response to alfa/RBV, its apparent impact on virologic suppression in alfa-sparing regimens is unexpected. However, given the small number of patients, any such correlation will require evaluation in a larger dataset.

The emergent RAVs at viral breakthrough or relapse (signature NS5A-L31 and -Y93 substitutions for DCV and NS3-D168 substitutions for ASV) were similar to observations from other clinical studies of DCV, and also with *in vitro* GT1b replicon resistance studies with ASV [19], although this study represents the first demonstration of emergent clinical ASV resistance. It is possible that signature resistance variants to both DCV and ASV pre-existed as minor species and subsequently enriched by selective pressure, as predicted by viral kinetic modeling [22]. Although a combination of these NS3 and NS5A variants were not detected by clonal sequencing at baseline, their low-level pre-existence cannot be ruled out. However, assessment of minor NS3 plus NS5A variants from the same RNA sequence is currently not feasible using available deep-sequencing technologies. Nevertheless, additional studies to assess the presence and dynamics of minority baseline variants under drug selection are indicated.

Interestingly, ASV-resistant NS3-D168 substitutions generally decayed during the off-drug follow-up period, implying a lack of replicative fitness relative to wild-type in the absence of selective drug pressure. Indeed, a reduction in replicative fitness has been observed for D168 variants in replicons [19]. Neither of the secondary variants associated with D168V in this study (Q80L or S122G) had an impact on fitness *in vitro* (replication capacity similar or higher than that observed for parental GT1b [Con1] replicon), with both double variants possessing replicative capacities similar to D168V alone [19]. However, clonal analysis indicated that ASV-resistant variants were still detectable in some post-treatment samples

as minority species, although not detectable by population sequencing. Deeper sequencing techniques will be required to fully establish the dynamics of decay and whether ASV-resistant strains remain enriched for long periods relative to baseline. Since the re-treatment of patients with prior NS3 protease inhibitor failure have only been assessed in small studies [23], it is not clear whether these NS3 RAVs will form a stable minority capable of rapid overgrowth on re-treatment. By contrast, NS5A variants associated with DCV resistance were observed to be linked and relatively stable through at least 48 weeks post-treatment, although change of DCV-resistance substitutions was noted in four of seven patient samples. As described above, the prevalence of the NS5A variant Y93H, that confers low level resistance to daclatasvir, is approximately 10% in the general HCV genotype 1b population. Linked NS5A RAVs conferring high level resistance to daclatasvir are less prevalent (<1%). While NS3 RAVs (substitutions at positions V36, T54, R155, or D168) associated with first-generation protease inhibitors have been reported to be present at ≤2.7% by population sequencing [5, 24], emergent NS3 RAVs have been shown to persist for up to 4 years in long-term follow-up studies [25]. Therefore, longer-term studies are indicated to assess what, if any, replicative impairment is conferred by these linked NS5A changes and how long these potentially transmissible drug-resistant strains persist without DCV selection pressure.

In conclusion, high response rates were achieved in this small Japanese study comprising GT1b null-responders and alfa/RBV ineligible/intolerant patients with limited treatment options. Among patients experiencing virologic failure, ASV- and DCV-resistant substitutions emerged together at the time of failure, which were similar to those reported previously. An analysis of persistence demonstrated that DCV-resistant substitutions appeared to have

greater fitness over the duration of the study. A loose association with a baseline NS5A polymorphism on virologic outcome was observed; however, further data from larger studies are required. Consequently, a greater understanding of the role and dynamics of pre-existing, emergent and persistent resistance variants to DCV and ASV will be sought from the planned Phase 3 global studies of this combination.

Acknowledgments

The authors wish to thank Arlene Carifa, Bernadette Kienzle, and Xin Huang for assistance with sequencing, and Nannan Zhou, Aaron Monikowski, Paul Falk, and Chaoqun Chen for assistance with drug-susceptibility analyses. Editorial assistance with the preparation of this manuscript was provided by Andrew Street, Ph.D. and Nick Fitch, Ph.D., of Articulate Science Ltd and was funded by Bristol-Myers Squibb.

References

[1] Jacobson IM, McHutchison JG, Dusheiko G, Di Bisceglie AM, Reddy KR, Bzowej NH et al. Telaprevir for previously untreated chronic HCV infection. N Engl J Med 2011;364:2405-2416.

[2] Poordad F, McCone J, Bacon BR, Bruno S, Manns MP, Sulkowski MS et al. Boceprevir for untreated chronic HCV genotype 1 infection. N Engl J Med 2011;364:1195-1206.

[3] Ghany M, Nelson DR, Strader DB, Thomas DL, Seeff LB. An update on treatment of genotype 1 chronic HCV infection: 2011 practice guidelines by the American Association for the Study of Liver Diseases. Hepatology 2011;54:1433-1444.

[4] Merck. VICTRELIS™ (boceprevir) [package insert] 2012.

[5] Vertex Pharmaceuticals. INCIVEK™ (telaprevir) [package insert] 2012.

[6] Gao M, Nettles RE, Belema M, Snyder LB, Nguyen VN, Fridell RA et al. Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect. Nature 2010;465:96-100.

[7] Pol S, Ghalib RH, Rustgi VK, Martorell C, Everson GT, Tatum HA et al. High rates of SVR24 for BMS-790052, an NS5A replication complex inhibitor, in combination with pegIFN-alfa-2A and ribavirin: phase 2A trial in treatment-naive HCV genotype 1 subjects [Abstract]. ICAAC 2011. Abstract H1-376.

[8] McPhee F, Sheaffer AK, Friborg J, Hernandez D, Falk P, Zhai G et al. Preclinical Profile and Characterization of the Hepatitis C Virus NS3 Protease Inhibitor Asunaprevir (BMS-650032). Antimicrob Agents Chemother 2012;56:5387-5396.

[9] Bronowicki JP, Pol S, Thuluvath P, Larrey D, Martorell CT, Rustgi VK et al. Asunaprevir (ASV; BMS-650032), an NS3 protease inhibitor, in combination with peginterferon and

ribavirin in treatment-naive patients with genotype 1 chronic HCV infection. J Hepatol 2012;56(Suppl2):S431-S432.

[10] Chayama K, Takahashi S, Toyota J, Karino Y, Ikeda K, Ishikawa H et al. Dual therapy with the NS5A inhibitor, daclatasvir, and the NS3 protease inhibitor, asunaprevir, in HCV genotype 1b-infected null responders. Hepatology 2012;55:742-748.

[11] Suzuki Y, Ikeda K, Suzuki F, Toyota J, Karino Y, Chayama K et al. Dual oral therapy with daclatasvir and asunaprevir for patients with HCV genotype 1b infection and limited treatment options. J Hepatol 2012. Submitted in parallel with this manuscript.

[12] Bronowicki JP, Pol S, Thuluvath PJ, Larrey D, Martorell CT, Rustgi VK et al. BMS-650032, an NS3 inhibitor, in combination with peginterferon alfa-2a and ribavirin in treatment-naive subjects with genotype 1 chronic HCV infection. J Hepatol 2011;54(Suppl1):S472.

[13] Fridell RA, Wang C, Sun JH, O'Boyle DR, Nower P, Valera L et al. Genotypic and phenotypic analysis of variants resistant to HCV NS5A replication complex inhibitor BMS-790052: in vitro and in vivo correlations. Hepatology 2011;54:1924-1935.

[14] Pasquinelli C, McPhee F, Eley T, Villegas C, Sandy K, Wendelburg P et al. Single and multiple ascending dose studies of the NS3 protease inhibitor asunaprevir in subjects with or without chronic hepatitis C. Antimicrob Agents Chemother 2012;56:1838-1844.

[15] Sheaffer AK, Lee MS, Hernandez D, Chaniewski S, Yu F, Falk P et al. Development of a chimeric replicon system for phenotypic analysis of NS3 protease sequences from HCV clinical isolates. Antivir Ther 2011;16:705-718.

[16] Fridell RA, Qiu D, Wang C, Valera L, Gao M. Resistance analysis of the HCV NS5A inhibitor BMS-790052 in an in vitro replicon system. Antimicrob Agents Chemother 2010;54:3641-3650.

[17] Sun JH, O'Boyle DR, Zhang Y, Wang C, Nower P, Valera L et al. Impact of a baseline polymorphism on the emergence of resistance to the HCV NS5A replication complex inhibitor BMS-790052. Hepatology 2012;55:1692-1699.

[18] Lenz O, Verbinnen T, Lin TI, Vijgen L, Cummings MD, Lindberg J et al. In vitro resistance profile of the HCV NS3/4A protease inhibitor TMC435. Antimicrob Agents Chemother 2010;54:1878-1887.

[19] McPhee F, Friborg J, Levine S, Chen C, Falk P, Yu F et al. Resistance analysis of the HCV NS3 protease inhibitor asunaprevir. Antimicrob Agents Chemother 2012;56:3670-3681.

[20] Kuiken C, Yusim K, Boykin L, Richardson R. The Los Alamos hepatitis C sequence database. Bioinformatics 2005;21:379-384.

[21] McPhee F, Hernandez D, Yu F, Ueland J, Chayama K, Toyota J et al. A description of virologic escape in HCV genotype 1-infected patients treated with daclatasvir (BMS-790052) in combination with ribavirin and peginterferon-alfa-2a or peginterferon-alfa-2b. J Hepatol 2012;56(Suppl2):S473.

[22] Guedj J, Rong L, Dahari H, Perelson AS. A perspective on modelling hepatitis C virus infection. J Viral Hepat 2010;17:825-833.

[23] Lenz O, de Bruijne J, Vijgen L, Verbinnen T, Weegink C, Van Marck H et al. Efficacy of Re-Treatment with TMC-435 as Combination Therapy in HCV-Infected Patients following TMC-435 Monotherapy. Gastroenterology 2012;143:1176-1178.e6.

[24] Kieffer TL, De Meyer S, Bartels DJ, Sullivan JC, Zhang EZ, Tigges A, et al. Hepatitis C viral evolution in genotype 1 treatment-naïve and treatment-experienced patients receiving telaprevir-based therapy in clinical trials. PLoS One 2012;7(4):e34372.

[25] Susser S, Vermehren J, Forestier N, Welker MW, Grigorian N, Füller C, et al. Analysis of long-term persistence of resistance mutations within the hepatitis C virus NS3 protease after treatment with telaprevir or boceprevir. J Clin Virol 2011;52:321-327.

Table 1: Baseline viral and host characteristics among genotype-1b null-responders and their virologic outcome

Patient	*IL28B* GT	HCV-RNA, log_{10} IU/mL	NS5A polymorphism(s)[a]	NS3 polymorphism(s)[a]	Virologic outcome
P-1	CT	7.2	Q54H/Q-Q62Q/E-Y93H/Y	T54S-Q80L	SVR
P-2	CT	7.0		Q80L-V170I/M	SVR
P-3	CT	7.4	Q54H		SVR
P-4	CT	6.7	R30Q		SVR
P-5	CT	7.0	L31L/M-P58P/S		SVR
P-6	CC	5.3	P58P/T-Q62E		D/C at WK2 due to SAE[b]
P-7	CC	7.2		S122S/G	SVR
P-8	CT	7.0	Q54H	Q80L	SVR
P-9	CT	7.1	Q54H-Y93H/Y	S122N	SVR
P-10	CT	6.4	L28M-R30Q		SVR
P-11	CT	6.8			D/C at WK12 due to AE; SVR
P-12	CT	6.4	Q54H-P58S-Q62E		SVR

P-13	CT	7.4	Q54H		D/C at WK6; PDR not achieved[c]
P-14	CT	6.5			SVR
P-15	CT	6.3	R30Q/R-Q62Q/R		SVR
P-16	CT	6.6	Q54H		SVR
P-17	CT	6.6	Q54H-Q62E		SVR
P-18	CT	6.9	Q54Y	Q80L	SVR
P-19	CT	6.6	Q54H-Y93H	N77A	SVR
P-20	CT	7.0	R30Q	S122G	SVR
P-21	CC	6.6	Q54L		SVR

[a]All NS3 and NS5A amino acids were examined with focus on polymorphisms at positions known to be associated with resistance to NS3 protease inhibitors (36,43,54,55,77,78,79,80,122,123,138,155,156,158,168,170,175) and NS5A inhibitors (21,23,24,28,30,31,32,54,58,62,92,93). When a mixture of substitutions is indicated, the most predominant is identified first.

[b]HCV-RNA undetectable at post-treatment week 24

[c]Alfa/RBV added; HCV-RNA undetectable at post-treatment week 24 following 52 weeks' therapy

D/C, discontinued; GT, genotype; HCV, hepatitis C virus; PDR, protocol-defined response; SAE, serious adverse event; SVR, sustained virologic response; WK, Week.

Table 2: Baseline viral and host characteristics among genotype-1b ineligible/intolerant patients and their virologic outcome

Patient	*IL28B* GT	HCV-RNA, log_{10} IU/mL	NS5A polymorphism(s) [a]	NS3 polymorphism(s) [a]	Virologic outcome
P-22	CC	7.1			SVR
P-23	CC	6.9	A92T	Q80L-S122G/S	SVR
P-24	CC	6.6	L28M-R30L-Q54H-A92T	Q80L-S122S/G	D/C at WK12 due to AE; SVR
P-25	CT	6.8	L31M/L-Y93H/Y		VBT (WK16)
P-26	CC	5.3			SVR
P-27	CC	6.9	Q54H-Y93H/Y	T54S	SVR
P-28	CC	6.8	Y93H/Y	Q80L	SVR
P-29	CT	6.7	Q54Y-Y93H/Y	Q80L	VBT (WK16)
P-30	CT	6.7	Q54H		SVR
P-31	CC	6.6	P58S/P-Y93Y/H	S122G	Relapse (FUWK12)
P-32	CT	6.7	P58L	S122G	Relapse (FUWK4)
P-33	CT	5.2	Q54H-Q62P/S		D/C at WK12 due to patient request; SVR
P-34	CC	6.6		Q80L	SVR
P-35	CC	6.4	Q54H-Q62E/A-A92T		SVR

P-36	CC	7.1		S122S/C	Relapse (FUWK4)
P-37	CC	6.6	Y93H		Relapse (FUWK4)
P-38	CC	7.5		S122T	SVR
P-39	CC	5.1	R30Q/R		SVR
P-40	CC	6.8	Q54H-A92A/T	Q80L	D/C at WK8[b]
P-41	CC	6.0		S122G	SVR
P-42	CC	6.5	A92T		SVR
P-43	CT	7.0	Q54Y-Y93H	S122G	VBT (WK10)

[a]All NS3 and NS5A amino acids were examined with focus on polymorphisms at positions known to be associated with resistance to NS3 protease inhibitors (36,43,54,55,77,78,79,80,122,123,138,155,156,158,168,170,175) and NS5A inhibitors (21,23,24,28,30,31,32,54,58,62,92,93). When a mixture of substitutions is indicated, the most predominant is identified first.

[b]Treatment discontinued at patient request; subsequently lost to follow-up

D/C, discontinued; FU, follow-up; GT, genotype; HCV, hepatitis C virus; SVR, sustained virologic response; VBT, viral breakthrough; WK, week.

Table 3: Emergence of resistance-associated variants among genotype-1b ineligible/intolerant patients experiencing viral breakthrough or relapse

Patient	Time point	DCV / ASV C_{trough} range, nM	NS5A RAVs				DCV EC_{90}, nM	NS3 RAVs			ASV EC_{90}, nM
			L31	Q54	P58	Y93		Q80	S122	D168	
VBT patients											
P-25	BL	190–261 / 25–41	M/L	–	–	H/Y	<137	–	–	–	
	WK16 (VBT)		M	–	A	H	>1000	–	–	A	540
	WK20		V	–	A	H		–	–	A	
	WK24		M	–	A	H		–	–	A	
	FUWK4		M	–	A	H		–	–	A	
	FUWK36		M	–	G	H	>5000	–	–	D/A	
	FUWK48		M	–	G/A	H		–	–	–	
P-29	BL	116–198 / 18–33	–	Y	–	H	0.04	L	–	–	1.6
	WK16 (VBT)		ND					ND			
	WK20		M/V	Y	–	H	750	L	–	V	55
	FUWK4		M	Y	–	H		L	–	V	
	FUWK36		M	Y	–	H		L	–	V	
	FUWK48		M	Y	–	H		L	–	V	
P-43	BL	243 / 69	–	Y	–	H	0.49	–	G	–	2.8
	WK10 (VBT)		M	Y	–	H	435	–	G	V	279
	FUWK4		M	Y	–	H		–	G	V	
	FUWK36		M	Y	–	H		–	G	–	
	FUWK48		M	Y	–	H		–	G	–	
Relapse patients											
P-31	BL	573–620 / 153–327	–	–	S/P	Y/H	0.02	–	G	–	
	FUWK16		ND					–	–	A	

	FUWK24		M	–	–	H	351	–	G	–	
	FUWK36		–	–	–	–		–	–	–	
	FUWK48		–	–	–	–		–	–	–	
P-32	BL		–	–	L	–	0.004	–	G	–	
	FUWK8	151–306 / 19–42	M	–	L	H		–	G	V/D	
	FUWK12		M	–	L	H	543	–	G	–	
	FUWK36		M	–	L	–	1.5	–	G	–	
	FUWK48		M	–	L	–		–	G	–	
P-36	BL		–	–	–	–		–	–	–	
	FUWK8		V/M	–	–	H		–	–	V	1190
	FUWK12	138 / 26	V	–	–	H	349	–	–	–	
	FUWK24		M/V	–	–	H		–	–	V/D	
	FUWK36		M	–	–	H	137	–	–	–	
	FUWK48		M	–	–	H		–	–	–	
P-37	BL		–	–	–	H	0.49	–	–	–	
	FUWK8		V	–	–	H		–	–	V	
	FUWK12	75–134 / 40–93	V/I	–	–	H		–	–	V	
	FUWK24		M	–	–	H		–	–	V	
	FUWK36		M	–	–	H		–	–	E/D	
	FUWK48		M	–	–	H		–	–	–	

ASV, asunaprevir; BL, baseline; DCV, daclatasvir; EC_{90}, 90% effective concentration; FU, follow-up; ND, not determined as multiple amplifications failed; RAV, resistance-associated variant; VBT, viral breakthrough; WK, week

When a mixture of substitutions is indicated, the most predominant is written first. ASV-resistant variants conferred no cross-resistance to DCV, and vice versa in a replicon assay. Dashes indicate consensus with control sequence GT1b (Con1)

Figure Legends

Fig. 1. HCV-RNA levels among genotype-1b null-responders. Treatment was initiated with (A) asunaprevir 600mg BID or (B) asunaprevir 200mg BID, in combination with daclatasvir 60mg QD. Individual patient HCV-RNA levels are shown in grey. Mean HCV-RNA levels are shown in black. BID, twice-daily; HCV, hepatitis C virus; LLOQ, lower limit of quantitation; QD, once-daily.

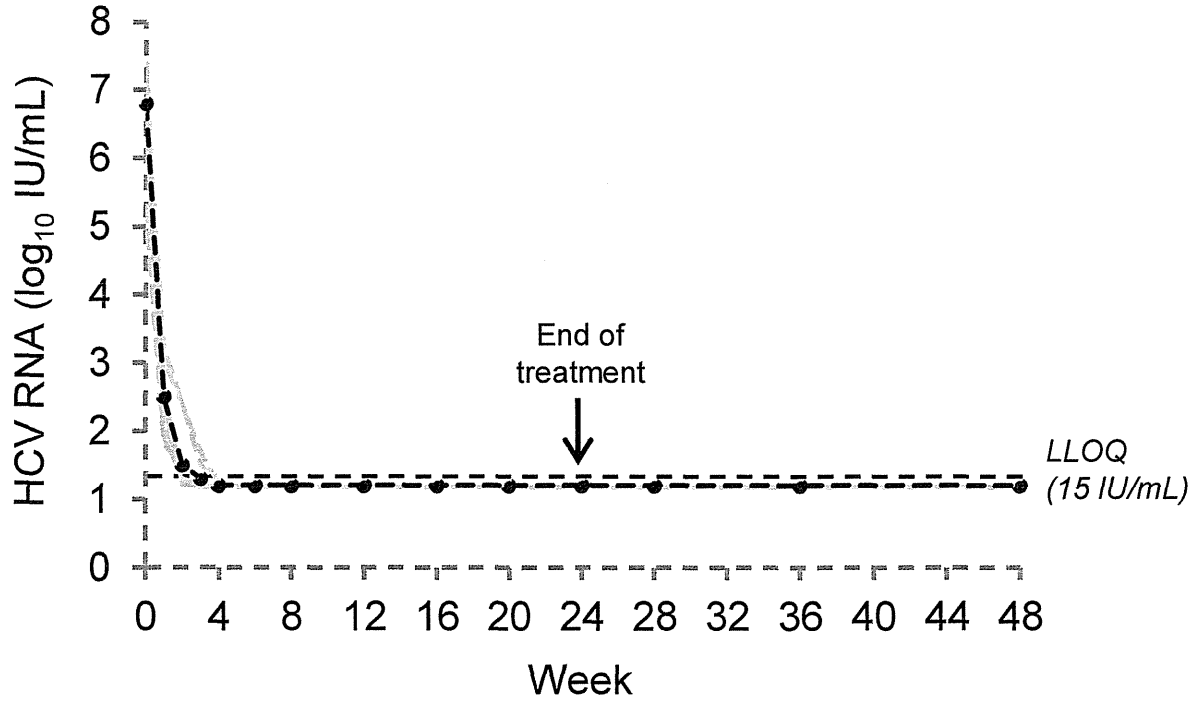
Fig. 2. Impact of baseline polymorphisms associated with resistance on virologic outcome among genotype-1b (A) null responders or (B) ineligible/intolerant patients. The ineligible/intolerant analysis excludes one patient (P-40) who discontinued therapy and was subsequently lost to follow-up. SVR, sustained virologic response.

Fig. 3. Early (Week 1) declines in HCV-RNA were similar among alfa/RBV ineligible or intolerant patients with and without baseline polymorphisms associated with resistance, virologic failure, and *IL28B* CT genotype. BL, baseline; GT, genotype; HCV, hepatitis C virus; RAV, resistance-associated variant; SVR, sustained virologic response; VBT, viral breakthrough.

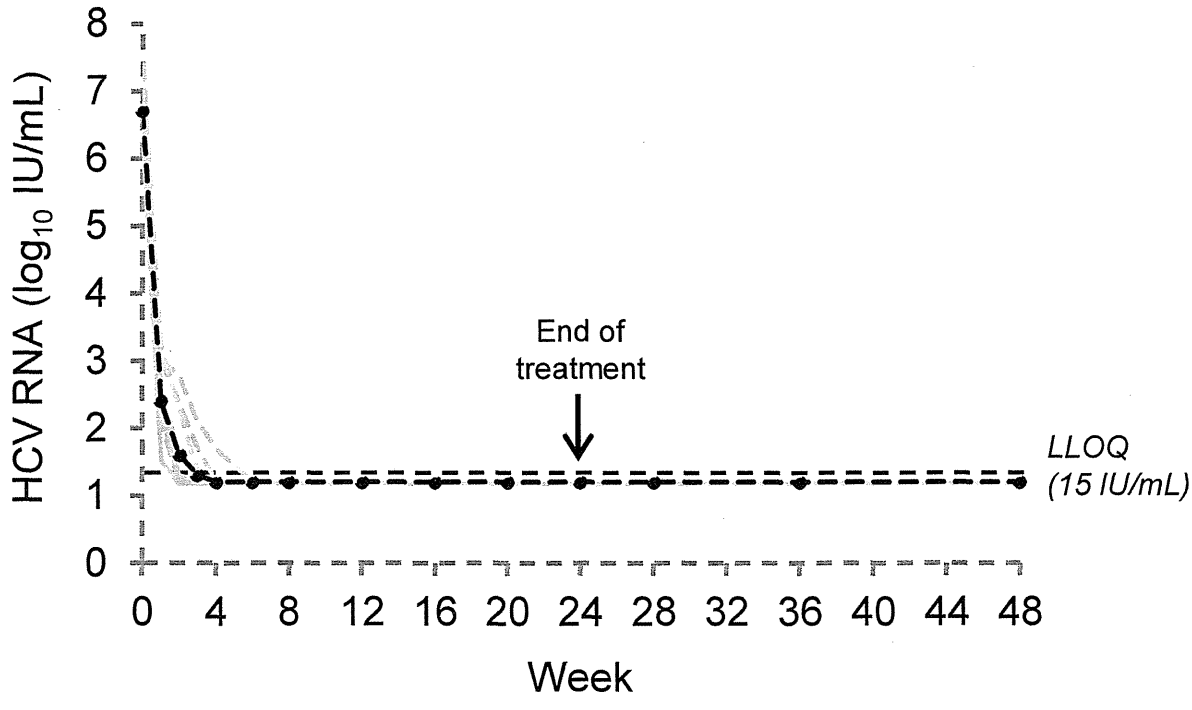
Fig. 4. HCV-RNA levels on-treatment and during post-treatment follow-up for genotype-1b ineligible/intolerant patients experiencing (A) viral breakthrough or (B) relapse. Solid lines indicate on treatment period. Dashed lines indicate post-treatment follow-up. HCV, hepatitis C virus; LLOQ, lower limit of quantitation.

Fig. 5. Clonal analysis of NS3 protease and NS5A resistance-associated variants in patients experiencing virologic breakthrough: (A) Patient P-25, (B) Patient P-29,and (C) Patient P-43. FU, follow-up; VBT, viral breakthrough.

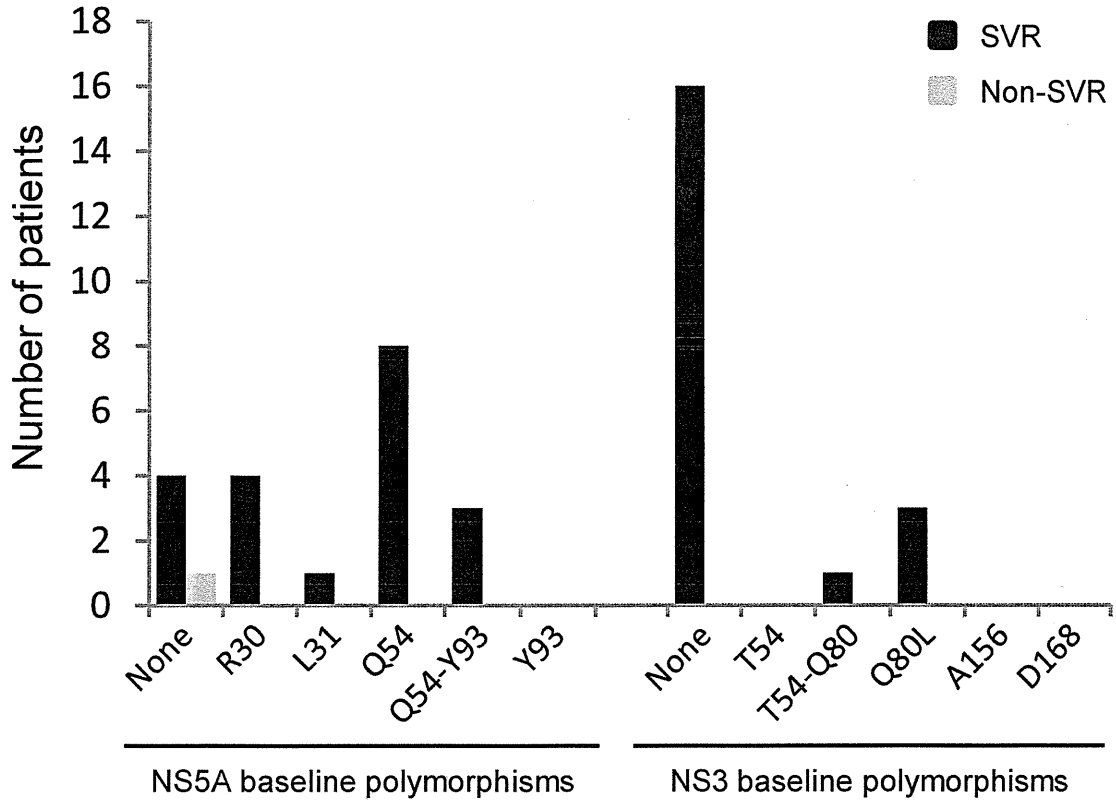
(A)


HCV RNA (log10 IU/mL)
8
7
6
5
4
3
2
1
0
End of treatment
LLOQ (15 IU/mL)
0 4 8 12 16 20 24 28 32 36 40 44 48
Week

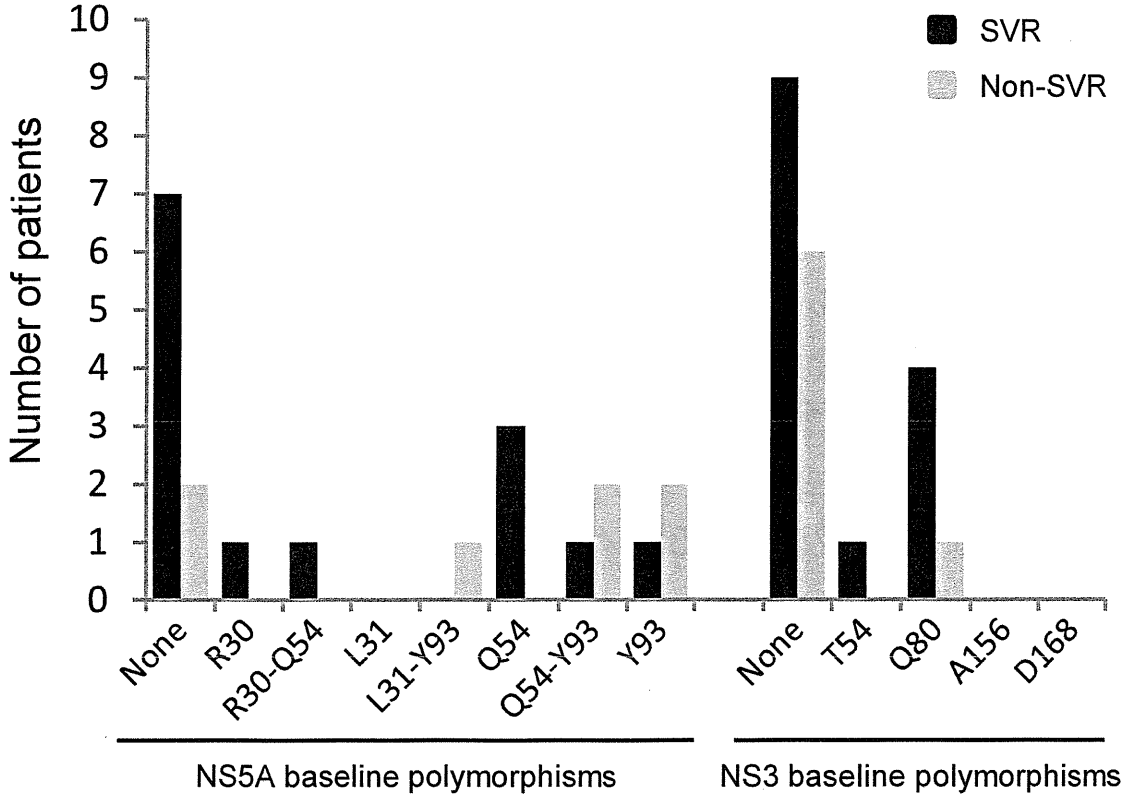
(B)

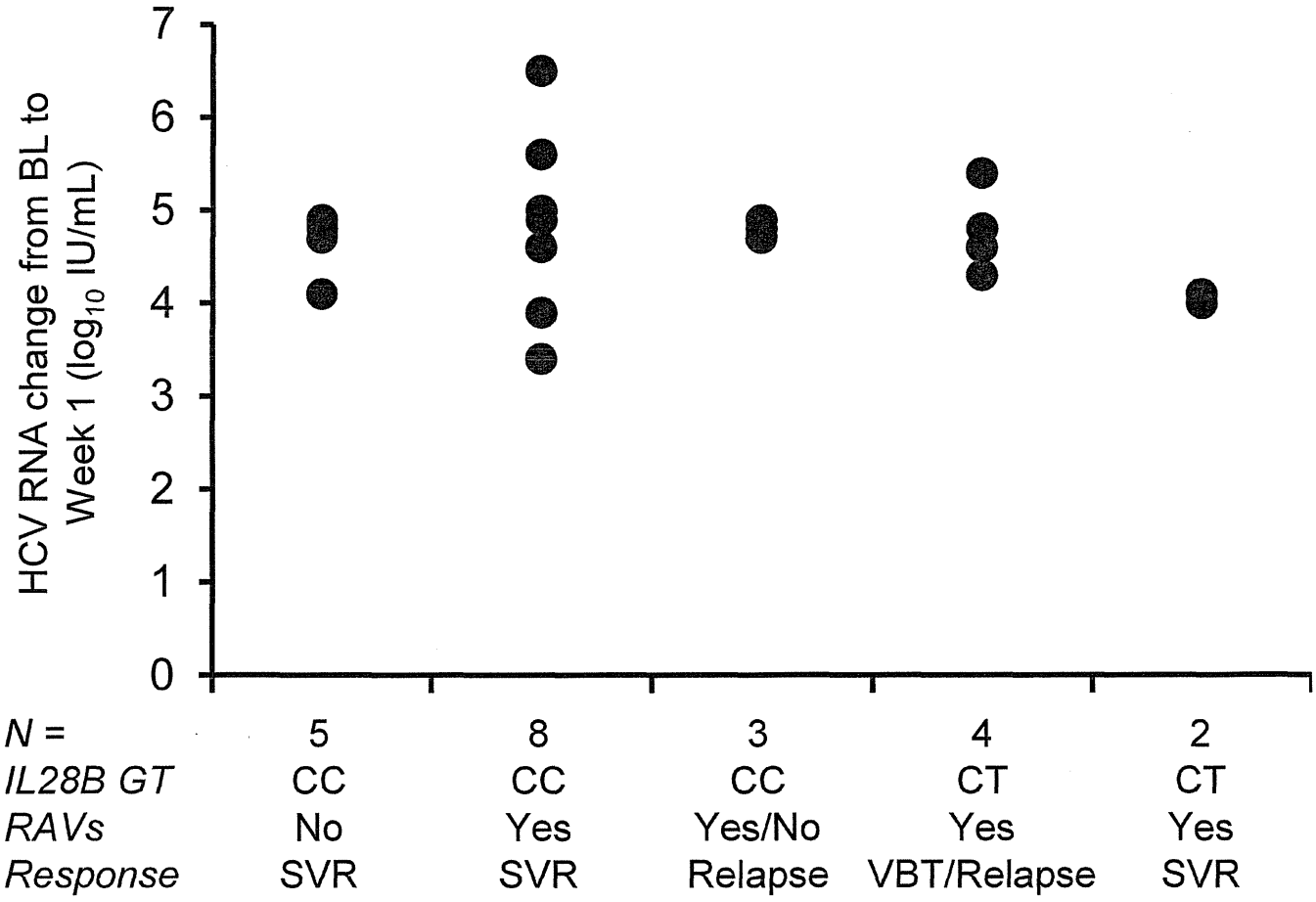

HCV RNA (log10 IU/mL)
8
7
6
5
4
3
2
1
0
End of treatment
LLOQ (15 IU/mL)
0 4 8 12 16 20 24 28 32 36 40 44 48
Week

(A)

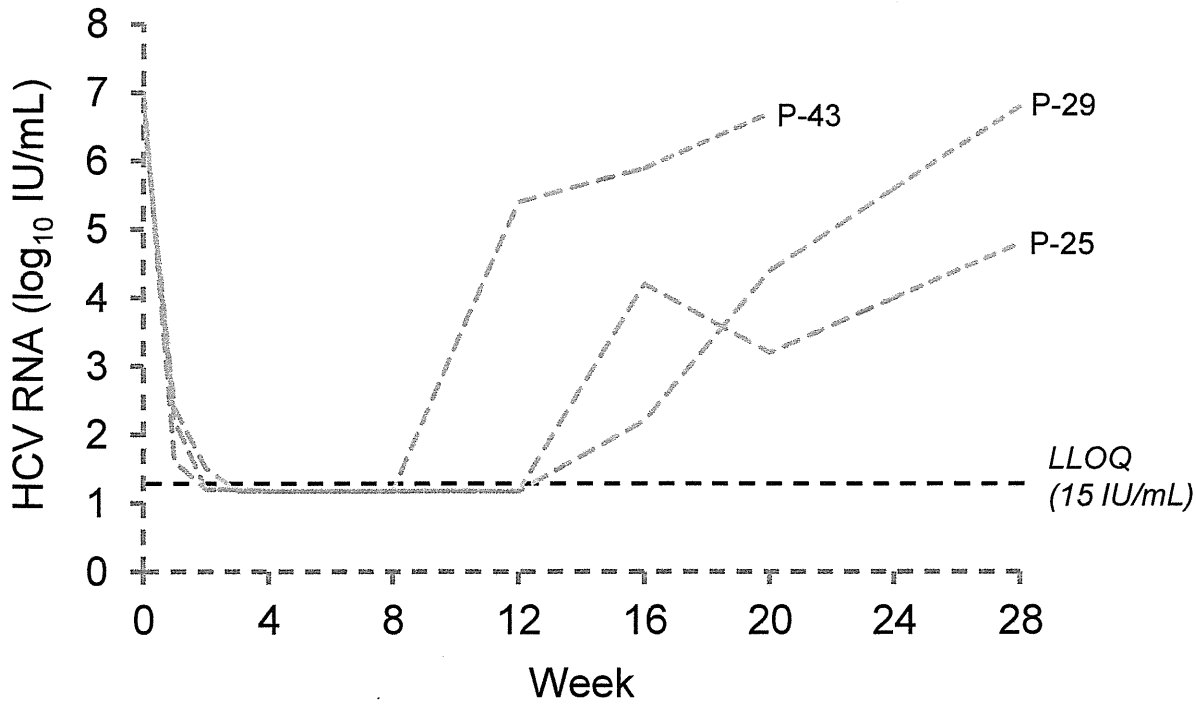

Number of patients
18
16
14
12
10
8
6
4
2
0
SVR
Non-SVR
None
R30
L31
Q54
Q54-Y93
Y93
None
T54
T54-Q80
Q80L
A156
D168
NS5A baseline polymorphisms
NS3 baseline polymorphisms

(B)

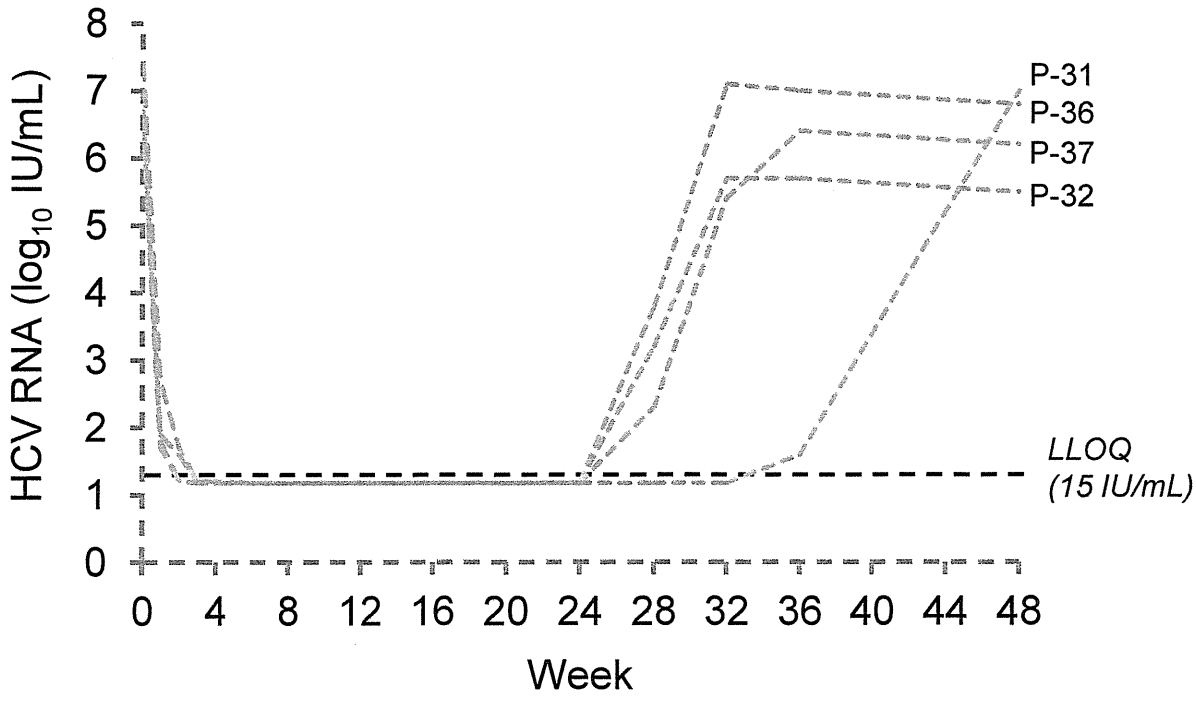

Number of patients
10
9
8
7
6
5
4
3
2
1
0
SVR
Non-SVR
None
R30
R30-Q54
L31
L31-Y93
Q54
Q54-Y93
Y93
None
T54
Q80
A156
D168
NS5A baseline polymorphisms
NS3 baseline polymorphisms

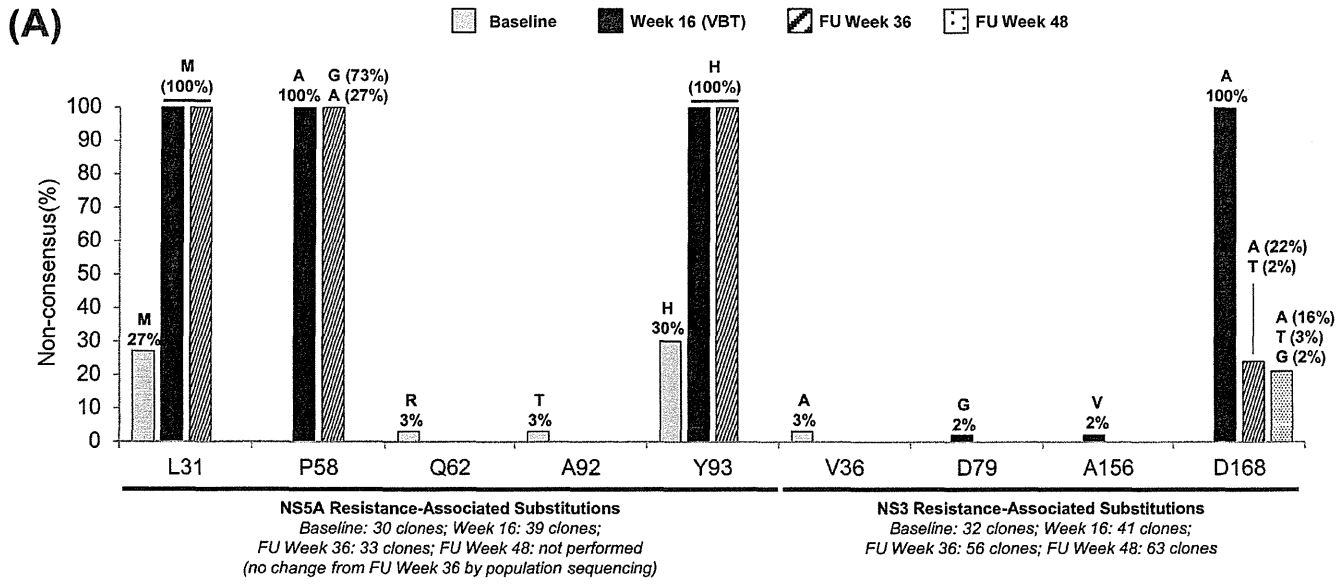

HCV RNA change from BL to Week 1 (log_{10} IU/mL)
7
6
5
4
3
2
1
0
N =
IL28B GT
RAVs
Response
5
CC
No
SVR
8
CC
Yes
SVR
3
CC
Yes/No
Relapse
4
CT
Yes
VBT/Relapse
2
CT
Yes
SVR

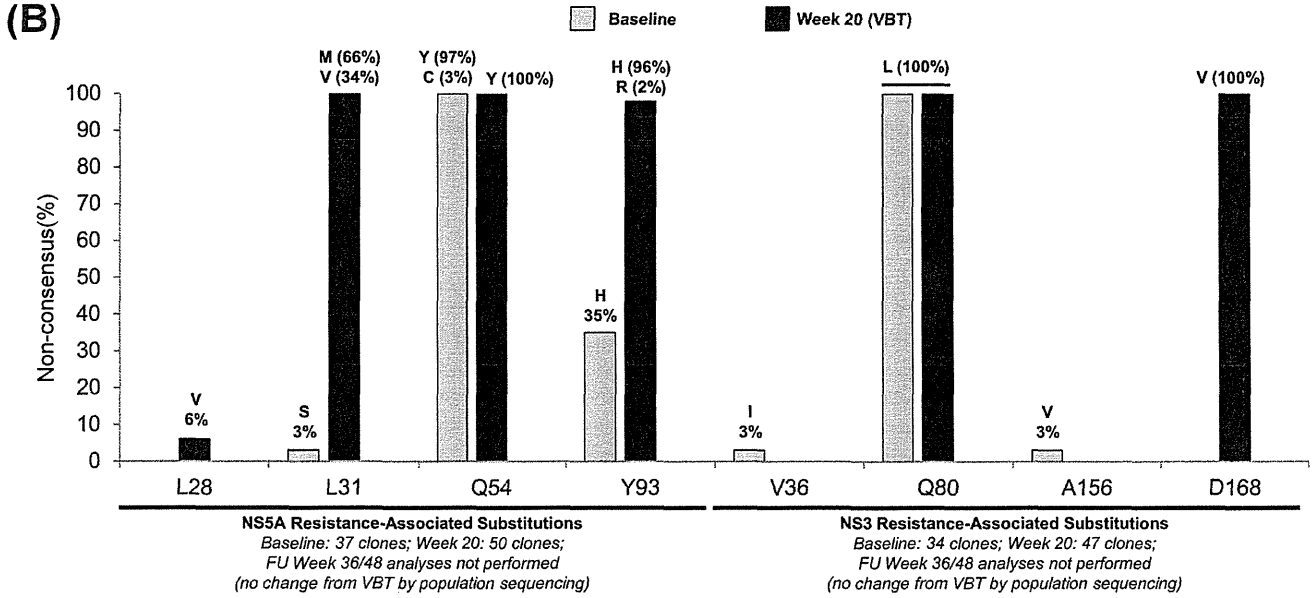
(A)

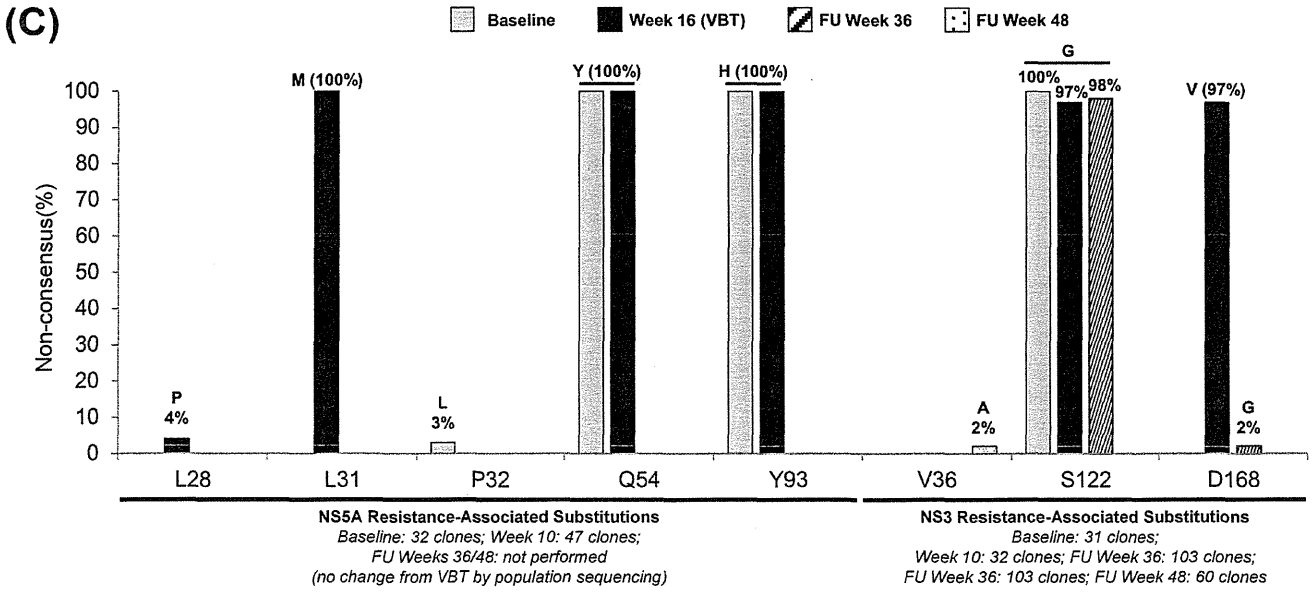

HCV RNA (log10 IU/mL)
8
7
6
5
4
3
2
1
0
P-43
P-29
P-25
LLOQ
(15 IU/mL)
0
4
8
12
16
20
24
28
Week

(B)


HCV RNA (log10 IU/mL)
8
7
6
5
4
3
2
1
0
P-31
P-36
P-37
P-32
LLOQ
(15 IU/mL)
0
4
8
12
16
20
24
28
32
36
40
44
48
Week


(A)
Baseline
Week 16 (VBT)
FU Week 36
FU Week 48
Non-consensus(%)
M (100%)
A 100%
G (73%)
A (27%)
H (100%)
A 100%
A (22%)
T (2%)
A (16%)
T (3%)
G (2%)
M 27%
R 3%
T 3%
H 30%
A 3%
G 2%
V 2%
L31
P58
Q62
A92
Y93
V36
D79
A156
D168
NS5A Resistance-Associated Substitutions
Baseline: 30 clones; Week 16: 39 clones;
FU Week 36: 33 clones; FU Week 48: not performed
(no change from FU Week 36 by population sequencing)
NS3 Resistance-Associated Substitutions
Baseline: 32 clones; Week 16: 41 clones;
FU Week 36: 56 clones; FU Week 48: 63 clones


(B)
Baseline
Week 20 (VBT)
Non-consensus(%)
M (66%)
V (34%)
Y (97%)
C (3%)
Y (100%)
H (96%)
R (2%)
L (100%)
V (100%)
V 6%
S 3%
H 35%
I 3%
V 3%
L28
L31
Q54
Y93
V36
Q80
A156
D168
NS5A Resistance-Associated Substitutions
Baseline: 37 clones; Week 20: 50 clones;
FU Week 36/48 analyses not performed
(no change from VBT by population sequencing)
NS3 Resistance-Associated Substitutions
Baseline: 34 clones; Week 20: 47 clones;
FU Week 36/48 analyses not performed
(no change from VBT by population sequencing)


(C)
Baseline
Week 16 (VBT)
FU Week 36
FU Week 48
Non-consensus(%)
M (100%)
Y (100%)
H (100%)
G
100%
97%
98%
V (97%)
P 4%
L 3%
A 2%
G 2%
L28
L31
P32
Q54
Y93
V36
S122
D168
NS5A Resistance-Associated Substitutions
Baseline: 32 clones; Week 10: 47 clones;
FU Weeks 36/48: not performed
(no change from VBT by population sequencing)
NS3 Resistance-Associated Substitutions
Baseline: 31 clones;
Week 10: 32 clones; FU Week 36: 103 clones;
FU Week 36: 103 clones; FU Week 48: 60 clones