



HAL
open science

Mixing particles from various HCV genotypes increases the HBV-HCV vaccine ability to elicit broadly cross-neutralizing antibodies

Elodie Beaumont, Benoit Joël Clément, Vanessa Guérin, Lucie Chopin, Emmanuelle Roch, Elsa Gomez-Escobar, Philippe Roingard

► To cite this version:

Elodie Beaumont, Benoit Joël Clément, Vanessa Guérin, Lucie Chopin, Emmanuelle Roch, et al.. Mixing particles from various HCV genotypes increases the HBV-HCV vaccine ability to elicit broadly cross-neutralizing antibodies. *Liver International*, 2020, 40 (8), pp.1865-1871. 10.1111/liv.14541 . hal-04112891

HAL Id: hal-04112891

<https://univ-tours.hal.science/hal-04112891>

Submitted on 1 Jun 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1

2 **Mixing particles from various HCV genotypes increases the HBV-HCV**
3 **vaccine ability to elicit broadly cross-neutralizing antibodies**

4

5 **Elodie Beaumont, Benoit Joël Clément, Vanessa Guérin, Lucie Chopin, Emmanuelle**
6 **Roch, Elsa Gomez-Escobar, and Philippe Roingear**

7

8 INSERM U1259, Université de Tours and CHRU de Tours, Tours, France

9

10 Correspondence: Philippe Roingear, INSERM U1259, Faculté de Médecine, Université de
11 Tours, 10 boulevard Tonnellé, 37032 Tours, France. E-mail: roingear@med.univ-tours.fr;
12 phone: (33) 2 34 37 96 46; fax: (33) 2 47 47 82 07.

13

14 Word count (without figure legends): 3397

15

16 Number of figures/tables: 2/0

17

18 Abbreviations: WHO, World Health Organization; HCV, hepatitis C virus; DAAs, direct-
19 acting antivirals; HBV, hepatitis B virus; HBsAg, hepatitis B virus S envelope protein; β -gal,
20 β -galactosidase; OD, optical density; HCVcc, hepatitis C virus in cell culture; FFU, focus-
21 forming unit.

22

23 Conflict of interest: Philippe Roingear is co-inventor of the patent “Fusion proteins and use
24 thereof for preparing hepatitis C vaccines” (U.S. No. US008765143B2) that is currently

25 exploited by the ViroCoVax company. Remaining authors declares that they have no conflict
26 of interest.

27

28 Financial support: This study was supported by the French National Agency for Research on
29 AIDS and Viral Hepatitis (ANRS), the French National League against Cancer (committees
30 of Indre-et-Loire, Maine-et-Loire and Sarthe) and the ARD2020 “Biomédicaments” grant
31 from the Centre-Val de Loire region.

32

33 Acknowledgments: The authors would like to thank Dr. Takaji Wakita (National Institute of
34 Infectious Disease, Tokyo, Japan) for providing the JFH-1 virus strain, Prof. Jens Bukh for
35 the chimeric JFH1 genomes H77/JFH1, J4/JFH1, S52/JFH1, and ED43/JFH1 (Hvidovre
36 Hospital and University of Copenhagen, Copenhagen, Denmark), and Prof. Charles M. Rice
37 (Rockefeller University, New York, USA) for the Huh7.5 cell line.

38

39 Author contributions: E.B. and P.R. conceptualized and designed the experiments, analyzed
40 the data, and wrote the manuscript. E.B., B.J.C., V.G. and L.C. performed the experiments.
41 E.R. and E.G.-E. provided technical support. All authors approved the final version of the
42 manuscript.

43

44

45

46

47

48

49

50 **Abstract**

51

52 The development of a safe, effective and affordable prophylactic vaccine against hepatitis C
53 virus (HCV) remains a medical priority. Hepatitis B-C subviral envelope particles, which
54 could be produced by industrial procedures adapted from those established for the hepatitis B
55 virus vaccine, appear promising for use for this purpose. The prototype HBV-HCV bivalent
56 vaccine bearing genotype 1a HCV envelopes can induce neutralizing antibodies against this
57 genotype, but is less effective against other genotypes. We show here, in a small-animal
58 model, that the use of a set of vaccine particles harboring envelopes from different HCV
59 genotypes in various association strategies can induce broad neutralizing protection or an
60 optimized protection against a particular genotype prevalent in a given region, such as
61 genotype 4 in Egypt. This vaccine could help to control the hepatitis C epidemic worldwide.

62

63 Abstract word count: 133

64

65 Keywords: hepatitis C virus, hepatitis B virus, subviral envelope particles, prophylactic
66 vaccine, neutralizing antibodies, HCV genetic variability.

67

68

69

70

71

72

73

74

75 1. INTRODUCTION

76 The World Health Organization (WHO) has estimated that hepatitis C virus (HCV) infection
77 could be eradicated by 2030 if the HCV epidemic worldwide decreases (i.e. the number of
78 patients cured by direct-acting antivirals (DAAs) consistently and significantly exceeds the
79 number of new infections) by at least 7% per year. However, recent studies have shown that
80 the global annual net regression, estimated at only 0.4%, is far from meeting this objective.¹

81 The cost of DAAs, which has recently decreased considerably, is less of a problem than the
82 screening logistics required to identify the 71 million chronic carriers of HCV worldwide that
83 have yet to be treated with DAAs. Indeed, due to the absence of symptoms in most HCV-
84 infected individuals, only a small fraction (probably around 5%) of these individuals are
85 aware that they are infected. Chronic carriers of HCV that are not diagnosed may, therefore,
86 transmit the virus to healthy people, and it is estimated that the world reservoir of HCV-
87 infected individuals increases by two million newly infected subjects each year. This increase
88 is not limited to low economic countries, as the Centers for Disease Control recently reported
89 a near tripling of HCV incidence in the US over the last five years, to reach a 15-year high of
90 41,200 new cases in 2016.² A recent mathematical model evaluating the impact on hepatitis C
91 eradication of scaling up prevention and treatment indicated that it would be extremely
92 difficult to achieve this goal.³ Improvements would be required in blood safety and infection
93 control, together with the expansion or creation of services for intravenous drug users, and
94 extensive screening and treatment, the cost of which has been estimated by the WHO to be at
95 least US\$12 billion.⁴ Furthermore, although antiviral drugs are highly effective at controlling
96 infection, they cannot prevent re-infection with HCV, a situation that is becoming
97 increasingly common among drug users in industrialized countries.⁵ Moreover, approximately
98 15% of subjects with chronic hepatitis C infection go on to develop cirrhosis or even liver

99 cancer after eradication of the virus by DAAs.⁶ For all these reasons, the development of a
100 prophylactic anti-HCV vaccine remains an important objective.

101 An original vaccine based on full-length HCV E1 or E2 envelope glycoproteins fused to
102 the heterologous hepatitis B virus (HBV) S envelope protein (HBsAg) has been
103 developed.⁷ These chimeric envelope proteins self-assemble into highly immunogenic,
104 noninfectious and secreted subviral envelope particles resembling to the HBV vaccine and
105 are able to elicit specific antibody responses against HCV and HBV envelope proteins.^{8,9}
106 More importantly, prototype vaccine particles bearing the E1 or E2 envelope proteins from
107 a genotype 1a HCV isolate induced antibodies capable of neutralizing heterologous
108 genotype 1a and 1b HCV strains efficiently in vitro. However, these antibodies appeared to
109 have a lower cross-neutralizing potential against viruses of genotypes 3 and 4, which are
110 genetically more distant.^{8,9} As genotypes 1, 3 and 4 are the most prevalent worldwide
111 (83.8% if considered together) and the most common in several parts of the world, the
112 objective of this study was to increase the broad neutralizing properties of antibodies
113 induced by such HBV-HCV bivalent vaccines through the development of new subviral
114 vaccine particles bearing envelope glycoproteins from genotype 3 and 4 HCV isolates.

115

116 2. METHODS

117 2.1 Production of the vaccine particles

118 Chimeric HBV (subtype *adw*) - HCV (genotype 1a) envelope particles bearing the full-length
119 HCV E1 (E1^{1a}S particles) or E2 proteins (E2^{1a}S particles) were produced and purified as
120 previously described.⁸ Using the same strategy, extensively described elsewhere,^{7,8} we
121 designed, produced and purified new vaccine particles bearing full-length genotype 3a or 4a
122 HCV E1 or E2 glycoproteins (envelope sequences provided in Figure S1 and S2) named
123 E1^{3a}S, E2^{3a}S, E1^{4a}S and E2^{4a}S.

124

125 **2.2 Immunization of New Zealand rabbits**

126 We immunized four groups of 12 rabbits subcutaneously on days 0, 14 and 28, with 15 μ g of
127 AddaVax-adjuvanted immunogens, quantified by HBsAg ELISA, as follows: (i) only E2^{1a}S
128 particles; (ii) equal amounts of E2^{1a}S, E2^{3a}S and E2^{4a}S particles (5 μ g of each particle); (iii)
129 equal amounts of E1^{1a}S, E2^{1a}S, E1^{3a}S, E2^{3a}S, E1^{4a}S and E2^{4a}S particles (2.5 μ g of each
130 particle); (iv) or equal amounts of E1^{4a}S and E2^{4a}S particles (7.5 μ g of each particle). Groups
131 of six rabbits immunized with the AddaVax adjuvant alone or with Engerix, a commercially
132 available HBV vaccine, were used as negative and positive controls, respectively. Serum
133 samples were collected from rabbits at various time points (days 0, 14, 28, 42, and 56) for the
134 characterization of antibody responses. Immunizations were performed by Agro-Bio (La
135 Ferté-Saint-Aubin, France), a company accredited by the French authorities for the
136 performance of protocols on laboratory animals in accordance with European Directive
137 2010/63/EU. The protocol was approved by the Agro-Bio Ethic Committee C2A-99 (French
138 Ministry of Research, agreement B45-146-01).

139

140 **2.3 Analysis of anti-HBsAg and anti-E1/E2 antibody responses**

141 Anti-HBsAg and anti-E1/E2 assays were conducted and analyzed as described in detail
142 elsewhere.^{8,9} Briefly, anti-HBsAg antibodies were quantified with the ARCHITECT system
143 (Abbott Laboratories). Anti-E1 and anti-E2 responses were evaluated with in-house ELISAs
144 based on the use of solid phases consisting of appropriate lysates (or mixtures of lysates) of
145 baby hamster kidney (BHK-21) cells expressing the same HCV E1 or E2 proteins of
146 genotypes 1a, 3a and 4a as were exposed at the surface of the vaccine particles, which were
147 compared with control cells expressing β -galactosidase (β -gal). For each serum sample tested,

148 the optical density (OD) value obtained for wells with capture by β -gal protein was subtracted
149 from that obtained for wells with capture by E1 or E2 proteins.

150

151 **2.4 Neutralization assays with HCV in cell culture**

152 HCV in cell culture (HCVcc) harboring HCV envelope glycoproteins derived from genotype
153 1a (JFH1/H77), 1b (JFH1/J4), 2a (JFH1), 3a (JFH1/S52) and 4a (JFH1/ED43) isolates were
154 used to assess and compare the neutralizing potential of the anti-HCV envelope protein
155 antibodies present in pre- and post-vaccination serum samples from rabbits. Virus stocks were
156 generated by transfecting hepatoma Huh7.5 cells with in vitro transcribed full-genomic HCV
157 RNA and viral infectivity was monitored using a focus-forming unit (FFU) staining assay, as
158 described elsewhere.⁸ FFU were counted under a microscope, supernatant infectivity titers
159 were determined as the number of FFU/ml and titrated viral stocks were diluted to obtain
160 2,000 FFU/ml in growth medium. A volume of 50 μ l, corresponding to 100 FFU, was then
161 incubated for 1 hour at 37 °C with 25 μ l of five-fold serial dilutions of each rabbit serum
162 collected on days 0 and 56. The virus/serum mixture was then used to infect 10,000 Huh7.5
163 cells plated the previous day on 96-well plates (Falcon) for 6 hours at 37°C. The virus-
164 containing medium was then removed, replaced with fresh medium and the infected cells
165 were incubated at 37°C. Infectivity was evaluated 48 hours after infection, as described
166 above. The assay was performed once in triplicate and the results are expressed as mean
167 values.

168

169 **2.5 Sequence analysis**

170 The amino-acid sequences encoding the E1 or E2 envelope proteins presented at the surface
171 of the HCVcc were downloaded from the GenBank database (deposited under the accession
172 numbers: JF343780.1 (H77/JFH1), FJ230881.1 (J4/JFH1), AB047639.1 (JFH1), EU204645.1

173 (S52/JFH1) and MK600383.1 (ED43/JFH1)) and aligned with those encoding the E1 or E2
174 envelope proteins presented at the surface of the vaccine particles (Figure S1 and S2).
175 Multiple sequence alignment was computed using ClustalW (BioEdit software) and edited
176 manually.

177

178 **2.6 Statistical analysis**

179 The observed differences in neutralizing antibody induction capacity between the vaccination
180 strategies for a given genotype were analyzed statistically with nonparametric Mann-Whitney
181 tests. These distribution comparison tests were conducted considering only the datasets
182 obtained for the six rabbit sera presenting the most sustained anti-HBV and anti-HCV
183 antibody responses and also containing the antibodies with the most significant cross-
184 neutralizing properties. A p value < 0.05 was considered to be statistically significant.

185

186 **3. RESULTS**

187 All HBV-HCV vaccine particles (whether used alone or in cocktails, Figure 1A) induced
188 specific antibody responses against the HCV and HBV envelope proteins (Figure 1B). After
189 the evaluation of the anti-HBsAg, anti-E1 and anti-E2 antibodies levels, the neutralizing
190 properties of antibodies induced by the various vaccination strategies in immunized rabbits
191 were investigated using HCVcc harboring HCV envelope glycoproteins derived from
192 different heterologous genotype 1a, 1b, 2a 3a and 4a strains (Figure S3). By paying particular
193 attention to the neutralizing properties of the six rabbit sera with the strongest humoral
194 responses to vaccination in each group (the rabbits with the highest levels of anti-HBsAg
195 antibodies were also those with the highest levels of anti-E1 and anti-E2 antibodies), we
196 demonstrated that immunization with a cocktail of E2S particles bearing HCV genotype 1a,
197 3a and 4a E2 envelope glycoproteins significantly increased the broad neutralizing properties

198 of the antibodies induced (p -values = 0.026 and 0.005 for HCV genotype 3 and 4
199 neutralization, respectively, when a comparison is made with animals immunized with E2^{1a}S
200 particles only) (Figure 2). More importantly, we clearly show that the antibodies produced in
201 response to vaccination with this cocktail were above all capable to neutralize infections with
202 an HCVcc of genotype 2a harboring an E2 envelope sequence not present in the vaccine
203 cocktail (Figures 2 and S3). The strength of these broad cross-neutralizing responses was
204 confirmed with additional neutralizing assays using serial dilutions of rabbit sera, showing
205 that these broad neutralizing properties of the antibodies induced were also observed for high
206 serum dilutions (Figure S4). The addition of particles bearing E1 glycoproteins to this
207 cocktail of particles bearing E2 proteins from genotypes 1a, 3a and 4a clearly reduced the
208 positive impact of this multi-genotypes strategy. By contrast and importantly, the use of a
209 cocktail of E2^{1a}S + E2^{3a}S + E2^{4a}S particles did not significantly decrease the ability of the
210 vaccine to induce neutralizing antibodies against HCV genotypes 1a and 1b, relative to the
211 prototype E2^{1a}S vaccine. Nevertheless, a cocktail of only two types of particles, bearing HCV
212 genotype 4 E1 and E2 glycoproteins separately, was identified as the best candidate vaccine
213 for protection against this particular genotype. It clearly gave the best protection in
214 comparisons of animals immunized with the E1^{4a}S + E2^{4a}S particle cocktail and animals
215 immunized with the prototype E2^{1a}S vaccine (p -value = 0.005, Figure 2). However, even
216 though the median value for neutralization obtained in animals immunized with E1^{4a}S +
217 E2^{4a}S particle cocktail was higher than that for animals immunized with the E2^{1a}S + E2^{3a}S +
218 E2^{4a}S particle cocktail (81.5% *versus* 70.5%, Figure 2), the difference was not significant,
219 probably due to the small number of animal.

220

221 4. DISCUSSION

222 DAAs have revolutionized the treatment of chronic HCV infection. Nevertheless, it has
223 become clear that it will be very difficult to eradicate hepatitis C without a prophylactic
224 vaccine against this virus.^{10,11} Various vaccination strategies are currently being tested for
225 HCV,¹²⁻¹⁴ but the development of such vaccines is difficult, and their place in vaccination
226 programs remains unclear. In this context, a bivalent HBV-HCV vaccine would be more
227 likely to be adopted, as its main function would be to provide protection against hepatitis B,
228 and any additional protection, even if only partial, against HCV would be a bonus. The
229 relevance of this vaccine approach is reinforced by the capacity of this vaccine candidate to
230 induce anti-HBsAg responses, although slightly lower than those induced by the
231 commercially Engerix vaccine. It cannot be excluded that this slight decrease in HBsAg
232 immunogenicity is due to the fusion of the envelope proteins, but this observation is probably
233 related to the difference in purity existing between the vaccine prepared in an academic
234 laboratory and the highly purified industrially produced Engerix vaccine. This approach
235 would also strengthen the notion of the hepatitis B vaccine being the first anti-cancer vaccine,
236 as it would result in a new vaccine providing protection against different virus-induced liver
237 cancers.

238 Our previous immunization studies, performed with chimeric HBV-HCV envelope
239 subviral particles, showed that particles containing E2^{1a} proteins gave the best neutralizing
240 coverage, but that particles containing E1^{1a} proteins could also induce neutralizing
241 antibodies against HCV genotype 1a and 1b, and, to a lesser extent, against other HCV
242 genotypes.⁸ In a second set of experiments, we showed that the most efficient strategy for
243 inducing both anti-E1 and anti-E2 neutralizing antibodies was to mix subviral HBV-HCV
244 envelope subviral particles containing E1^{1a} proteins with particles containing E2^{1a}
245 proteins.⁹ In the multi-genotype strategy presented here, the addition of a cocktail of
246 particles bearing E1 proteins to the cocktail of particles bearing E2 proteins clearly

247 decreased the positive impact of this multi-genotype approach, probably by reducing the
248 amount of each immunogen in the dose of vaccine administered and imposing conflicting
249 selection forces which frustrate the affinity maturation of the B lymphocytes. However, if
250 limited to a cocktail of multi-genotype particles containing only E2 proteins, this approach
251 clearly yielded a much better broad cross-neutralizing response to the main HCV
252 genotypes 1a, 1b, 2a, 3a and 4a. The strength of this broad cross-neutralizing response was
253 greatly amplified by the fact that antibodies produced in response to vaccination with this
254 cocktail were capable to neutralize HCV of genotype 2a, and this also for high serum
255 dilutions. Nevertheless, a cocktail of particles bearing HCV E1 or E2 glycoproteins of a
256 particular genotype separately would be the best strategy for protection against a given
257 genotype (reference 9 for the genotype 1; this study for the genotype 4). These data
258 suggest that a set of HBV-HCV envelope subviral vaccine particles bearing HCV envelope
259 glycoproteins of different genotypes could be used, in various combination strategies, to
260 induce a broad neutralizing protection against different HCV genotypes or to provide
261 protection against a particular genotype being prevalent in a given region. This second
262 approach would clearly be relevant for genotype 4 in Egypt, which has the highest
263 prevalence of HCV infection in the world, where more than 90% of HCV-infected subjects
264 are infected with genotype 4 viruses.¹⁵

265
266 In conclusion, the mixing of different envelope-based subviral particles can be used to
267 optimize the bivalent HBV-HCV prophylactic vaccine, particularly for use in cases of
268 potential exposure to different HCV genotypes. As the neutralizing breadth of anti-HCV
269 antibodies was investigated in this study using a limited panel of historical important
270 reference strains which are known for some to be highly sensitive to neutralization,^{16,17} and
271 as the replication-competent cell culture viruses have been described varied widely in

272 terms of sensitivity to neutralization by monoclonal antibodies,^{16,18,19} it will be important in
273 future experiments to investigate these phenotypic differences. Further studies, including
274 the immunization of humans, will, of course, also be required before definitive conclusions
275 can be drawn. In addition, it will be interesting to conduct further studies to analyze
276 precisely the genotype-specific or more conserved epitopes targeted by the antibodies
277 induced by the different vaccination strategies. However, this vaccine would be simple to
278 produce, as it could be processed by industrial procedures adapted from those established
279 for the HBV vaccine, and it could potentially help to control the global hepatitis C
280 epidemic.

281

282 REFERENCES

283 Hill AM, Nath S, Simmons B. The road to elimination of hepatitis C: analysis of cures
284 versus new infections in 91 countries. *J Virus Erad* 2017;3:117-123.

285 Chhatwal J, Sussman NL. Universal screening for hepatitis C: an important step in virus
286 elimination. *Clin Gastroenterol Hepatol* 2019;17:835-837.

287 3. Heffernan A, Cooke GS, Nayagam S, Thursz M, Hallett TB. Scaling up prevention and
288 treatment towards the elimination of hepatitis C: a global mathematical model. *Lancet*
289 2019;393:1319-1329.

290 4. Wiktor S. How feasible is the global elimination of HCV infection? *Lancet*
291 2019;393:1265-1267.

292 5. Midgard H, Weir A, Palmateer N, et al. HCV epidemiology in high-risk groups and the
293 risk of reinfection. *J Hepatol* 2016;65:S33-S45.

294 6. Hamdane N, Jühling F, Crouchet E, et al. HCV-induced epigenetic changes associated
295 with liver cancer risk persist after sustained virologic response. *Gastroenterology*
296 2019;156:2313-2329.

- 297 7. Patient R, Hourieux C, Vaudin P, Pagès JC, Roingeard P. Chimeric hepatitis B and C
298 viruses envelope proteins can form subviral particles: implications for the design of new
299 vaccine strategies. *N Biotechnol* 2009;25:226-234.
- 300 8. Beaumont E, Patient R, Hourieux C, Dimier-Poisson I, Roingeard P. Chimeric hepatitis B
301 virus/hepatitis C virus envelope proteins elicit broadly neutralizing antibodies and constitute
302 a potential bivalent prophylactic vaccine. *Hepatology* 2013;57:1303-1313.
- 303 9. Beaumont E, Roch E, Chopin L, Roingeard P. Hepatitis C virus E1 and E2 proteins used
304 as separate immunogens induce neutralizing antibodies with additive properties. *PLoS One*
305 2016;11:e0151626.
- 306 10. Bartenschlager R, Baumert TF, Bukh J, et al. Critical challenges and emerging
307 opportunities in hepatitis C virus research in an era of potent antiviral therapy:
308 considerations for scientists and funding agencies. *Virus Res* 2018;248:53-62.
- 309 11. Lombardi A, Mondelli MU. ESCMID study group for viral hepatitis (ESGVH).
310 Hepatitis C: Is eradication possible? *Liver Int* 2019;39:416-426.
- 311 12. Kinchen VJ, Cox AL, Bailey JR. Can broadly neutralizing monoclonal antibodies lead to
312 a hepatitis C virus vaccine? *Trends Microbiol* 2018;26:854-64.
- 313 13. Bailey JR, Barnes E, Cox AL. Approaches, progress, and challenges to hepatitis C
314 vaccine development. *Gastroenterology* 2019;156:418-430.
- 315 14. Laidlaw SM, Dustin LB. An HCV vaccine on the fly. *J Infect Dis* 2019;
316 <https://doi.org/10.1093/infdis/jiz231>
- 317 15. Omran D, Alborai M, Zayed RA, et al. Towards hepatitis C virus elimination: egyptian
318 experience, achievements and limitations. *World J Gastroenterol* 2018;24:4330-4340.
- 319 16. Carlsen TH, Pedersen J, Prentoe JC, et al. Breadth of neutralization and synergy of
320 clinically relevant human monoclonal antibodies against HCV genotypes 1a, 1b, 2a, 2b, 2c,
321 and 3a. *Hepatology* 2014;60:1551-1562.

322 17. Giang E, Dorner M, Prentoe JC, et al. Human broadly neutralizing antibodies to the
323 envelope glycoprotein complex of hepatitis C virus. *Proc Natl Acad Sci USA*
324 2012;109:6205–6210.

325 18. Urbanowicz RA, McClure CP, Brown RJ et al. A diverse panel of hepatitis C virus
326 glycoproteins for use in vaccine research reveals extremes of monoclonal antibody
327 neutralization resistance. *J Virol* 2016;90:3288-3301.

328 19. Wasilewski LN, Ray SC, Bailey JR. Hepatitis C virus resistance to broadly neutralizing
329 antibodies measured using replication-competent virus and pseudoparticles. *J Gen Virol*
330 2016;97:2883-2893.

331

332 **FIGURE LEGENDS**

333

334 **FIGURE 1** Immunization protocol design and analysis of the antibody responses induced by
335 various vaccination strategies. (A) Groups of twelve rabbits were immunized three times
336 subcutaneously with AddaVax-adjuvanted chimeric HBV-HCV envelope subviral particles
337 harboring a genotype 1a E2 envelope glycoprotein (15 μ g) or with different cocktails
338 consisting of equal amounts of chimeric particles harboring genotype 1a, 3a and 4a HCV E1
339 or E2 envelope glycoproteins, on days 0, 14 and 28. Groups of six rabbits immunized with the
340 AddaVax adjuvant alone or with Engerix, a commercially available HBV vaccine (15 μ g)
341 were used as negative and positive controls, respectively. Serum samples were collected from
342 rabbits at various time points (days 0, 14, 28, 42 and 56), for the characterization of antibody
343 responses and evaluation of the neutralizing potential of the antibodies induced by
344 vaccination. (B) Specific anti-E1/anti-E2 and anti-HBsAg responses were evaluated in rabbit
345 sera with in-house ELISAs^{8,9} and a routine immunoassay (Abbott Laboratories), respectively.

346 Results are expressed as the difference in optical density (anti-E1- or anti-E2- β -Gal) and anti-
347 HBsAg titer (mIU/ml), respectively.

348

349 **FIGURE 2** Neutralizing potential of antibodies induced by the various vaccination strategies.
350 Cross-neutralizing properties against HCVcc of the anti-HCV antibodies induced by the
351 various vaccination strategies evaluated on the six rabbit serum samples with the strongest
352 humoral responses to vaccination in each group. Five-fold dilutions of rabbit serum samples
353 collected on days 0 and 56 were first incubated with HCVcc harboring HCV envelope
354 glycoproteins derived from genotype 1a, 1b, 2a, 3a and 4a isolates for 1 hour at 37°C, which
355 were then used to infect Huh7.5 cells for 6 hours. Infection levels were determined after 48
356 hours of incubation at 37°C, in a FFU staining assay⁸. FFU were counted under a microscope,
357 and the percentage neutralization in the presence of the postimmune serum (Day 56) was
358 compared with that in the presence of preimmune serum (Day 0) from the same rabbit. The
359 assay was performed once in triplicate, and the results are expressed as mean values.
360 Statistical analyses were performed with nonparametric Mann-Whitney tests. * $p < 0.05$; **
361 $p < 0.005$; *ns* = nonsignificant.

362

363 **FIGURE S1** Comparison of the amino-acid sequences of the E1 proteins harbored at the
364 surface of the vaccine particles and the HCV in cell culture used to conduct the neutralization
365 assays. (A) Multiple sequence alignment of the E1 proteins. Amino-acid sequences of the E1
366 proteins from genotype 1a, 3 and 4 viruses selected for the development of the vaccine
367 particles (represented in blue) were aligned with the amino-acid sequences of the E1 proteins
368 presented at the surface of the genotype 1a, 3 and 4 HCV in cell culture neutralized by the
369 antibodies induced through vaccination (shown in black). Signal sequence responsible for
370 translocation of the E1 ectodomain into the endoplasmic reticulum lumen is boxed in red.

371 Amino-acid identities with the uppermost sequence (.) and substitutions are indicated. (B)
372 Comparative analysis of the sequence homologies. The numbers indicate the similarities at
373 the amino-acid level in percentage.

374

375 **FIGURE S2** Comparison of the amino-acid sequences of the E2 proteins harbored at the
376 surface of the vaccine particles and the HCV in cell culture used to conduct the neutralization
377 assays. (A) Multiple sequence alignment of the E2 proteins. Amino-acid sequences of the E2
378 proteins from genotype 1a, 3 and 4 viruses selected for the development of the vaccine
379 particles (represented in blue) are aligned with the amino-acid sequences of the E2 proteins
380 presented at the surface of the genotype 1a, 3 and 4 HCV in cell culture neutralized by the
381 antibodies induced through vaccination (shown in black). Signal sequence responsible for
382 translocation of the E2 ectodomain into the endoplasmic reticulum lumen is boxed in red.
383 Amino-acid identities with the uppermost sequence (.), gaps (-) and substitutions are
384 indicated. (B) Comparative analysis of the sequence homologies. The numbers indicate the
385 similarities at the amino-acid level in percentage.

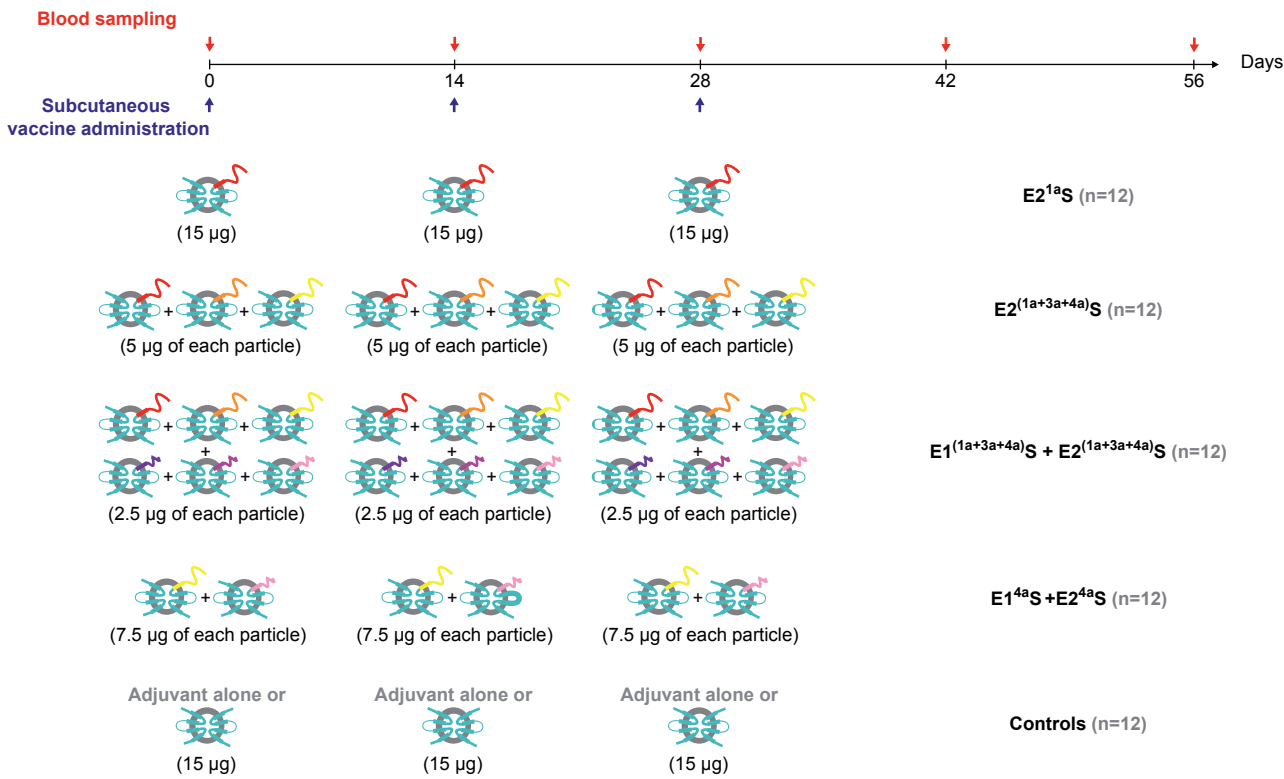
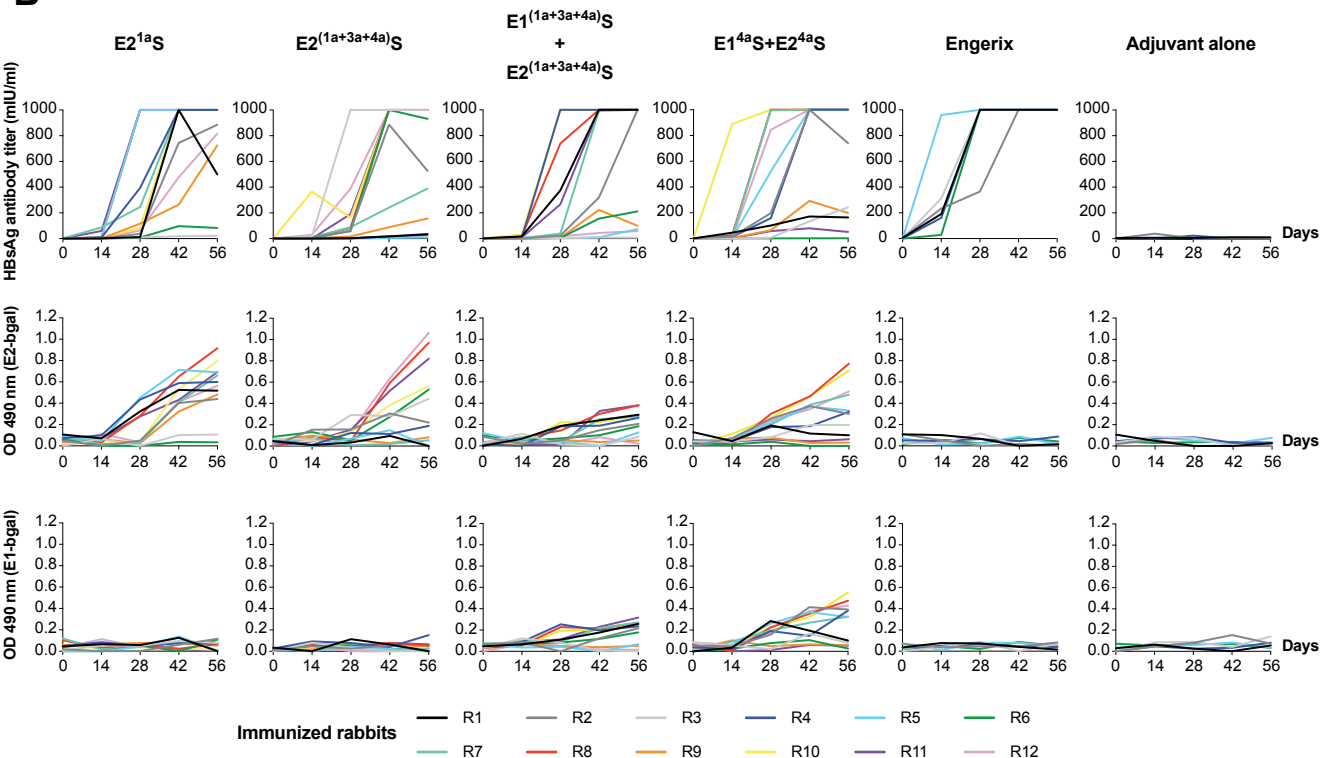
386

387 **FIGURE S3** Neutralizing potential of antibodies induced by the various vaccination
388 strategies in all immunized rabbits. Cross-neutralizing properties against HCVcc of the anti-
389 HCV antibodies induced by the various vaccination strategies evaluated on all rabbit serum
390 samples. Five-fold dilutions of rabbit serum samples collected on days 0 and 56 were first
391 incubated with HCVcc harboring HCV envelope glycoproteins derived from genotype 1a, 1b,
392 2a, 3a and 4a isolates for 1 hour at 37°C, which were then used to infect Huh7.5 cells for 6
393 hours. Infection levels were determined after 48 hours of incubation at 37°C, in a FFU
394 staining assay⁸. The percentage of neutralization was determined by subtracting the infectious
395 titer obtained with the pre-immune serum (day 0) from that obtained with the post-immune

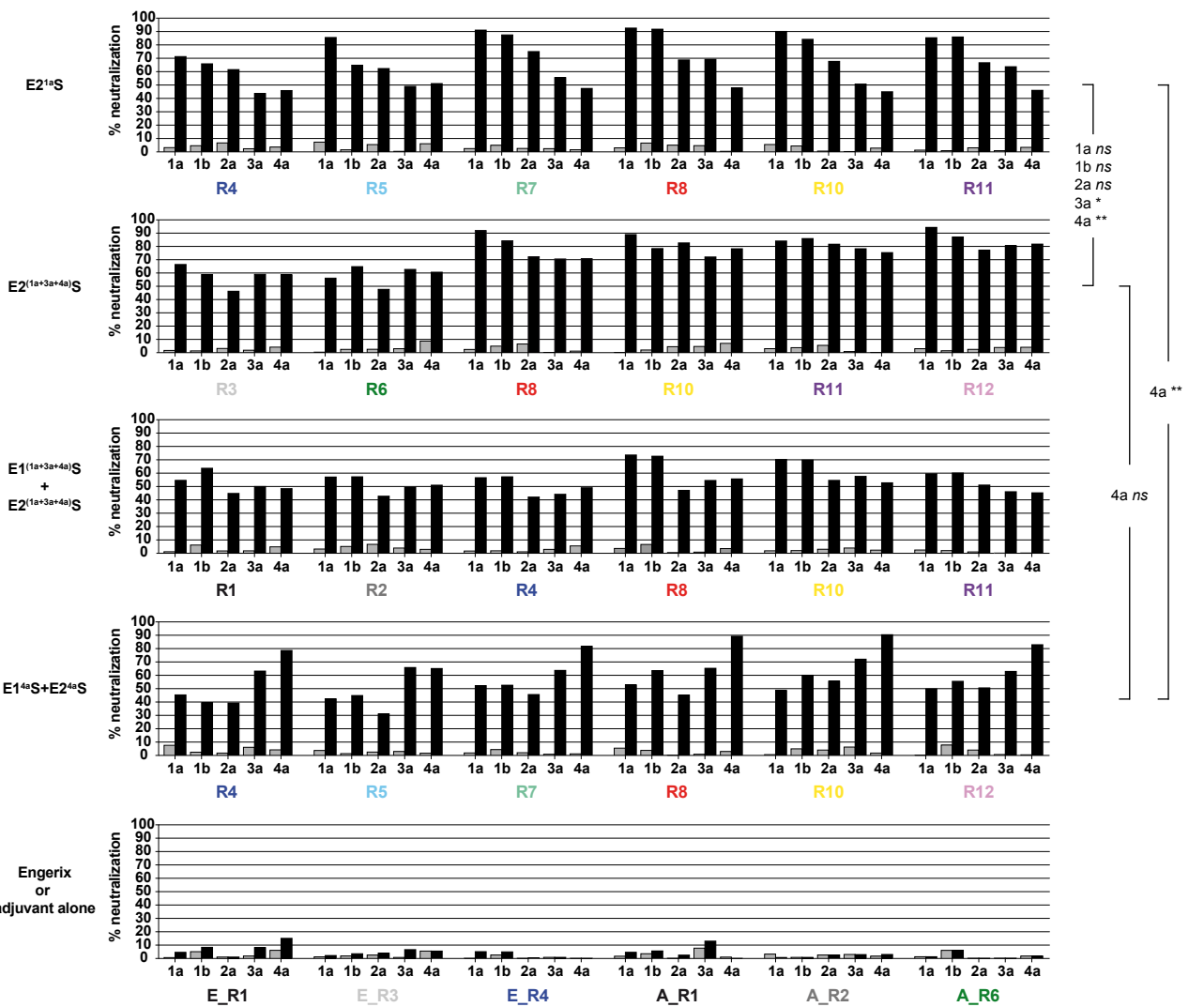
396 serum (day 56) from the same rabbit. The assay was performed once in triplicate, and the
397 results are expressed as mean values. Each colored point represents the neutralization value
398 observed for the serum sample from an individual immunized rabbit. The horizontal line
399 represents the median value. The conservation of the color code between the main and
400 supplementary figures allows a close correlation of these data with those resulting from the
401 evaluation of the antibody responses.

402

403 **FIGURE S4** Strength of the broad cross-neutralizing antibody response. Evaluation of the
404 cross-neutralizing properties against HCVcc of 5-fold serial serum dilutions of the six rabbits
405 with the strongest humoral responses to vaccination in each group. Dilutions of rabbit sera
406 collected on days 0 and 56 were first incubated with HCVcc harboring HCV envelope
407 glycoproteins derived from genotype 1a, 1b, 2a, 3a and 4a isolates for 1 hour at 37°C, which
408 were then used to infect Huh7.5 cells for 6 hours. Infection levels were determined after 48
409 hours of incubation at 37°C, in a FFU staining assay⁸. FFU were counted under a microscope,
410 and the percentage of neutralization was determined by subtracting the infectious titer
411 obtained with the pre-immune serum (D0) from that obtained with the post-immune serum
412 (D56) from the same rabbit. The assay was performed once in triplicate, and the results are
413 expressed as mean values.

A**B**

Serum collected on Day 0
 Serum collected on Day 56 post-immunization



A

```

      10      20      30      40      50      60      70      80
Genotype 1a E1 vaccine protein MTGNLPGCSFSIFLLALLSCLTVPASAYQVRNSSGLYHVTNDCPNSSIVYETADTILHSPGCVPCVREGNASKCWAVAAP
Genotype 3a E1 vaccine protein ..IH.ASLEW.V.VL.....AD.V.T.....QD.I.T.TS.T.
Genotype 4a E1 vaccine protein .....VNY.T.I.I.....ADHH.L.....KT.R.R.....LT.
Genotype 1a H77 E1 protein A.....A.A.T.....R.....T.
Genotype 1b J4 E1 protein A.....I.....E.V.I.....S.....A.V.M.T.....Q.S.R.....LT.
Genotype 2a JFH1 E1 protein A.....FP.....I.V.A.K.T.SS.M.....S.D.TWQLEAAV.V.....E.V.T.R.....P.S.
Genotype 3a S52 E1 protein A.....F.VH.ASLEW.T.VL.....S.....AD.V.T.....QDD.T.T.TP.T.
Genotype 4a ED43 E1 protein A.....VNY.V.I.....ADHH.L.....Q.R.....LT.

      90      100     110     120     130     140     150     160
Genotype 1a E1 vaccine protein TVATRDKGLPTTQLRRHIDLVRSATLCSALYVGDLCGSVFLVGLFTTSPRRHWTTQDCNCSMPYGHITGHRMAWMMMM
Genotype 3 E1 vaccine protein ...V.YLGAT.ASV.S.V...GA.M.....M.A.....A.R.....Q.V.T...L...LS...
Genotype 4 E1 vaccine protein ...APYIGA.LHS.S.V.M.GA.V...I...GL.A.V.S.R.....I.T.....G...
Genotype 1a H77 E1 protein .....G.....I.....
Genotype 1b J4 E1 protein .L.A.NASV..TI..V...GT.AF..M.....I.S.....E.V.....I...VS...
Genotype 2a JFH1 E1 protein NM.V.QPGAL.QG.T...MV.M...F.....G.M.AA.V.IV.QY.FV.E...I...T.
Genotype 3a S52 E1 protein ...V.YVGAT.ASI.S.V...GA.....M.A.....A.R.....Q.V.T...L...VS...
Genotype 4a ED43 E1 protein ...APYIGA.LES.S.V.M.GA.V.G.I...GL...M.S.R.....I.T.....

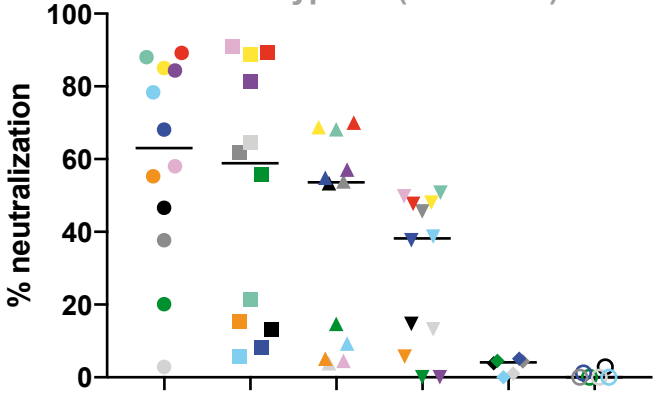
      170     180     190     200     210
Genotype 1a E1 vaccine protein NWSPTTALVVAQLLRVPAILDMIAGAHWGVLAGIAYFMSVGNWAKVLVVLLLFASVDA
Genotype 3 E1 vaccine protein ...AVGM...HV.L.TLF.I.....I.L.Y.Q...A.IMIM.SG...
Genotype 4 E1 vaccine protein ...T...LS.IM.I.GTLV.LL.G...V.V.Y.QA...IL.F.G...
Genotype 1a H77 E1 protein ...A.....I...M.....G...
Genotype 1b J4 E1 protein .....S.I...VV.V.....L.Y.....I.A...G.G
Genotype 2a JFH1 E1 protein .....ATMIL.YVM...EV.I.IVS...MF.L...Q.A...I.I.A.G...
Genotype 3a S52 E1 protein ...AVGM...HI.L.TLF.LL...I.L.Y.Q...AI.MIM.SG...
Genotype 4a ED43 E1 protein ...T.L.VM.I.TTLA.LLS.G...V.V...QA...IL.F.G...

```

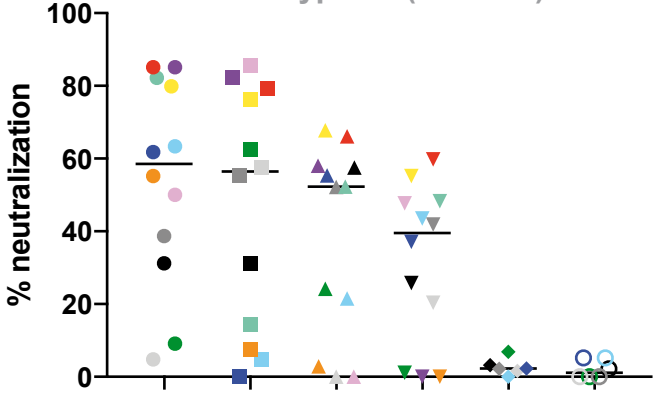
B

E1 amino acid sequence (% homology)	Genotype 1a vaccine candidate	Genotype 3a vaccine candidate	Genotype 4a vaccine candidate
Genotype 1a vaccine candidate		69.4	70.8
Genotype 3a vaccine candidate	69.4		67.6
Genotype 4a vaccine candidate	70.8	67.6	
Genotype 1a H77/JFH1 HCVcc	94.5	70.3	72.1
Genotype 1b J4/JFH1 HCVcc	78.1	68.5	69.9
Genotype 2a JFH1 HCVcc	63.5	54.8	58.9
Genotype 3a S52/JFH1 HCVcc	67.6	92.7	66.7
Genotype 4a ED43/JFH1 HCVcc	72.6	67.1	92.7

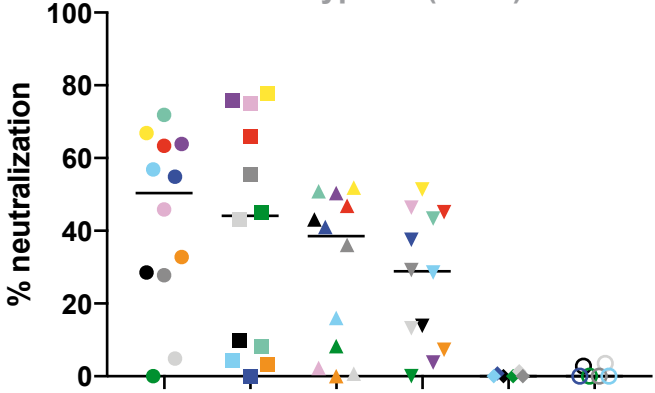
Genotype 1a (H77/JFH1)



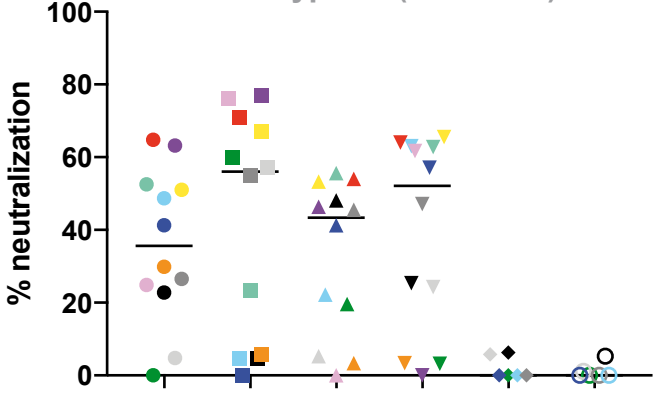
Genotype 1b (J4/JFH1)



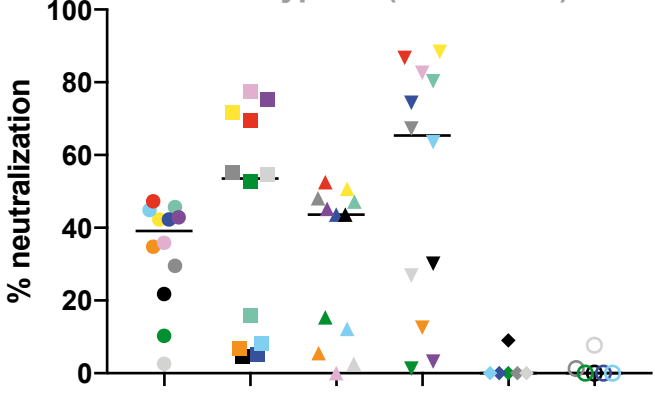
Genotype 2a (JFH1)



Genotype 3a (S52/JFH1)



Genotype 4a (ED43/JFH1)



E2^{1a}S

E2^(1a+3a+4a)S

E1^(1a+3a+4a)S + E2^(1a+3a+4a)S

E1^{4a}S + E2^{4a}S

Engerix

AddaVax

