PR open-reading frame. Amplifications were performed in a Perkin-Elmer 9600 thermocycler, using a protocol provided by J. Condra (Merck) that included denaturation at 95°C for 5 min, followed by 35 cycles consisting of 94°C for 15 s, 56°C for 1 min, and 72°C for 2 min, and a final elongation cycle of 72°C for 7 min. Amplified products were cloned by using the pGEM-T Easy vector system (Promega) and a competent DH5α strain of *Escherichia coli*. Recombinant plasmids were purified by use of a QIAprep Miniprep kit (QIAGEN), and the presence of an insert was verified by digestion with restriction enzymes. Approximately 10 (range, 5–12) plasmids were isolated, and the presence of an insert was verified by digestion with restriction enzymes. Approximately 10 (range, 5–12) plasmids were sequenced after amplification reaction. Sequences were generated with primers p3 and p4, using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) and ABI 377 automated sequencer instrument.

Nucleic acid sequences of HIV-1 RT and PR were edited, translated, and analyzed with GeneObjects v3.1 (Visible Genetics). Any detectable amino acid polymorphisms at positions in RT or PR that are associated with reduced sensitivity to drugs were scored as resistant. The GenBank accession numbers for the nucleotide sequences generated are AF320515–AF320559.

**Genetic analysis.** Phylogenetic analysis was performed by using neighbor-joining and maximum parsimony in the PHYLIP software package [41]. Bootstrap values were based on generation of 100 replicate trees. The integrity of genetic data was verified by using amino acid alignments and construction of phylogenetic trees, to compare each sequence with all pol sequences generated in our laboratory. Distances in RT and PR nucleic acid sequences within or between therapy response groups were determined by a Jukes-Cantor algorithm.

Amino acid substitutions in RT (M41L, A62V, K65R, D67N, T69A/S/D, K70R, L74V, V75I/T, F77L, A98G, L100I, K101E, K103N/T, V106A, V108I, Y115F, F116Y, Q151M, I178M, V179D, Y181C/I, M184V/I/T, Y188L/C, G190A/S, L210W, T215Y/F/C, K219Q/E, and P236L) or in PR (R5Q, L10I/V/F/R, K20R/M, L24I, D30N, V32I, L33F, M36I, M46I/L, I47V, G48V, I50V, I54L/M/V, L63P, A71V/T, G73S, V77I, V82A/F/T/I/S, I84V, N88D, and L90M) that are associated with reduced drug sensitivity were evaluated as specific or nonspecific for particular drugs, by using data summarized by Schnitz et al. [42]. Substitutions associated with reduced sensitivity to a particular drug administered are considered therapy specific, whereas therapy-nonspecific substitutions would confer decreased sensitivity to a drug not administered to the patient. Analysis of PR polymorphisms included primary positions, in which initial mutations develop in drug-treated patients that have discernible effects on HIV-1 drug resistance in vitro, and secondary changes, which usually arise after primary mutations in drug-treated patients and have more subtle effects on decreased sensitivity to inhibitors in in vitro assays [23, 43].

**Statistical analysis.** Clinical variables were summarized among therapy responses as median and range or quartiles. Comparisons between RT or PR genotype and therapy outcome were performed using Fisher’s exact test (Sigma Stat software package; Jandel Scientific). Statistical significance was set as $P < .05$.

Univariate ordinal regression models (SAS Institute) were used to examine the relationship between each variable (clinical or genetic) and therapy response, by combining viral and immune responses into a single ordered outcome variable with 3 levels: viral and immune failure (VFIF), viral failure but with marked improvement in CD4 T cell counts (VFIS), and viral and immune success (VSIS). Individual odds ratios for viral or immune response were estimated from the regression coefficients of this model.

An exploratory and descriptive receiver operating characteristic (ROC) analysis was performed, using SAS software (SAS Institute), to examine whether viral and/or immune outcome could be predicted as a function of clinical variables, pretherapy PR genotype, or a combination of clinical and genetic parameters [44, 45]. A least-squares model was employed to carry out the ROC analysis, to avoid numerical fitting problems typically associated with the logistic regression approach in smaller samples [45]. Area under the ROC curve (AUC) was calculated by the trapezoidal rule and was used as the summary measure of fit for each model. An AUC value of 1.0 corresponded to a perfectly fitting predictive model, whereas an AUC value of 0.5 corresponded to a poor predictive model [45]. The best 1-, 2-, and 3-variable models were developed from the univariate information, using a forward stepwise selection procedure. One-variable models for immune or viral outcomes were determined independently, using the best individual predictors of success—i.e., the variable with the highest AUC. Two-variable models were developed from 1-variable models by adding the variable that provided the greatest percentage of increase in AUC. The procedure was repeated for development of 3-variable models.

The outcome obtained from the fitted model for each patient consisted of 2 probability estimates (P1 and P2), which corresponded to viral or immune responses after initiation of therapy. Optimal classification of actual versus predicted outcomes, using viral and immune criteria, was generated from P1 and P2, on the basis of the sensitivity and specificity given by the 2 ROC curves.

**Results**

**Viral and immune responses to therapy.** IS in response to combination therapy was achieved by 80% (21/26) of the cohort, whereas VS occurred in only 35% (9/26) of the patients. When changes in virus levels were evaluated in combination with CD4 T cell counts, patients displayed one of 3 therapy responses (figure 1). Nine patients (35%), who experienced sustained suppression of viral replication and significant improvement in CD4 T cell counts, were classified as VSIS. Five children (19%), who failed to maintain suppression of viral replication or to demonstrate improvement in CD4 T cell counts, were classified as VFIF. Twelve children (46%), who demonstrated only transient suppression of viral replication but marked improvement in CD4 T cell counts, were classified as VFIS. No pediatric patient displayed sustained viral suppression in the absence of increased CD4 T cells (VSIF), which can develop in adults and most likely reflects an age-related potential for immune cell reconstitution [16].

VSIS children demonstrated a dramatic reduction in virus
burden over the first 4 weeks of therapy, with a median decline of 1.82 log_{10} copies/mL (quartile range, –2.84 to –1.80 log_{10}; figure 1, top). A second-phase decline in virus levels to –2.35 log_{10} copies/mL (quartile range, –3.02 to –1.83 log_{10}) had occurred by 16 weeks of therapy, followed by sustained suppression of plasma virus levels to <400 copies/mL. CD4 T cell counts in the VSIS group had increased by a median of 110 cells/μL at 4 weeks and continued to increase during 24 weeks of treatment (figure 1, top). Median increases of 186 CD4 T cells/μL above baseline were sustained through 44 weeks of therapy.

The VFIF group displayed an initial decline in median plasma virus levels of 1.27 log_{10} (quartile range, –1.86 to –0.68 log_{10}), which was transient and had rebounded to baseline levels by 16 weeks of therapy (figure 1, center). CD4 T cell numbers remained essentially unchanged during therapy.

A discordant VFIS response to therapy was characterized by a decline in median plasma virus levels of 2.0 log_{10} copies (quartile range, –3.17 to –0.76 log_{10}) per mL by 4 weeks of therapy, which had rebounded to pretherapy levels by 16 weeks (figure 1, bottom). Viral responses among the individuals in the VFIS group were virtually identical to those among the VFIF group of patients but were clearly distinguishable by 16 weeks of therapy from those of the VS patients. Despite poor viral responses to therapy, VFIS patients displayed sustained increases in CD4 T cell counts (figure 1). Median CD4 T cell counts increased above pretherapy levels by 198 cells/μL after 4 weeks, 296 cells/μL after 24 weeks, and 772 cells/μL after 44 weeks of combination therapy. The increased CD4 T cell numbers displayed by the VFIS group paralleled closely the VSIS group’s immune response (figure 1). The magnitude of CD4 T cell reconstitution differed between IS and immune failure groups and was evident after only 4 weeks of therapy.

Long-term clinical outcomes between 24 and 48 weeks of therapy differed significantly among the therapy response groups. All VFIF patients experienced clinical decline, including new AIDS-defining diseases or death. In contrast, VSIS and VFIF patients experienced no significant decline in CD4 T cells, new AIDS-defining diseases, or death.

Pretherapy clinical characteristics among the therapy response groups. Clinical variables were related to therapy response by ordinal regression models (table 1). Although VFIF patients (median age, 10 years) were slightly older than those in the VSIS and VFIF groups (median ages, 8 and 6 years, respectively), age at and years of infection among the response groups were similar (P = .73). Median pretreatment virus levels of 4.7 log_{10} copies/mL in the VSIS group did not differ statistically from 5.5 and 5.2 log_{10} copies/mL among the VFIS and VFIF groups, respectively (P = .07). Most patients (17/26) received combination treatments that included ritonavir as the PI, but the distribution of ritonavir therapy among the response groups (67% of VSIS and VFIS patients and 60% of VFIF patients) was similar (data not shown). Therapy response was unrelated to CD4 T cell count (P = .46) and CD4:CD8 ratios (P = .16). Disease stage was the only clinical variable that displayed an association with therapy outcome (P = .02).

Genetic relationships among viruses. Phylogenetic analysis of viral sequences in RT or PR before initiation of combination therapy was assessed for each patient. Nucleotide sequences from individual patients formed distinct monophyletic branches, which confirmed the integrity of both RT and PR data sets (figure 2). Branches with sequences from patients with different responses were interspersed, rather than clustered together, in the trees, indicating that similar therapy response were independent of pretherapy RT or PR quasi species.

Genetic diversity (genetic distance among viruses) in both RT and PR within the 3 groups of patients was similar. For example, genetic distance in RT among VS patients (mean ± SE, 5.6% ± 0.6%) was indistinguishable from the mean distance among VF patients (4.4% ± 0.5% in the VFIS group and 6.0% ± 0.9% in the VFIF group). Diversity in PR within VSIS patients (5.3% ± 0.7%) was similar to the diversity found within VFIS (4.6% ± 0.6%) and VFIF patients (3.9% ± 0.7%). Pairwise comparisons of RT and PR sequences between response
Table 1. Clinical characteristics of study population at entry.

<table>
<thead>
<tr>
<th>Therapy response group</th>
<th>No. of subjects (N = 26)</th>
<th>Median (range)</th>
<th>Disease stage, no. of subjects</th>
<th>CD4 T cells/µL</th>
<th>CD4-CD8 ratio</th>
<th>Log₁₀ plasma viral RNA, copies/µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSIS</td>
<td>9</td>
<td>8 (6–13)</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>256 (78–273)</td>
</tr>
<tr>
<td>VFIF</td>
<td>5</td>
<td>10 (9–14)</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>25 (3–45)</td>
</tr>
<tr>
<td>VFIS</td>
<td>12</td>
<td>6 (4–9)</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>210 (41–410)</td>
</tr>
</tbody>
</table>

NOTE. VFIF, viral failure and immune failure; VFIS, viral failure and immune success; VSIS, viral success and immune success.

a Median value (25th–75th interquartile range).
b P values were based on ordinal regression models.

groups revealed similar genetic distances (data not shown), which indicated that relationships in RT or PR quasi species among the patients at the initiation of combination therapy were independent of outcome.

Amino acid polymorphisms within RT. A potential relationship between pretherapy genotypic resistance in RT, as measured by amino acid substitutions, and viral and immune outcomes was evaluated in 23 patients from the cohort (figure 3). All patients had received nucleoside RTI therapy before initiation of combination therapy, which was evident by mutations in RT amino acid positions 41, 62, 67, 69, 70, 74, 178, 184, 210, 215, or 219 in 20 patients. Similar to patients in other studies [46, 47], 3 patients, VSIS 17, VSIS 21, and VFIF 09, displayed no RT resistance mutations, despite a history of RTI medications.

Amino acid substitutions in RT were classified as therapy specific or therapy nonspecific for the RTI(s) included in the new combination treatment for each patient [42]. RT genotypes were defined as sensitive, if amino acid changes were nonspecific for all RTIs administered; as resistant, if mutations were specific for all RTIs received; or as mixed, if RT mutations were sensitive for one, but resistant to the second RTI in the treatment combination (figure 3A). RT displayed sensitive amino acid profiles in 10 (44%) patients, mixed genotypes in 9 (39%) patients, and resistant genotypes in 4 (17%) patients.

RT genotypic profiles were evaluated as variables in outcome to combination therapy (figure 3B). Most patients (19/23) were sensitive to one or, in many cases, both RTIs. Among 9 VSIS patients, 3 had sensitive RT genotypes, whereas 4 had mixtures of sensitive and resistant RT genotypes. Among 10 VFIS patients, 6 had RT-sensitive genotypes and 2 had mixed genotypes. RT among VFIF patients displayed either sensitive or mixed genotypes. In contrast, resistant RT genotypes were detected in only 4 patients: 2 VSIS and 2 VFIS patients. No significant relationship between the genotype of RT and response to combination therapy was apparent by these analyses.

Figure 2. Phylogenetic relationship of reverse transcriptase (A) and protease (B) sequences among the cohort of subjects with human immunodeficiency virus (HIV) type 1 infection. Phylogenetic relationships were assessed by using a neighbor-joining algorithm and nucleic acid sequences from pretherapy plasma samples. HIVLAI (*) was used as the outlier sequence. Sequences for each subject are designated by therapy outcome. ▲, Viral success and immune success; ■, viral failure and immune failure; ○, viral failure and immune success. “0.01” Indicates genetic distance.
Amino acid polymorphisms within pretherapy PR. A contribution by pretherapy PR genotypes to combination therapy outcome was assessed. Analysis focused on amino acid positions in PR that are related to reduced sensitivity to PIs [42]. Amino acid substitutions in PR at positions other than those related to decreased sensitivity to inhibitors were detected but were found to be unrelated to therapy outcomes (data not shown). Predominant amino acid polymorphisms among the cohort were restricted to 6 positions: 10, 36, 63, 71, 77, and/or 82. Even though all patients were infected before widespread treatment of adults by PIs, 94% (24/26) of the cohort had pretherapy PR genotypes that contained >1 amino acid substitution at a position in PR that could diminish sensitivity to inhibitors. PR genotypes with >2 substitutions were detected in more than half the patients (58% [15/26]).

The frequency of polymorphisms differed among the amino acid positions. L63P was the most frequent substitution and appeared in 68% (17/26) of patients, followed by M36I in 36% (9/26), V71I in 32% (8/26), L10I in 20% (5/26), and A71T and V82I each in 8% (2/26) of patients. Differences in mean numbers of substitutions at the 4 most frequent polymorphic amino acid positions in PR (10, 36, 63, and 77) between VF and VS groups were not significant.

Therapy-specific amino acid substitutions in pretreatment PR genotypes. Amino acid substitutions in pretreatment PR were characterized as sensitive, if none of the polymorphisms would reduce sensitivity to the administered PI, or as resistant, if polymorphisms would reduce sensitivity to the administered PI [42] (figure 4A). PI-sensitive or -resistant genotypes were equally distributed among the 26 patients in the cohort. Amino acid polymorphisms appeared at no more than one position in PR in 9 (69%) of 13 patients with PI-sensitive genotypes but in only 2 (15%) of 13 patients with PI-resistant genotypes. In contrast, PR alleles with >1 amino acid polymorphism were found in 4 (31%) of 13 patients with PI-sensitive genotypes but in 11 (85%) of 13 patients with PI-resistant genotypes ($P = .015$, Fisher’s exact test).

The frequency of PI-sensitive or -resistant genotypes differed among the clinical response groups (figure 4B). Among patients who had PI-sensitive PR genotypes, 100% achieved IS, and more than half (61% [8/13]) achieved durable viral suppression. The reliability of a PI-sensitive genotype as a marker for therapy success was diminished somewhat by 5 VFIS patients (19% of the cohort) whose viruses failed to respond to therapy, although their PR genotypes were sensitive to the PI administered. In contrast, PI-resistant genotypes were restricted almost exclusively to VF patients. Among patients with PI-resistant amino acid substitutions, 12 (92%) of 13 had viral failure,
Figure 4. Genotypes of protease (PR) before PR inhibitor (PI) combination therapy for human immunodeficiency virus (HIV) type 1 infection. 

A, PR genotype. Patients are designated by the same numbers as in figure 3. IDV, indinavir; NFV, nelfinavir; RTV, ritonavir. White box, no resistance substitution; shaded box, therapy-nonspecific amino acid substitution; black box, therapy-specific amino acid substitution [42]. Patients had viral success and immune success (VSIS), viral failure and immune failure (VFIF), or a mixed response (viral failure and immune success [VFIS]). 

B, Pretherapy PR genotypes relative to therapy outcome. White bars, VSIS response; striped bars, VFIS response; black bars, VFIF response.

whereas only patient VSIS 04 achieved a successful viral response to combination ritonavir therapy, despite a pretherapy PR genotype that included M36I. PR genotype determined on the basis of population sequencing was confirmed by allele-specific sequencing (data not shown).

Overall, PR genotype as a single variable was related correctly to viral outcome in 76% (20/26) of patients. Among patients who demonstrated immune reconstitution, 62% (13/21) had sensitive genotypes, whereas 100% (5/5) of those with immune failure had resistant genotypes. Sensitive or resistant PR genotype was associated with viral or immune outcome within the response groups (P = .01 or .04, respectively, Fisher’s exact test).

Viral and immune responses were combined into a single ordered outcome variable with 3 levels (VFIF, VFIS, and VSIS) for analysis by univariate ordinal regression models. PR-sensitive or -resistant genotype, as a variable, was related significantly to viral and immune responses to therapy (P = .005). Odds ratios for viral or immune response were estimated from the regression coefficients of the model. The odds for viral failure relative to success for PR-resistant genotypes were 2.5 to 1. In contrast, the odds for immune failure versus IS for PR-resistant genotypes were 17.7 to 1, reflecting that all immune failures occurred in patients with PR-resistant genotypes.

Clinical and genetic variables that predict viral and immune outcomes. To develop a more sensitive and specific model to predict therapy response, pretherapy clinical and PR genetic variables were evaluated by multivariate analysis. The best single pretherapy clinical variable for predicting immune outcome by ROC analysis was CD4 percentage (AUC, 0.83), whereas disease stage (AUC, 0.75) provided the best single clinical predictor of viral response. Other clinical variables, such as age, sex, or virus burden, had AUC values of 0.54–0.69, with limited predictive value. PR genotype as a single variable was similar for immune and viral outcomes (AUC, 0.81 or 0.80, respectively). When the 2 best variables, either CD4 percentage plus PR genotype or disease stage plus PR genotype, were combined, the AUC increased to 0.91 for immune outcome and 0.88 for viral outcome. The best predictive models for therapy outcome with AUC close to the maximum of 1.0 included 3 variables, 2 clinical and 1 genetic. PR genotype plus CD4 percentage and age resulted in an AUC of 0.97 (95% confidence interval [CI], 0.89–0.99) for immune response. PR genotype combined with disease stage and CD4 percentage resulted in an AUC of 0.92 (95% CI, 0.82–0.99) for viral outcome. CD4 T cell count, plasma virus levels, and sex were also evaluated in 3-variable models but failed to improve the predictive value for either immune or viral outcome (data not shown).

Immune and viral models were combined to determine the ability to predict outcome on the basis of VFIF, VFIS, or VSIS responses. When the best clinical variables (CD4 percentage plus disease stage) were used, therapy response was predicted correctly for 58% of the patients (15/26; data not shown). In contrast, when PR genotype was combined with the 2 best clinical variables for immune or viral outcome, therapy response was predicted correctly for 81% (21/26) of the patients (table 2). Among 5 patients who were misclassified, 2 VFIS patients were predicted to have VSIS responses, whereas 3 (2 VSIS and 1 VFIS) patients displayed responses that were better than predicted by the model. Overall, the combined model that included PR genotype plus clinical variables predicted correct therapy response in 100% (5/5) VFIF, 78% (7/9) VSIS, and 75% (9/12) VFIS patients.
secondary mutations, in combination with mutations in the active site, clearly enhance PR resistance to drugs [43, 55]. Even if amino acid polymorphisms in secondary sites in PR fail to reduce detectable drug sensitivity, secondary substitutions present before the initiation of therapy may provide a replication advantage when the virus is placed under the selective pressure of drug therapy in patients [29]. Independent of function, natural polymorphisms in amino acid positions 10, 36, 63, and/or 77 in PR served as accurate biomarkers for therapy outcome.

Several factors were essential for the success of the overall model to predict response. First, stratification of therapy outcome on the basis of both viral and immune parameters was critical. If viral response to treatment had been used to the exclusion of immune response in classifying outcome, the relationship between pretherapy protease genotype and treatment response would have been less obvious, and the predictive value of the model would have been diminished. Second, natural polymorphisms in any amino acid position in PR that could be associated with diminished sensitivity to inhibitors were considered. Natural polymorphisms occurred predominantly in any of 4 positions in PR that were localized outside the active site of the enzyme. If analysis had focused only on polymorphisms in primary sites, to the exclusion of secondary sites, in PR, pretherapy PR genotype would have limited, if any, predictive value. Finally, defining PR genotype as sensitive or resistant relative to the particular PI received provided a parameter that enhanced both the univariate regression model and the multivariate model.

A striking finding of the study was the value of the multivariate model to predict therapy outcome not only for children who had viral and immune failure or success, but for children who developed a discordant response as well. Even though the repertoire of viral and host factors that can lead to immune success with viral failure is undefined, use of viral genotype combined with pretherapy clinical parameters resulted in successful prediction of outcome in 75% of VFIS patients. Pediatric patients were particularly well suited for the analysis, because of high pretherapy virus burden and greater potential for immune reconstitution, in comparison with adults [15, 16]. Nevertheless, restoration of CD4 T cell counts, despite rebound of virus burden, develops in both HIV-infected children and adults treated with combination antiretroviral therapy and occurs in as many as 50% of patients in some cohorts [15–17, 56].

Although the multivariate model provided a significant level of accuracy in prediction of outcome for most patients, ~20% of the patients in the cohort were misclassified, which indicates that variables in addition to disease stage, immune status, and PR genotype affect therapy response. Two patients had PR-sensitive genotypes and clinical parameters that predicted a VSIS response, in contrast to their actual VFIS therapy response. Genetic determinants outside PR—for example, in gag or in PR cleavage sites—that modulate PR activity could account for these results [57–59]. Alternatively, viral failure in

### Table 2. Combination of clinical and genetic variables to predict therapy outcome in subjects with human immunodeficiency virus type 1 infection.

<table>
<thead>
<tr>
<th>Actual response</th>
<th>Predicted response</th>
<th>VSIS</th>
<th>VFIS</th>
<th>VFIF</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSIS</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>VFIS</td>
<td>2</td>
<td>9</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>VFIF</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** VFIF, viral failure and immune failure; VSIS, viral failure and immune success; VFIS, viral success and immune success. Boldface numbers represent patients whose predicted response to therapy was identical to their actual response.

### Discussion

Genetic characteristics of PR or RT, which are targets in the viral genome for drug action, provide prognostic value when combination antiretroviral therapy is switched for patients who fail to maintain viral suppression [18, 21, 23, 48]. In our study HIV-1 PR genotype provided accurate prognostic value for patients who were naive to PI combination therapy. The findings indicate that a spectrum of naturally occurring amino acid polymorphisms in PR can be used as biomarkers to predict both immune and viral outcomes to initial combination therapy in HIV-infected patients.

Disease stage, virus load, and CD4 T cell counts, which are generally applied as prognostic measures of treatment outcome [3, 5, 6], predicted response to combination antiretroviral therapy among 58% of patients in the cohort, which was only slightly better than chance. The limited value of clinical variables was not unexpected, in view of inclusion criteria that focused on patients with advanced disease. Pretherapy RT genotype provided little prognostic value for predicting therapy response in the cohort, most likely because the patients had received extensive RT therapy over a period of years [46, 47]. Nonetheless, patients who had RT-sensitive genotypes were no more likely than patients who were resistant to new RTIs to achieve a successful outcome to combination therapy. In contrast to RT genotype, pretherapy PR genotype, either alone or in combination with clinical variables, led to development of a reliable and sensitive model, which predicted both viral and immune outcomes in >80% of the cohort of PI-naive patients.

Similar to pretherapy PR genes in other data sets [26, 27, 49–53], PR amino acid profiles that included PI-resistant polymorphisms were detected at some frequency in virtually all patients in our cohort. These polymorphisms are localized predominantly in amino acid positions classified as secondary mutations, persist for years before initiation of PI therapy, and are independent of primary mutations that appear in response to drug therapy [26, 52, 54]. Although a role for secondary mutations alone in diminishing PR sensitivity to inhibitors can be difficult to demonstrate in biochemical or replication assays,
these patients might result from suboptimal drug levels or from immune failure to control viral replication. Three patients experienced viral and/or immune outcomes that were better than those predicted by the model. Host factors, such as cytotoxic T cell responses or polymorphisms in chemokines or their receptors, could affect control of viral replication and contribute to the unexpected successful therapy outcome in these patients.

Opportunities to control viral replication with combination drug therapies are limited. Initial intervention before the development of cross-resistance and further immune decline is preferable to salvage therapy, but it requires identification of biomarkers, in addition to the standard clinical variables, that can contribute to prediction of response to combination antiretroviral therapy. Currently, little information supports general use of pretherapy genotyping in clinical practice [23]. Our data clearly indicate enhanced prognostic significance for naturally occurring amino acid polymorphisms in PR, combined with clinical variables, among PI-naive patients with advanced disease and provide a foundation for design of clinical studies that use pretherapy PR genotype to optimize combinations of antiretroviral therapeutics.

References


