

# Dynamic change in the NS3 protease domain in HIV/HCV-coinfected patients naïve to anti-HCV protease inhibitors

Sabrina Bagaglio<sup>1,2</sup>, Emanuela Messina<sup>1</sup>, Hamid Hasson<sup>1</sup>, Marco Merli<sup>1</sup>, Andrea Andolina<sup>1</sup>, Adriano Lazzarin<sup>1,2</sup>, Caterina Uberti-Foppa<sup>1</sup>, Giulia Morsica<sup>1</sup>

<sup>1</sup>Department of Infectious Diseases, Ospedale San Raffaele, Milan, Italy;

<sup>2</sup>Vita-Salute University, Milan, Italy

## SUMMARY

The genetic analysis of THE natural protease inhibitors (PI) resistance of HCV genotype (GT)1 involves subtypes 1a and 1b. NS3 protease domain was sequentially analysed in 10 HIV/HCV GT1-coinfected individuals naïve to HCV treatment. Analysis at different time points showed that 2/3 GT1b patients were infected by a GT1a clade1 during follow-up. In one patient a switch from clade1 to clade2 and in one other patient a switch from clade2 to clade1 was revealed. Four out of ten patients had resistance-associated substitutions (RASs) at baseline. The dynamics of the dominant infecting subtype suggests the presence of mixed infection in some patients.

Received June 13, 2016

Accepted November 6, 2016

HIV-infected patients benefit from the interferon (IFN)-free combination treatment with new anti-hepatitis C virus (HCV) direct-acting antivirals (DAAs) including 2nd generation protease inhibitors because these regimens are characterized by high efficacy, good tolerability, a low pill burden and a shorter treatment duration than IFN-based regimens including first generation linear PI.

Differences in the activities of NS3/4A protease inhibitors against different HCV-1 subtypes have been reported. A number of studies (Cento *et al.*, 2012; Sarrazin *et al.*, 2015) showed that a natural polymorphism Q80K is present in 5-40% of subtype 1a strains depending on geographical region and conferring natural resistance to macrocyclic anti-HCV PI. Recently, Pickett *et al.* (Pickett *et al.*, 2011) showed that HCV subtype 1a isolates can be separated into at least two distinct clades, designated 1 and 2 with different susceptibility to HCV-PI.

Baseline HCV NS3 amino acid (aa) substitution among PI treatment-naïve patients has been addressed in many countries (Morsica *et al.*, 2009; Bae *et al.*, 2010; Trimoulet *et al.*, 2011; Vallet *et al.*, 2011; Palanisamy *et al.*, 2013; Sarrazin *et al.*, 2015) showing discordant results. In this context, only case reports (Kim *et al.*, 2009; Colson *et al.*, 2011; Bagaglio *et al.*, 2013) are available on the natural mutational change in the NS3 protease over time in the absence of antiviral treatment.

We aimed to evaluate the temporal dynamics of the HCV NS3 protease domain in isolates from HIV/HCV (GT1)-coinfected individuals.

Ten HIV/HCV-coinfected patients were recruited among

HIV/HCV-coinfected individuals attending our Department of Infectious Diseases as outpatients [7 males and 3 females, aged 40 years, IQR 38-43 years (median, inter quartile range)] with confirmed diagnosis of HIV/HCV coinfection, all naïve to anti-HCV treatment, and selected on the basis of availability of at least 2 sequential plasma samples. The median interval between specimens was 30 months (range: 5-84 months).

These patients harbored HCV genotype 1a or 1b, assessed by standard methods (Inno-lipa, Innogenetics, Pomezia, Italy or direct sequence analysis of the 5'untranslated region, 5'UTR, when Innolipa gave an indeterminate result on subtype assignment).

The patients were part of a study on virological and immunological evaluation of HIV/HCV coinfection performed in the years 2003-2010. The study conformed to the ethical guidelines of the Declaration of Helsinki and the subjects gave written informed consent. Some of the data were generated as part of the routine laboratory and clinical work performed for standard care of outpatients. Among the risk factors, nine patients were ex-intravenous drug users (IVDU), one had sexual exposure.

Nine patients were on antiretroviral treatment (ART) at baseline, 5 of them had a PI-based regimen, 4 had a NN-RTI/NRTI-based regimen while one patient was not on ART. The median HIV-RNA load was 2.76 Log copies/mL (IQR 2.27-4.05 Log copies/mL), median CD4+ cells count was 598 cells/mm<sup>3</sup> (IQR 348-753 cells/mm<sup>3</sup>). The median HCV-RNA value was 6.08 Log IU/mL (IQR 5.81-6.19 Log IU/mL); median alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were 135 IU/L (IQR 107-160 IU/L) and 108 IU/L (IQR 85-156 IU/L) respectively. Baseline and sequential plasma samples were obtained from these 10 HIV/HCV-coinfected patients, with a total of 24 specimens analysed.

HCV-RNA extraction (QIAamp Viral RNA mini kit, QIAGEN, Milan, Italy), RT-nested PCR and population sequencing of NS3 protease gene were performed as pre-

### Key words:

NS3 protease inhibitors, HIV/HCV coinfection, RAS.

### Corresponding author:

Bagaglio Sabrina

E-mail: bagaglio.sabrina@hsr.it

viously described (Lodrini *et al.*, 2003). Briefly, the complete protease domain (181 aa) was amplified by means of an RT-nested PCR using selected primers specific for GT1a and GT1b subtypes (Lodrini *et al.*, 2003). The PCR products (543 base pairs) obtained from the NS3 protease gene were purified using a micro column system (Amicon, Millipore, Bedford, MA, USA). Primers used for the second round of the nested PCR were employed for direct sequencing by means of an ABI 3730 XL (Applied Biosystems, Life Technologies, Italy).

PCR fragments were sequenced bi-directionally and only mutations present in both strands were considered.

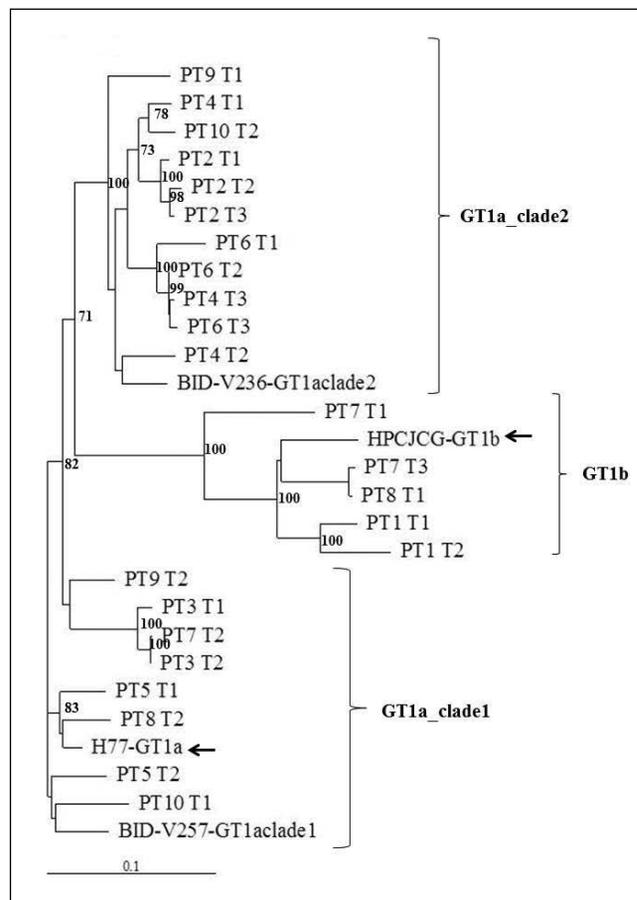
The multiple alignment of the nucleotide sequences was inferred by Clustal X version 1.6.4. A maximum likelihood phylogenetic rooted tree containing the isolates obtained at each time point from HIV/HCV-coinfected patients was constructed. The nucleotide distances were calculated by generating a distance matrix using the DNADIST program (PHYLIP 3.5c package) maximum-likelihood model. A phylogenetic tree was then constructed using NEIGHBOR (PHYLIP) with random addition, and drawn using TreeViewPPC version 1.5.3. Bootstrap analysis was performed using SEQBOOT (100 resampling) in order to place approximate confidence limits on individual nodes. A bootstrap value  $\geq 70$  was indicated in *Figure 1*. To define the infecting genotype were added to phylogenetic tree GT1a (GenBank accession number AF009606, isolate H77) and GT1b (GenBank accession number D90208, isolate HPCJCG) prototype sequences.

To identify different clades within GT1a were included in phylogenetic analysis isolates belonging to GT1a clade1 (accession number EU155237, isolate BID-V257) and isolates identified as GT1a clade2 (accession number EU155346, isolate BID-V236) by Pickett *et al.* (Pickett *et al.*, 2011). Sequences from baseline (T1) and from follow-up (T2, T3) plasma samples were submitted to GenBank under accession numbers KM592011, KM591988, KM592002, KM591971, KM591972, KM591999, KM592028, KM591996, KM591983, KM592007 and KT934335-KM954348. Distinct clades were identified by phylogenetic analysis and PI resistance pattern was evaluated by amino acid sequences alignment. HCV genotype, GT1a clades and RASs were confirmed by Geno2Pheno algorithm (<http://hcv.geno2pheno.org>).

Drug resistance to NS3 inhibitors in clinical use, simprevir (SMV), asunaprevir (ASV), paritaprevir (PTV) and grazoprevir (GZV) were considered. On the basis of the results of clinical studies, the following were identified to be NS3 resistance-associated amino acid positions: positions 36, 56, 122, 80, 155 and 168 in GT1a; 155, 156, and 168 in GT1b for SMV, ASV and PTV. On the basis of the results obtained "in vitro" for GZV, the resistance-associated amino acid positions were 168 in GT1a; 155, 156 and 168 in GT1b. Interpretation of fold-change resistance to NS3 candidate drugs were done from already published phenotypic resistance data.

Phylogenetic analysis of the protease domain at baseline showed that 3 patients were infected by GT1b, 3 other patients were infected by GT1a clade1 and the remaining 4 individuals by GT1a clade2. Follow-up examination showed that the same HCV GT as that found at baseline was detected in six patients: 5 of them harbored GT1a and the remaining patient harbored GT1b (*Figure 1*). Interestingly, 2/3 patients exhibiting GT1b at baseline, were infected by GT1a clade1 at the follow-up evaluation.

In addition, a switch from GT1a clade 1 to clade 2 or vice versa was revealed in follow-up samples of two patients infected with HCV GT1a at baseline (*Figure 1*). Sequence analysis showed that of 6 individuals harboring a strain without RASs at baseline, 4 patients (PT1, PT2, PT3, PT8) maintained the PI susceptible virus during the follow-up evaluation; 2 patients (PT4 and PT5) developed RAS (R155K and Q80K respectively, that confer 10-100 fold-change resistance to PIs). The presence of RASs was revealed at baseline in 4/10 (40%) HIV/HCV coinfecting patients (*Table 1*). In all these patients these natural baseline RASs were lost in follow-up samples. Of note, 3 patients



**Figure 1** - The maximum-likelihood phylogenetic rooted tree of hepatitis C virus subtypes 1a and 1b from NS3 protease sequences showing relationships between strains obtained at different time points in HIV/HCV-coinfected individuals. The "PT" code followed from a number identified each patient and T1, T2 and T3 indicate different time points. Sequences from the study of Pickett *et al.* (Pickett *et al.*, 2011), clustering with GT1a clade1 isolates and GT1a clade2 are indicated as following: GT1a clade1 isolate BID-V457 identified by GenBank accession numbers: EU155237 and GT1a clade2 isolate BID-V236 identified by GenBank accession numbers: EU155346. Prototype sequence of Genotype 1a (GenBank accession number AF009606, isolate H77) and prototype sequence of Genotype 1b (GenBank accession number D90208, isolate HPCJCG) are indicated by a black arrow. Evolutionary distance units are indicated in the scale. Bootstrap analysis was performed using SEQBOOT (100 re-sampling) in order to place approximate confidence limits on individual nodes. A bootstrap value  $\geq 70$  was indicated.

**Table 1** - Dynamic of HCV infecting genotype and PI resistant variants in HIV/HCV coinfecting patients.

	T1		T2		T3	
	Genotype	RAVs	Genotype	RAVs	Genotype	RAVs
PT1	1b	-	1b	-	NA	NA
PT2	1a clade 2	-	1a clade 2	-	1a clade 2	-
PT3	1a clade 1	-	1a clade 1	-	NA	NA
PT4	1a clade 2	-	1a clade 2	-	1a clade 2	R155K
PT5	1a clade 1	-	1a clade 1	Q80K	NA	NA
PT6	1a clade 2	R155K	1a clade 2	-	1a clade 2	-
PT7	1b	A156T+D168V	1a clade 1	-	1b	-
PT8	1b	-	1a clade 1	-	NA	NA
PT9	1a clade 2	Q80E	1a clade 1	-	NA	NA
PT10	1a clade 1	Q80K	1a clade 2	-	NA	NA

PT = patient, - = wild type virus, NA = plasma sample not available. HCV subtype or clade switch is in bold.

(PT7, PT9 and PT10) showed a peculiar pattern of mutations; PT7 was infected by HCV GT1b at baseline that harbored A156 + D168 substitutions; six months later (T2) this patient had a GT1a subtype without RASs and 1 year later (T3) had GT1b without RASs (Table 1).

PT9, infected by GT1a clade2 had Q80E mutant at baseline that disappeared at follow-up evaluation with concomitant subtype change (GT1b, Table 1). PT10 had GT1a clade1 at baseline with Q80K substitution that changed to a GT1a clade 2 without RASs at the follow-up evaluation. The emergence of a different genotype from that detected at baseline in these 3 HIV/HCV coinfecting patients, suggests a mixed infection with dominance of one virus over the other. The dominance of one genotype in mixed infection must be due to host and/or virus adaptation (i.e. change of host's environmental condition, different fitness of the virus). Since in two cases (PT9, PT10) information on risk factors during follow-up was not available, reinfection with another virus could not be fully excluded. In patient 7 "de novo" infection with a different genotype (GT1a) was unlikely because the patient denied unprotected sexual intercourse and active use of illicit drugs.

One limitation of the present study is that mixed infection was not evaluated by clonal analysis or deep sequencing, at least at baseline. However, 40% of HIV/HCV-coinfecting patients had a dynamic change in infecting genotype (that suggests a mixed infection) and this finding is in line with that of our previous study that proved by deep sequencing the presence of mixed genotypes in 58% of HIV/HCV-coinfecting individuals (Bagaglio *et al.*, 2015).

In conclusion, we showed evidence of dynamic changes in GT and natural RASs in HIV/HCV-coinfecting patients with disappearance/reappearance of RASs over time in some patients.

The dynamic pattern of RASs could be due to a mixed infection with a different virus. Whether mixed infection may influence the response to PI-based DAAs remains to be established.

#### Disclosure of Conflict of Interest

A.L. has acted as a consultant or participated in advisory board as a speaker or in the conduct of clinical trials for

Abbott, BMS, Gilead, MSD, ViiV Health, Pfizer, AbbVie All other authors: none to declare.

#### References

- Bae A., Sun S.C., Qi X., Chen X., Ku K., Worth A., et al. (2010). Susceptibility of treatment-naïve hepatitis C virus (HCV) clinical isolates to HCV protease inhibitors. *Antimicrob Agents Chemother.* **54**, 5288-5297.
- Bagaglio S., Uberti-Foppa C., Di Serio C., Trentini F., Andolina A., Hasson H., et al. (2015). Dynamic of Mixed HCV Infection in Plasma and PBMC of HIV/HCV Patients under Treatment with Peg-IFN/Ribavirin. *Medicine.* **94**, e1876.
- Bagaglio S., Messina E., Uberti-Foppa C., Merli M., Torre L.D., Lazzarin A., et al. (2013). Reversion of naturally occurring high-level resistance mutations to NS3 protease inhibitors in two treatment-naïve individuals infected with hepatitis C virus. *J Antimicrob Chemother.* **68**, 1448-1450.
- Cento V., Mirabelli C., Salpini R., Dimonte S., Artese A., Costa G., et al. (2012) HCV genotypes are differently prone to the development of resistance to linear and macrocyclic protease inhibitors. *PLoS One.* **7**, e39652.
- Colson P., Gérolami R. (2011). Two years' persistence of naturally present substitution R155K within hepatitis C virus NS3 protease in the absence of protease inhibitor-based therapy. *J Infect Dis.* **203**, 1341-1342.
- Kim A.Y., Timm J., Nolan B.E., Reyor L.L., Kane K., Berical A.C., et al. (2009). Temporal dynamics of a predominant protease inhibitor-resistance mutation in a treatment-naïve, hepatitis C virus-infected individual. *J Infect Dis.* **199**, 737-741.
- Lodrin S., Bagaglio S., Canducci F., De Mitri M.S., Andreone P., Loggi E., et al. (2003). Sequence analysis of NS3 protease gene in clinical strains of hepatitis C virus. *J Biol Regul Homeost Agents.* **17**, 198-204.
- Morsica G., Bagaglio S., Uberti-Foppa C., Galli L., Lazzarin A. (2009). Detection of hepatitis C mutants with natural resistance to NS3/4A protease inhibitors in HIV/HCV coinfecting individuals treated with antiretroviral therapy. *J Acquir Immune Defic Syndr.* **51**, 106-108.
- Palanisamy N., Danielsson A., Kokkula C., Yin H., Bondeson K., Wesslén L. et al. (2013). Implications of baseline polymorphisms for potential resistance to NS3 protease inhibitors in Hepatitis C virus genotypes 1a, 2b and 3a. *Antiviral Res.* **99**, 12-17.
- Pickett B.E., Striker R., Lefkowitz E.J. (2011). Evidence for separation of HCV subtype 1a into two distinct clades. *Journal of Viral Hepatitis.* **18**, 608-618.
- Sarrazin C., Lathouwers E., Peeters M., Daems B., Buelens A., Wittek J., et al. (2015). Prevalence of the hepatitis C virus NS3 polymorphism Q80K in genotype 1 patients in the European region. *Antiviral Res.* **116**, 10-16.
- Trimoulet P., Belzunce C., Faure M., Wittkop L., Reigadas S., Dupon M., et al. (2011). Hepatitis C virus (HCV) protease variability and anti-HCV protease inhibitor resistance in HIV/HCV coinfecting patients. *HIV Med.* **12**, 506-509.
- Vallet S., Viron F., Henquell C., Le Guillou-Guillemette H., Lagathu G., Abravanel F., et al.; ANRS AC11 HCV Group. (2011). NS3 protease polymorphism and natural resistance to protease inhibitors in French patients infected with HCV genotypes 1-5. *Antivir Ther.* **16**, 1093-1102.