

Predictive values of the human immunodeficiency virus phenotype and genotype and of amprenavir and lopinavir inhibitory quotients in heavily pretreated patients on a ritonavir-boosted dual-protease-inhibitor regimen.

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1 Revised version 2

2 **Predictive value of the HIV phenotype and genotype and of amprenavir and lopinavir**
3 **inhibitory quotients in heavily pretreated patients on a ritonavir-boosted dual protease**
4 **inhibitor regimen**

5
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24 Abstract

25 The inhibitory quotient (IQ) of human immunodeficiency virus (HIV) protease inhibitors
26 (PI) that is the ratio of drug concentration to viral susceptibility is considered to be
27 predictive of the virological response. We used several approaches to calculate the IQs of
28 amprenavir and lopinavir in a subset of heavily pretreated patients participating in the
29 ANRS-104 trial, then compared their potential for predicting changes in the plasma HIV-
30 RNA level. Thirty-seven patients were randomly assigned to receive either amprenavir
31 (600mg BID) or lopinavir (400mg BID) plus ritonavir (100 or 200mg BID) for two
32 weeks, before combining the two PIs. The IC_{90} was measured using a recombinant assay
33 with or without additional human serum ($IC_{90+serum}$). Total and unbound PIs
34 concentrations in plasma were measured. Univariate linear regression was used to
35 estimate the relation between the change in viral load and the IC_{90} or IQ values. The
36 amprenavir phenotypic IQ values were very similar when measured with the standard
37 and protein binding-adjusted IC_{90} . No relationship was found between the viral load
38 decline and the lopinavir IQ. During combination therapy the amprenavir and lopinavir
39 genotypic IQ values were predictive of the viral response at week 6 ($p=0.03$). The
40 number of protease mutations ($<$ or ≥ 5) was related to the virological response
41 throughout the study. These findings suggest that the combined genotypic IQ and the
42 number of protease mutations are the best predictors of virological response. High
43 amprenavir and lopinavir concentrations in these patients might explain why plasma
44 concentrations and the phenotypic IQ have poor predictive value.

45 **Introduction**

46 The virological efficacy of protease inhibitors (PI) in patients infected by the Human
47 Immunodeficiency Virus (HIV) is dependent on both their pharmacodynamic and
48 pharmacokinetic properties. *In vitro* high and sustained plasma (or cell) concentrations are
49 needed to maximally suppress viral replication. The dose administered in HIV-infected patients
50 should provide such concentrations.

51 The inhibitory quotient (IQ), defined as the ratio of the trough drug concentration to viral
52 susceptibility expressed as an inhibitory concentration (ideally the IC_{90}), has been used to
53 estimate the antiviral potency of PIs *in vivo* (24). This parameter has also been proposed to
54 optimize the dosing regimen of treatment-experienced patients (12, 20). Although there is a
55 strong theoretical rationale for using the inhibitory quotient, the practical value of this parameter
56 is controversial. Firstly, there are few prospective studies of the relationship between IQ and
57 virological response (22). Secondly, there is no consensus method for calculating this parameter
58 (1, 24, 27). Standard calculations estimate IQ from both the plasma drug concentration and virus
59 susceptibility. However, several pharmacokinetic and virologic issues remain unsolved. In most
60 pharmacokinetic studies the total drug concentration in plasma is measured, whereas the active
61 component is the free (protein-unbound) fraction. Furthermore, the addition of human albumin to
62 the cell culture medium increases the IC_{90} *in vitro*. In summary, either the total concentration or
63 the protein-adjusted concentration, and either the standard or the serum-adjusted IC_{90} , can be used
64 to calculate the IQ. More recently, a “genotypic inhibitory quotient” was proposed, as the ratio of
65 the plasma concentration to the number of mutations on the viral protease gene. Indeed,
66 genotypic resistance assays can be performed rapidly and are less costly than phenotypic
67 resistance assays (11). Both genotypic and phenotypic IQs are predictive of changes in the HIV

68 RNA level in treatment-experienced patients (5, 9, 11, 17, 22, 23, 31), but only one study of the
69 predictive value of the IQ is available in patients treated with two ritonavir-boosted PIs (8).

70 We aimed at estimating the relationship between IQs of amprenavir and lopinavir and virological
71 response after 2, 6 and 26 weeks of treatment in a group of heavily pretreated patients who
72 participated in the ANRS-104 trial. Several methods for calculating IQs were compared with the
73 viral phenotype and genotype for their ability to predict changes in plasma viral load.

74 **Materials and methods**

75 *Study design and study population*

76 The ANRS 104 study was a prospective, randomized, open-label, multicenter trial involving
77 patients with CD4 < 500/mm³ and plasma HIV RNA > 10 000 copies/mL after receiving
78 successive antiretroviral treatments including at least two PIs and one non nucleoside reverse
79 transcriptase inhibitor (NNRTI). The main objective of this trial was to compare the clinical
80 efficacy and tolerability of a combination of amprenavir and lopinavir/ritonavir in treatment-
81 experienced patients (26). The study was divided into two periods. For the first two weeks
82 (period 1), patients were randomized to receive, in addition to their ongoing nucleoside reverse
83 transcriptase inhibitors (NRTI), 1) lopinavir/ritonavir (400/100mg BID) or lopinavir/ritonavir
84 (400/100 mg BID) + ritonavir (100 mg BID), 2) amprenavir (600 mg BID) + ritonavir (100 mg
85 BID), or amprenavir (600 mg BID) + ritonavir (200 mg BID). From week 3 to week 26 (period
86 2), all the patients received amprenavir + lopinavir/ritonavir, with or without an additional boost
87 of ritonavir 100 mg BID. The NRTIs were optimized on the basis of viral genotyping results and
88 previous antiretroviral exposure. The genotype was interpreted for each inhibitor by using the
89 2001 update of the French National Agency for AIDS Research (ANRS) algorithm. Patients were
90 recruited from 16 French clinical AIDS units. All patients signed an Ethics Committee-approved

91 informed consent form. Patients were instructed to take their medication in the morning and
92 evening, with a light meal. Physical examinations, CD4 and CD8 cell counts, hematologic and
93 clinical chemistry measurements were performed at each study visit (weeks -2, 0, 2, 4 and 6, then
94 monthly for the subsequent 20 weeks). Blood samples were also drawn, at weeks -2, 0, 2, 6, and
95 26, for plasma HIV-1 RNA, viral genotyping and phenotyping and drug assays in plasma.

96 97 **Laboratory measurements**

98 **Virological parameters**

99 Plasma HIV-1 RNA was assayed locally at weeks -2, 0, 2, 4, 6, 14 and 26, by using the Roche
100 Amplicor HIV-1 Monitor kit (Roche, France; limit of quantitation (LOQ) 200 copies/mL) or the
101 Quantiplex[®] HIV-RNA 3.0 assay (Bayer diagnostics, France; LOQ 50 copies/mL).

102 **HIV-1 protease genotyping and phenotyping**

103 Viral genotyping was performed at weeks -2, 2, 6 and 26, based on direct sequencing of the HIV-
104 1 protease coding region, using the consensus technique of the ANRS AC11 Resistance Group or
105 the TruGene HIV-1 genotyping kit (Visible Genetics, Bayer). The genotype was taken into
106 account only if a complete protease sequence (amino acids 1-99) was obtained. Protease
107 sequences from each patient were examined for the presence of mutations associated with
108 protease resistance at 21 relevant codons (11): 10, 20, 24, 30, 32, 33, 36, 46, 47, 48, 50, 53, 54,
109 63, 71, 73, 77, 82, 84, 88 and 90 (The Stanford HIV drug resistance database, 2004.
110 <http://hivdb.stanford.edu/>). The genotype for lopinavir/ritonavir and amprenavir/ritonavir was
111 interpreted by using the 2006 update of the French National Agency for AIDS Research (ANRS)
112 algorithm.

113 Resistance phenotyping was performed at screening (week-2) in all the patients and at week 26 in
114 those patients who failed drug regimen and had plasma HIV-RNA above 50 copies/mL (20/37

115 patients), using a recombinant virus assay (Phenoscript^R, Viralliance) (25). Results were
116 expressed as the PI concentration inhibiting virus spread by 90% (IC₉₀), in a standard method
117 without added human serum (IC₉₀). In a subgroup of 12 patients who started their study regimen
118 with amprenavir, the amprenavir IC₉₀ was also determined after adding 40% human serum to the
119 growth medium which contains 10% fetal bovine serum in order to reach a total protein
120 concentration close to that found in human plasma (IC_{90+serum}).

121 **Drug assays in plasma**

122 Blood samples were drawn at week 2 and week 6, just before the scheduled drug intake (C_{min}).
123 The total and unbound amprenavir and total lopinavir C_{min} values were measured by HPLC with
124 separation on a C18 column after liquid/liquid extraction of alkaline plasma and UV detection as
125 described elsewhere (2, 26, 29). Bound and unbound amprenavir were separated by ultrafiltration
126 using Centrifree[®] devices (Amicon, YM-300 filter system, Millipore Corp., Bedford,
127 Massachusetts, USA). Amprenavir was then measured in the ultrafiltrate as previously described.
128 The overall day-to-day coefficient of variation was below 12 % (2). All concentrations were
129 expressed in ng/mL or μ mol/L for inhibitory quotient calculations.

130 **Calculation of IQs**

131 Phenotypic IQs were calculated as the ratio of the plasma C_{min} of each PI to the IC₉₀ measured at
132 baseline. For amprenavir, the IC₉₀ values were determined with or without added protein
133 (IC_{90+serum} or IC₉₀), and both the total and unbound C_{min} (C_{min_u}) values were measured. Two
134 methods to adjust for protein binding were tested, namely the C_{min_u} and the IC_{90+serum} (24).
135 Amprenavir IQs were therefore calculated as the ratios of C_{min_u}/IC₉₀ (IQ_u) and C_{min}/IC_{90+serum}
136 (IQ_{serum}).

137 The genotypic inhibitory quotient (GIQ) of each PI was calculated as the ratio of the plasma
138 Cmin corrected for protein binding ($C_{min,u}$) to the baseline number of protease resistance
139 mutations. The following mutations on the viral protease were considered: L10I/F/R/V, K20M/R,
140 L24I, D30N, V32I, L33F, M36I, M46I/L, I47A/V, G48V, I50V, F53L, I54L/T/V, L63P,
141 A71I/L/V/T, G73S, V77I, V82A/F/T/S, A84V, N88D/S, and L90M.

142 During the second treatment period, in which patients received two ritonavir-boosted PIs, the
143 combined IQs were calculated as the sum of the phenotypic IQ_u for each PI. $C_{min,u}$ was not
144 measured but was calculated as the total Cmin corrected by the average protein binding of
145 amprenavir (10%) (2, 15) and lopinavir and ritonavir (1%) (3, 4). The genotypic combined IQ
146 was calculated as the sum of the ratios of $C_{min,u}$ /number of protease resistance mutations.

148 *Statistical analysis*

149 The median (range) was used to describe the distribution of amprenavir, lopinavir and ritonavir
150 parameters and for the different IQ calculations. Univariate linear regression was used to estimate
151 the relation between the decline in viral load (difference between baseline and weeks 2, 6 or 26)
152 and the different Cmin, IC90 and IQ values. The higher the proportion of explained variance (r^2)
153 of viral load, the better the model. All statistical tests were run on SAS software (version 8.2;
154 SAS Institute).

155 **Results**

156 Overall, 37 patients were enrolled in the ANRS 104 study. Their baseline characteristics are
157 shown in table 1. Twenty three patients started their study antiretroviral drug regimen with
158 lopinavir/ritonavir (group 1) and 14 patients started with amprenavir (group 2) .

159 Amprenavir and lopinavir Cmin values are reported in detail elsewhere (26, 29) and were not
160 related to the virologic response. The median unbound amprenavir Cmin_u at week 2 was 177
161 ng/mL and was not predictive of the virologic response at week 2.

162 In a subgroup of 12 patients who had phenotypic studies (group 2), the amprenavir IC₉₀ was 57.8
163 ng/mL (8.7-150.9), and increased to 453 ng/mL (33-1105) when measured in the presence of
164 50% human serum (IC_{90+serum}). The IC_{90+serum} was a good predictor of the early virologic response
165 (w2 p=0.006), whereas the IC₉₀ was not. Amprenavir IQs adjusted for protein binding (IQ_u and
166 IQ_{serum}) were rather similar (2.5 and 3.6, respectively). The relationship with viral load decline at
167 week 2 was slightly better with IQ_u than IQ_{serum} (r²=0.45, p=0.02 versus r²=0.31, p=0.06),
168 although those results might be explained by an outlier patient (r²=0.24, p=0.12 versus r²=0.11,
169 p=0.31 without the outlier patient).

170 In the 21 patients treated with lopinavir during the first period, the lopinavir IC₉₀ was 34.2 ng/mL
171 (0.7-330.4) and was a good predictor of the virologic response at weeks 2, 6 and 26 (r²=0.37,
172 p=0.003; r²=0.23, p=0.03; r²=0.33, p=0.006 respectively).

173 The number of protease mutations (< or ≥5) was related to the virologic response throughout the
174 study (at week 2, r²=0.18, p=0.008; at week 6 r²=0.11, p=0.046; and at week 26 r²=0.12, p=0.034
175 - figure 1).

176 Table 2 shows the virologic responses throughout the study as a function of the amprenavir and
177 lopinavir phenotypic IQ and GIQ values measured at week 2 or week 6. The amprenavir
178 genotypic IQ (corrected amprenavir Cmin at week 2/number of protease mutations at week -2)
179 was related to the virologic response at week 2 (r²=0.66, p=0.0004) and at week 6 (r²=0.38,
180 p=0.02). Patients who had a Cmin corrected GIQ above 75 had a median decline in HIV-RNA of
181 1.23 log₁₀ copies/mL (-2.27 ; -0.97) versus 0.19 log₁₀ copies/mL (-1.07 ; 0.15) in patients with a

182 GIQ below 75 ($p=0.005$). In contrast, none of the lopinavir IQs was predictive of antiretroviral
183 activity.

184 None of the phenotypic or genotypic IQs of amprenavir or lopinavir (determined with the Cmin
185 corrected for protein binding and measured at week 6) was predictive of the viral load decline.
186 However, a combination of the amprenavir and lopinavir genotypic IQs, whether measured either
187 with the standard equation or adjusted for protein binding, and with or without ritonavir, was
188 predictive of the viral response at week 6 but not at the end of the study as shown on figure 2.
189 Patients who responded at week 6 (plasma HIV-RNA $<$ vs \geq 10000 copies/mL) had a higher
190 median combined genotypic IQ measured at week 6 (65 vs 29 $p=0.01$).

191 Discussion

192 A significant proportion of antiretroviral-experienced patients never have optimal viral
193 suppression or experience a viral rebound shortly after starting a new treatment. Our population
194 was heavily pretreated with a high proportion of baseline NRTIs genotypic resistance,
195 consequently, the efficacy of the combination results essentially from both PIs. Treatment thus
196 needs to be optimized according to viral susceptibility and the plasma PI concentration. This
197 study provides IQ data for two currently used ritonavir-boosted PIs, amprenavir and lopinavir,
198 when administered alone (first two weeks of the study) or in combination, in heavily pretreated
199 HIV-infected patients. This is one of the few studies to show that the combined IQ can be a
200 useful predictor in patients who receive ritonavir-boosted dual-PI therapy (8).

201 We compared two methods for calculating the phenotypic IQ, which incorporates protein
202 binding. Amprenavir and lopinavir are highly bound to plasma proteins, and especially alpha1
203 acid glycoprotein (90% and 98-99% respectively). As only free drug inhibits viral replication,
204 protein binding affects the potency of these two PIs. ICs measured *in vitro* must be adjusted for

205 protein binding before extrapolation to the clinical setting. Moreover, ICs suffer from variability
206 due to a lack of standardisation of phenotyping methods (14). There are three methods for
207 adjusting the *in vitro* IC for protein binding: multiplication of the IC by the free fraction
208 measured *in vivo*; measurement of the IC in the presence of 50% human serum albumin; and
209 multiplication of the IC by a constant to adjust for assay variations (20), but none of these
210 methods is clinically validated. Our results show that, whatever the equation used to calculate IQ
211 ($C_{min_u}/\text{standard IC}_{90}$ or $C_{min}/\text{IC}_{90+\text{serum}}$), the relationship with viral load decline is very similar.
212 The best correlation was obtained with the IQ_u (C_{min_u}/IC_{90}). It has previously been
213 demonstrated that measuring and calculating the C_{min_u} gives similar results (29). However, it
214 remains to be determined whether these findings can be extrapolated to PIs other than
215 amprenavir.

216 We found no relationship between lopinavir or amprenavir exposure and the decline in viral load,
217 in agreement with previous studies (6, 7, 13, 16, 30). Amprenavir and lopinavir C_{min} values
218 obtained with the ritonavir boost were far higher than the IC_{50} reported for wild-type virus (28)
219 and the IC_{90} measured at screening. This might explain why plasma concentrations were poorly
220 predictive of the decline in plasma HIV RNA. Compliance with medication was not measured in
221 our study; the amprenavir and lopinavir C_{min} values suggested that it was maximal at week 2
222 and week 6 but declined thereafter. Thus, none of the phenotypic IQ values based on
223 concentrations measured at week 6 was predictive of antiviral efficacy.

224 The genotypic IQ is simpler to measure than the phenotypic IQ. We found that the genotypic IQ
225 of amprenavir was associated with the virologic response at week 2 and week 6 but not at week
226 26. Furthermore the amprenavir GIQ cut off of 75 could be a useful tool in clinical practice as
227 previously demonstrated by Marcelin et al. (18). Our findings confirm that the genotypic IQ,
228 which incorporates both baseline viral resistance and the level of drug exposure in plasma, is

229 superior to drug exposure alone in predicting the virologic response to a salvage regimen (18).
230 However, lopinavir IQs did not correlate with virologic efficacy, and our data do not support
231 those reported elsewhere (5, 9, 16). This is probably because the lopinavir C_{min} values in our
232 patients were sufficiently high not to restrict efficacy (13), whereas lopinavir concentrations were
233 lower in other studies (5, 9). In these latter studies, the C_{min} was determined as part of routine
234 therapeutic drug monitoring or observational studies, settings in which compliance is important
235 for overall exposure (5, 9, 16). One limitation of therapeutic drug monitoring is the wide intra-
236 patient variability of trough concentrations, as recently demonstrated by Nettles et al.(21) and
237 Goujard et al. (10). Poor compliance and an effect of food may be involved in this variability. We
238 acknowledge that one limitation of our study is that compliance was not measured. However,
239 comparison of trough concentrations measured at weeks 6, 14 and 26 clearly shows that
240 compliance is decreasing from week 6, where concentrations of lopinavir and amprenavir were in
241 the expected range in all patients, in contrast to weeks 14 and 26 where 7 and 6 patients
242 respectively, had concentrations below the limit of quantification of the assay (26).
243 Interestingly, the combined GIQ was predictive of the virologic response at week 6, as in the
244 GigHAART trial (8). This suggests that a combination of two PIs has a strong antiviral effect that
245 might overcome resistance in these strongly pretreated patients. As expected, ritonavir did not
246 participate in the drug effect, as the concentrations used to boost PIs are too low (even though
247 some patients received 200 mg BID). The combined IQ of amprenavir+lopinavir+ritonavir was
248 close to the combined IQ of amprenavir+lopinavir and had the same predictive potential.
249 However, the predictive value of this parameter disappeared at the end of the study, for several
250 possible reasons: in particular, the C_{min} tends to fall, as a negative initial interaction between the
251 two PIs and a decreased compliance tends to reduce their concentrations and exposure levels;
252 second, the viral resistance profile is continually evolving (19). Further studies are needed to

253 determine which viral mutations have the biggest impact on the genotypic IQ, and how these
254 mutations can be taken into account in the calculations (30).

255 The combined genotypic IQ did not have better predictive value than the number of mutations. In
256 these highly pretreated patients with high PI Cmin values, the number of PI resistance mutations
257 is a major determinant of virologic outcome (5, 13, 16) and, in our study, was the only factor
258 predictive of the virologic response at week 26. Finally, as previously demonstrated (26), patients
259 with more than five protease resistance mutations or a lopinavir mutation score (13,14) higher
260 than 5 at baseline had a significantly poorer virological response than other patients ($p=0.04$ and
261 $p=0.006$, respectively).

262 Thus, this study suggests that when treatment compliance is optimal and high PI concentrations
263 are achieved, the viral genotype is the best predictor of virologic outcome.

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268 **Appendix**

269 Puzzle1 Study Group: The following institutions and investigators participated in the Puzzle 1
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400 antiretroviral-experienced HIV-infected patients. *J Med Virol* **77**:460-4.

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401 **TABLE 1: Baseline characteristics of the patients participating in the ANRS 104 Study.**
 402 **Patients from group 1 started with amprenavir/ritonavir for the first 2 weeks and patients**
 403 **from group 2 started with lopinavir/r**

Parameters	Group 1, amprenavir	Group 2, lopinavir
	n=14	n=23
Median age, years (range)	47 (32-53)	41 (27-65)
Males, n (%)	12 (86)	21 (91)
CDC clinical stage,	n (%)	n (%)
A	6 (42)	10 (43)
B	4 (29)	3 (13)
C	4 (29)	10 (43)
Median CD4+ cells/mm ³ (range)	195 (65-385)	185 (3-509)
Median HIV1-RNA, log ₁₀ copies/mL (range)	4.9 (3.6-5.7)	4.6 (3.8-5.6)
Median number of previous antiretrovirals (range)	7.5 (4-12)	7.5 (4-12)
Median number of antiretrovirals taken prior to inclusion and still in use at inclusion (range)	9.5 (7-13)	10 (8-13)
Genotypic resistance	n (%)	n (%)
Amprenavir/r	7 (50)	9 (56)
Lopinavir/r	7 (50)	7 (30)
Median (range) number of protease mutations	7.0 (1.0-9.0)	7.0 (1.0-10.0)
Median (range) number of reverse transcriptase mutations	6.5 (0-11.0)	7.0 (0.0-11.0)
Median (range) phenotypic resistance index		

Amprenavir	2.8 (0.5-24.3)	2.5 (0.5-19.5)
Lopinavir	8.7 (0.3-84.0)	10.7 (0.2-95.3)

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405 **TABLE 2: Relationships between the viral load decline at weeks 2, 6 and 26 and the**
 406 **amprenavir, lopinavir and ritonavir inhibitory quotients (univariate analysis)**

Inhibitory quotients	Week	Number of patients	Median (min-max)	p			
				Week 2	Week 6	Week 26	
Amprenavir	IQsd	2	4.3 (0.62-25.9)	<i>0.04</i>	0.24	0.70	
		6	1.8 (0.20-11.7)		0.80	0.26	
	GIQ	2	81.2 (17.9-291.1)	<i>0.0004</i>	<i>0.02</i>	0.16	
		6	26.4 (9.8-69.1)		0.56	0.59	
Lopinavir	IQsd	2	4.0 (0.16-153.3)	0.97	0.65	0.66	
		6	2.0 (0.15-157.5)		0.70	0.80	
	GIQ	2	23.9 (6.6-177.6)	0.37	0.20	0.20	
		6	17.8 (3.6-182.5)		0.15	0.36	
Ritonavir	IQsd	2	0.01 (0.0004-0.5)	0.55	0.18	0.28	
		6	0.005 (0.0004-0.8)		0.34	0.87	
	GIQ	2	1.7 (0.12-19.1)	0.18	0.10	0.12	
		6	1.3 (0.12-20.4)		0.16	0.45	
Amprenavir+	IQsd	6	34	4.9 (0.36-166.5)		0.34	0.71
Lopinavir	GIQ	6	37	46.9 (15.9-543.4)		<i>0.03</i>	0.15
Amprenavir+	IQsd	6	34	4.9 (0.37-167.3)		0.34	0.71
Lopinavir+	GIQ	6	37	48.8 (16.0-549.3)		<i>0.03</i>	0.15
Ritonavir							

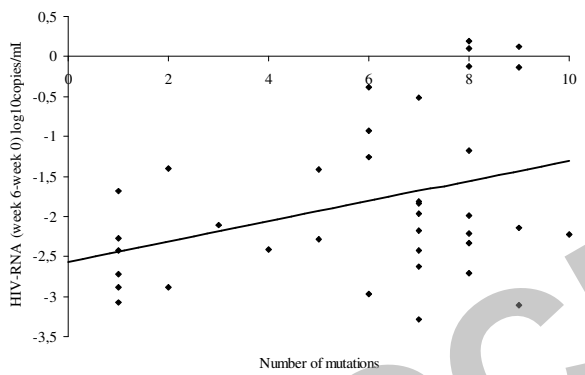
407 IQsd: standard inhibitory quotient: trough concentration (C_{min}) at week 2 or 6/IC90 standard at
408 week -2, GIQ: genotypic inhibitory quotient: corrected C_{min} (calculated protein unbound $C_{min,u}$)
409 at week 2 or 6/number of protease mutations at week -2.

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1 Figure 1: relationship between viral load decline at week 26 and the number of protease
2 mutations at screening (r^2 0.13, $p=0.03$).

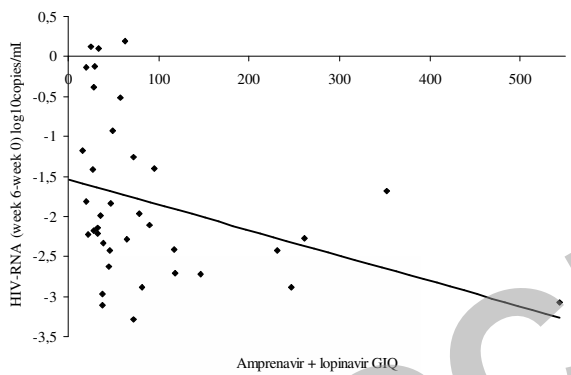
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- 1 Figure 2: relationship between viral load decline at week 6 and combined (lopinavir and
- 2 amprenavir) genotypic inhibitory quotient at week 6 ($r^2= 0.12$, $p=0.03$).

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