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1 **Net Emergence of Substitutions at Position 28 on NS5A of Hepatitis C Virus Genotype 4 in**
2 **Patients Failing Direct-Acting Antivirals by Next-Generation Sequencing**

3 **Running title: Emergence of Substitutions at Position 28 on NS5A in HCV Genotype 4**

4 Thuy NGUYEN^{1*}, Sepideh AKHAVAN², Fabienne CABY³, Luminita BONYHAY⁴, Lucile LARROUY⁵,
5 Anne GERVAIS⁶, Pascal LEBRAY⁴, Thierry POYNARD⁷, Yvon CALMUS⁸, Anne SIMON⁹, Marc-
6 Antoine VALANTIN³, Vincent CALVEZ¹, Anne-Geneviève MARCELIN¹, Eve TODESCO¹

7 ¹ Sorbonne Université, INSERM, Institut Pierre Louis d'Epidémiologie et de Santé Publique
8 (iPLESP), AP-HP, Hôpital Pitié-Salpêtrière, Laboratoire de virologie, F-75013 Paris, France.

9 ² Sorbonne Université, INSERM, CIMI-Paris, AP-HP, Hôpital Pitié-Salpêtrière, Laboratoire de
10 virologie, F-75013 Paris, France.

11 ³ Sorbonne Université, INSERM, Institut Pierre Louis d'Epidémiologie et de Santé Publique
12 (iPLESP), AP-HP, Hôpital Pitié-Salpêtrière, Services de maladies infectieuses et tropicales, F-
13 75013 Paris, France.

14 ⁴ AP-HP, Hôpital Pitié-Salpêtrière, Service d'Hépatogastroentérologie, F-75013 Paris, France.

15 ⁵ IAME, UMR 1137, INSERM, Université Paris Diderot, Sorbonne Paris Cité, AP-HP, Hôpital
16 Bichat Claude Bernard, Laboratoire de Virologie, F-75018 Paris, France.

17 ⁶ AP-HP, Hôpital Bichat Claude Bernard, Service des maladies infectieuses et tropicales, F-
18 75018 Paris, France.

19 ⁷ Sorbonne Université, INSERM, Institute of Cardiometabolism and Nutrition (ICAN), APHP,
20 Groupe Hospitalier Pitié Salpêtrière, F-75013 Paris, France.

21 ⁸ APHP, Hôpital Pitié-Salpêtrière, Unité Médicale de Transplantation Hépatique, Hépatogastro-
22 Gastro-Entérologie, F-75013 Paris, France.

23 ⁹ AP-HP, Hôpital Pitié-Salpêtrière, Département de Médecine Interne, F-75013 Paris, France.

24

25 ***Corresponding author:**

26 Thuy Nguyen

27 Service de virologie, Bât CERVI, Hôpital Pitié-Salpêtrière, 83 Bd de l'Hôpital, 75013 Paris

28 Email: thithu-thuy.nguyen@aphp.fr

29 Fax: 33 1 42 17 74 11 ; Phone: 33 1 42 17 58 42

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34 **ABSTRACT (250 words)**

35 **Background:** More data on resistance of HCV genotype (GT) 3 and 4 to Direct-Acting
36 Antivirals (DAAs) are still needed. We investigated presence of Resistance-Associated
37 Substitutions (RASs) pre- and post-treatment and their emergence under DAAs in HCV GT3
38 and GT4 infected patients failing DAA regimens by next-generation sequencing (NGS).

39 **Methods:** Sanger sequencing and NGS were performed on NS5B and NS5A for plasma
40 samples prior- and post-treatment of 13 patients. Positions implicated in resistance to anti-
41 NS5A and anti-NS5B in literature were analysed.

42 **Results:** No baseline RASs was detected on NS5B but one GT4r virus developed the mutation
43 S282T at failure. On NS5A, we detected pre-existing RASs or polymorphisms in viruses of
44 6/10 patients (L28M for a GT4a, M28V for a GT4r, L30R for a GT4a, 2 GT4d ,and 1 GT4r, T58P
45 for a GT4d) by Sanger sequencing and in viruses of 7/10 patients by NGS. Additional baseline
46 minority substitutions detected by NGS were Y93H in a GT3a, L28M in a GT4a and a GT4d,
47 and L28F in a GT4d virus. At failure, these substitutions were found at frequency of 100%.
48 The Y93H was detected alone at baseline while the L28M and L28F were accompanied by
49 polymorphisms L30R or L30R + T58P.

50 **Conclusions:** The use of NGS in patients failing DAAs and infected by HCV GT3 and GT4
51 revealed the emergence of specific patterns of substitutions on NS5A and NS5B, in particular
52 substitutions at position 28 on NS5A in GT4 virus, highlighting the need to list these
53 substitutions in guidelines for resistance interpretation.

54 **Keywords:** Direct-acting antivirals, resistance-associated substitutions, substitutions at
55 position 28, next generation sequencing, HCV genotype 3 and 4

56

57

58 INTRODUCTION

59 Hepatitis C virus (HCV) is a leading cause of morbidity and deaths worldwide. Despite the
60 considerable reduction in number of HCV infections recently, there are still approximately
61 399 000 deaths each year largely due to hepatitis C-related liver diseases [1]. Remarkable
62 advances have been made in treatment of HCV infection, notably with the introduction of
63 direct-acting antivirals (DAAs). However, as a highly variable virus with many quasispecies,
64 HCV can select *in vitro* and *in vivo* Resistance-Associated Substitutions (RASs) to antivirals in
65 particular to anti-NS5A owing to their low genetic barrier [2]. Several studies have been
66 performed to study prevalence of RASs at baseline, their emergence under anti-NS5A
67 treatment but mainly in HCV genotype (GT) 1 infected patients. RASs have been shown to be
68 present at 10-15% in HCV GT1 prior to treatment and negatively impact response to anti-
69 NS5A containing regimen [3]. In addition, GT3 and non-a-GT4 especially GT4r are well known
70 as hard-to-treat genotypes in particular due to some pre-existing polymorphisms associated
71 with resistance in cases of GT4r infection [4,5]. However, more data on resistance of HCV
72 GT3 and 4 to DAAs, especially on pre-existing RASs at low frequency and their impact on
73 NS5A and NS5B inhibitors, are still needed. In real life, the failure rate under GT3 and GT4-
74 specific DAA treatment fluctuates in a range of 3%-5% in treatment-naïve patients without
75 cirrhosis but could be higher up to 10% in treatment-experienced and cirrhotic patients
76 [6,7].

77 Furthermore, studies often utilized Sanger sequencing and thus possibly underestimated
78 RASs prevalence. Next-generation sequencing (NGS) allows detection of minority RASs
79 representing less than 20% of viral population which are under detection threshold of
80 Sanger sequencing. Therefore, in this study, we evaluated in patients failing anti-NS5B with

81 or without anti-NS5A regimens the prevalence of RASs at baseline and at failure, and the
82 emergence dynamics of pre-existing minority RASs by NGS in a context of HCV GT3 and GT4
83 infection.

84 **STUDY DESIGN AND METHODS**

85 ***Study design***

86 Available samples prior and post-treatment of 13 HCV patients failing an anti-NS5B
87 (Sofosbuvir (SOF)) with or without anti-NS5A regimen (Ledipasvir (LDV) or Daclatasvir (DCV))
88 were collected at the Pitié-Salpêtrière (n = 12) and the Bichat (n = 1) hospitals, Paris, France
89 between January 2014 and June 2016.

90 Patients were defined as failing HCV therapies if they met the following criteria: 1) They had
91 a detectable viral load within 6 months after completing treatment; 2) they harboured the
92 same consensus viral sequence at baseline and at failure; and 3) they had no evidence of
93 reinfection from the same viral sources.

94 All patients gave their written informed consent to release the data in their electronic
95 medical records and for using their samples in the conduct of clinical research.

96 ***Methods***

97 ***RNA extraction, polymerase chain reaction and subtyping***

98 Sanger sequencing and NGS were performed on samples at baseline and at DAA failure on
99 NS5A (10 subjects) and NS5B (13 subjects) depending on the DAAs received (SOF ± anti-NS5A
100 ± RBV). Briefly, 80 µl of HCV RNA were extracted from 1 ml of plasma using NucliSENS®
101 easyMAG® (bioMérieux Clinical Diagnostics). The extracted RNA underwent reverse
102 transcription to produce complementary DNA, and NS5B fragment was amplified by
103 polymerase chain reaction in a one-step process (Superscript III One-step RT-PCR with

104 platinum Taq kit; Invitrogen, Carlsbad, CA, USA) according to the manufacturers' protocol
105 and sequenced by Sanger method (BigDye Terminator, Applied Biosystems, Foster City,
106 USA). We determined HCV subtypes by constructing phylogenetic trees with reference
107 sequences cited in the article of Smith DB *et al* [8] using neighbour joining method in Clustal
108 W (version 2.0 available at <http://www.clustal.org/clustal2/>). We used also
109 *geno2pheno[HCV]* (<http://hcv.bioinf.mpi-inf.mpg.de>) to verify viral subtype of each sample
110 from NS5B sequences [9]. For the resistance tests, NS5A (amino acid 1 to 143) and NS5B
111 (amino acid 117 to 565) fragments were amplified using Superscript III kit as described above
112 using genotype-specific primers following specific protocols (supplementary tables S3 and
113 S4).

114 ***Next-generation sequencing (NGS) and computational method***

115 The NS5A and NS5B amplicons were sequenced using Illumina MiSeq platform. Samples
116 were purified by SPRIselect beads (Beckman Coulter, France), then "tagmented"
117 (fragmented and tagged), and prepared for libraries using Nextera[®] DNA Sample Preparation
118 and Index Kit (Illumina, USA) according to the manufacturer's protocol. Resulting libraries
119 were quantified on a 2100 Bioanalyzer (Agilent Technologies), normalized, and pooled
120 equimolarly. Pooling libraries were diluted to 10 pM for cluster generation and subjected to
121 standard Illumina paired-end sequencing at 2 x 150 bp on Miseq platform.

122 The commercial and fully automated SmartGene IDNS ASP service (www.smartgene.com,
123 SmartGene, Zug, Switzerland) was used to analyse NGS data. Positions considered in
124 literature to be implicated in resistance to DAAs therapies such as those mentioned in
125 *geno2pheno* rules (updated February 2017) [9], Pawlotsky JM's review [10], and Sorbo *et al.*
126 [11] study were analysed. Briefly, positions 159, 282, 237, 289, 320, and 321 were taken into
127 account for resistance interpretation to SOF; positions 24, 26, 28, 29, 30, 31, 32, 38, 58, 62,

128 92, and 93 were considered for resistance interpretation to anti-NS5A. Amino acid
129 substitutions in other positions were also analysed and shown if there was emergence of
130 these substitutions from pre- to post-treatment.

131 References used for resistance interpretation are listed in supplementary table 5.

132 ***Fibrosis evaluation***

133 Fibrosis stage was assessed using fibrosis biomarker (FibroTest®) [12] and elastography
134 (Fibroscan®) [13].

135 ***Error rate control***

136 Synthetized double stranded DNA of the same NS5A gene fragment of isolate H77 from
137 MWG-Biotech (Eurofins, France) was also amplified and sequenced with the same procedure
138 as a control for error rate.

139 **RESULTS**

140 ***Patients' characteristics***

141 Ninety two percent of patients were male with a median age of 56 years (IQR = 51-60). The
142 median HCV viral load was 5.8 log IU/mL (IQR = 5.5-6.6) at baseline and 6.3 log IU/mL (IQR =
143 5.6-6.5) at failure. Five patients (38%) were cirrhotic (F4 Metavir). Seven patients (54%) were
144 co-infected with HIV. Patients were infected with HCV GT3a (4/13, 31%), GT4a (3/13, 23%),
145 GT4d (5/13; 38%), and GT4r (1/13, 8%). For HCV treatment history, 8 patients (61.5%) were
146 HCV treatment-naïve and 5 (38.5%) were previously treated by pegylated interferon alfa
147 (PEG-IFN) and ribavirine (RBV). Regarding DAA therapies, 10 patients (78%) received
148 sofosbuvir (SOF) + NS5A inhibitors ± RBV (3 patients treated by daclatasvir (DCV) and 7 by
149 ledipasvir (LDV)) and 3 (18%) received SOF + RBV ± PEG-IFN. Virological failure occurred after
150 a median time of 4 months following the end of DAA treatment.

151 ***Reads coverage and error rate control for NGS***

152 A median of 77163 reads per amplicon (IQR: 66563-90018) was obtained. The mean \pm SD of
153 error rate per bp (%) realized on synthesized dsDNA was 0.02 ± 0.13 . Therefore, a cut-off of
154 1% was used for calling mutations and interpreting drug resistance.

155 ***Prevalence of pre-existing substitutions at baseline***

156 No baseline RASs was detected on NS5B gene for all 13 patients. On NS5A gene, we detected
157 pre-existing RASs or polymorphisms in viruses of 6/10 patients (L28M for a GT4a, M28V for a
158 GT4r, L30R for a GT4a, 2 GT4d ,and 1 GT4r, T58P for a GT4d) by Sanger sequencing and in
159 viruses of 7/10 patients by NGS. Additional baseline minority substitutions detected only by
160 NGS were Y93H (at frequency of 1%) in a GT3a virus, L28M in a GT4a and a GT4d virus (at
161 1.8% and at 1.1%, respectively), and L28F (at 1%) in a GT4d virus. Y93H was detected alone
162 at baseline while L28M or L28F were accompanied by naturally occurring polymorphisms,
163 known to be implicated in anti-NS5A resistance, such as L30R and T58P.

164 Another polymorphism associated with GT4 virus-specific resistance was also detected by
165 both techniques at baseline, such as the substitution at codon 30 (L30R) in 1/3 GT4a and in
166 3/3 GT4d and 1/1 GT4r viruses.

167 Details of substitutions detected and patients' clinical information are shown in
168 supplementary tables S1 and S2.

169 ***Prevalence and emergence of RASs at failure***

170 For the NS5B region, we identified by Sanger sequencing and NGS the RAS S282T (at 98.8%)
171 in plasma of one GT4r virus. This mutation was not detected at baseline even in minority.
172 Interestingly, by NGS, we detected the emergence of pre-existing substitutions, which have
173 not yet known to be associated with resistance against anti-NS5B, in viruses of 2 patients
174 (N300S in one GT4a and K270R + I523M in one GT4d virus). Furthermore at failure, the GT4d
175 virus with K270R and I523M on NS5B also acquired Y93H on NS5A.

176 For the NS5A region, both NGS and Sanger sequencing detected presence of RASs or
177 polymorphisms implicated in resistance in viruses of 8/10 patients (two GT3a and six GT4).
178 Viruses for two of them (one GT3a and one GT4a) carried the Y93H at failure in majority
179 which were not detected at baseline even by NGS. Interestingly, the emergence of baseline
180 pre-existing minority substitutions on NS5A was found in viruses of four patients. The RAS
181 Y93H emerged from 1% at baseline to 98.7 % at failure in one HCV GT3a infected patient
182 treated by SOF + DCV + RBV. In three other patients infected by a GT4 virus and treated by
183 SOF + LDV, we detected the emergence from around 1% (1%-1.8%) at baseline up to nearly
184 100% (88%-100%) at failure of three substitutions at position 28 (L28M and L28F) which
185 were accompanied by polymorphisms L30R in two patients and L30R + T58P in the third
186 patient.

187 The pre-existing polymorphism such as the substitution at codon 30 (L30R) in 1/3 GT4a and
188 in 3/3 GT4d and 1/1 GT4r viruses was also detected at failure by both techniques.

189 Details of substitutions detected and patients' clinical information are shown in
190 supplementary tables S1 and S2.

191 **DISCUSSION**

192 In this study, we aimed to study RASs to DAAs in patients infected with HCV GT3 or 4 by NGS
193 at a detection threshold of 1% which is more sensitive than that used in other clinical studies
194 [10]. This threshold allowed a better characterization of mutant dynamics before and after
195 DAA failure and strong dynamics of RAS emergence could somehow reflect their potential
196 impact on DAA response. In accordance with other studies about RASs on NS5B region, we
197 detected at failure a low rate (1/13 patients) of RASs (S282T) to NS5B inhibitors or precisely
198 to SOF. No RASs, including minority substitutions, were detected at baseline even by UDS.
199 Similarly, a study on 1459 sequences (91% from DAA-naïve patients) extracted from the

200 Nucleotide NCBI database by Chen *et al.* reported a low global prevalence of RASs to SOF
201 (3.9%) or lower if considering the S282T (occurred in one sequence) [14]. A more recently
202 published study by Gane *et al.* reported that in 8598 patients, no S282T substitution was
203 detected at baseline and only 10 of 901 (1%) patients had the S282T detected at virological
204 failure [15]. In our study, mutations which have never been described in resistance to NS5B
205 inhibitors were also analysed. We detected the emergence of mutants such as N300S in one
206 GT4a and K270R + I523M in one GT4d virus at failure under SOF containing regimen.
207 Importantly, the K270R substitution has been previously described to be selected together
208 with other mutants in HCV GT1b-replicon cells in an *in vitro* study [16]. Hence, the impact of
209 these substitutions on response to NS5B inhibitors should be further investigated by
210 mutagenesis or on a larger group of patients.

211 Emergence of pre-existing minority substitutions on NS5A was detected in viruses of 4/10
212 patients such as Y93H, which is known to confer medium to high level of resistance to NS5A
213 inhibitors, in one GT3a virus and substitutions at position 28 (L28M, L28F) in three GT4
214 viruses. Furthermore, emergence of substitutions L28M and L28F were accompanied by
215 baseline polymorphisms L30R alone or by L30R + T58P in three HCV GT4-infected patients
216 treated by SOF + LDV. To our knowledge, few studies have evidenced emergence and impact
217 of substitutions at position 28 with resistance to LDV in GT4 virus. A study by Dietz J *et al.* on
218 18 patients infected with HCV GT4 and failing LDV/SOF regimen showed that RASs L28M/V
219 became prominent in 39% of patients [17]. Furthermore, an *in vitro* study demonstrated that
220 L28M + L30R could confer a 350-fold decrease in DCV susceptibility in GT4 virus. In the same
221 study, DCV was shown to be 45 fold more potent than LDV against L28M and 15 fold more
222 potent against L30R [18]. Last but not least, the impact of substitutions at position 28 on LDV
223 response has been well described for GT1 virus [19] and substitution at this position could

224 even impact newly approved DAA molecules such as pibrentasvir [20]. Although the new
225 substitution L28F was not tested in phenotypic *in vitro* model in this study, our finding gives
226 a perspective for further investigations of this substitution by mutagenesis and highlights the
227 need to list substitutions at codon 28 of NS5A protein in resistance interpretation guidelines
228 for GT4 virus.

229 One of the limitations in our study is the low number of patients enrolled on one hand
230 because of high and increasing SVR rate under DAA therapy and on the other hand because
231 of amplification failure or absence of samples at baseline. In addition, the investigated group
232 is heterogeneous making it difficult to draw a robust conclusion for a specific genotype or a
233 specific profile of patients. As the control group of patients responding to DAA therapy was
234 not investigated in parallel, no inference about the clinical significance of baseline minority
235 RASs on treatment response has been conclusively established. However, this study using a
236 highly sensitive technique (NGS) revealed the emergence of specific patterns of substitutions
237 on NS5A and NS5B in patients infected by HCV GT3 and 4 and failing a DAA regimen. This
238 finding proves the usefulness of NGS compared to Sanger sequencing in detecting minority
239 pre-existing RASs which could emerge under treatment pressure and impact the treatment
240 outcome. RASs screening by NGS might not be beneficial enough in first-line therapy thanks
241 to the high SVR rate of DAA treatment. However, in the case of failure on previous DAA
242 regimens, RASs in particular on NS5A gene could be accumulated under treatment pressure
243 and gradually disappear after the end of treatment. NGS, when feasible and more accessible
244 in clinical practice, should be performed right before retreatment by new DAA regimens
245 especially if a same class of DAAs is considered, to choose an optimized strategy of
246 retreatment.

247

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254

255 **DECLARATIONS**

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258 **Competing Interests :** None to declare

259 **Ethical Approval :** Not required

260

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