HepaMAb Final Scientific Activity Report

Research objectives for the period:

(Overview of Scientific objectives for the period 01/01/12 - 31/12/17.)

OBJECTIVE 1 Pre-clinical and clinical development of lead anti-viral and anti-receptor antibodies for use in the liver transplant setting.

- **Objective 1.1 Pre-clinical development of a lead anti-HCV human monoclonal antibody**
- **Objective 1.2 Pre-clinical development of the lead monoclonal antibody targeting the HCV entry receptor Scavenger Receptor Class B Type I (SR-BI)**
- **Objective 1.3 In vitro efficacy testing and GLP/GMP lot validation**
- **Objective 1.4 *In vivo* efficacy testing of lead and back-up antibodies**
- **Objective 1.5 GMP Scale-up and production**
- **Objective 1.6 Clinical Trial of one anti-viral and one anti-receptor monoclonal antibody**

OBJECTIVE 2 Securing the therapeutic antibody pipeline: isolation and characterisation of back-up anti-viral and anti-receptor antibodies (WP2, 4, 5 & 6)

- **Objective 2.1 Development and i*n vitro* characterisation of an anti-viral back-up antibody**
- **Objective 2.2 Development and characterization of humanized tight junction-specific mAbs (CLDN-1)**

Summary of progress towards objectives and details for each task:

OBJECTIVE 1 Pre-clinical and clinical development of lead anti-viral and anti-receptor antibodies for use in the liver transplant setting, (WP1, 2, 3, 6, 7 & 8)

Objective 1.1 Pre-clinical development of a lead anti-HCV monoclonal antibody

The original plan was to advance mAb 1-7 to clinical trial. However, the patent for mAb 1-7 was to expire in April 2016. In order to provide a solid basis for eventual commercialization, we therefore made substantial efforts to generate improved antibodies, sufficiently different from the 1-7 clone so that they could be anticipated to be protected by new patents. Thus, we worked to make *i.)* affinity improved variant of 1-7 by light chain shuffling (clone G8), that showed improved anti-viral potency (vs. 1-7). *ii.)* We have also made additional improvement to G8, as well as *iii.)* bi-specific anti-HCV antibodies that showed synergistic improvements in virus neutralization capacity.

Several *in vitro* assays were performed, showing that IgG1 of clone G8 was superior to 1-7 in its anti-viral activity. Accordingly, G8 was chosen as the mAb to advance through process development and eventual clinical trial, and a high-producing CHO-cell line is being established for this antibody. Please note that G8 has the same heavy chain as 1-7, and thus bind to the same epitope on the E2 glycoprotein of HCV.

Significant results include:

- Development of a protocol for purification to 98-99% purity. It comprises a combination of affinity chromatography (Protein G), size exclusion (Sephadex), and ion-exchange (Mono Q).
- A purification method resulting in antibody preparation of >99% purity. The protocol for purification mentioned above was later improved to include a “polishing” step to achieve 99% purity; It now comprises a combination of affinity chromatography, ion-exchange, and size exclusion.
- Purified antibody assessed for aggregation and pH stability. Aggregation and pH stability are very good for clone 1-7, and equally very good for its improved daughter clone G8.
- In vitro resistance investigation completed. Reported by Partner 1.
- Potential antibody synergy. Partner 1 has shown clear synergy for a combination of anti-HCV clone 1-7 and the humanized SRB1 antibody from Partner 4.
- Tissue cross-reactivity of mAbs 1:7 and G8. While waiting for the GMP lot of G8 to be used for assessment of tissue cross reactivity, we have performed test with clone 1-7 to nine different human tissues: No cross reactivity was found. Unfortunately, the tissues were formalin fixed and the results is therefore not valid from a regulatory standpoint. The antibody clone G8 has recently been tested in array experiments for binding, if any, to 19 000 human proteins: No cross reactivity was observed.
- In vivo characterisation of the lead anti-HCV antibody. A preliminary test with clone 1-7 has been performed by Partner 3, but clone G8 has yet to be tested in the mouse model.

A major undertaking was generation of a cell line expressing the anti-HCV antibody that can be used for eventual GMP production. Numerous attempts had been performed previously in-house, but antibody yields were below target productivity. Therefore MoMAB worked with Celonics in Basel, Switzerland and with Trenzyme in Germany, to perform cell line establishment using a proprietary cell line from Celonic (CHO-K1). This included a period of negotiation around IP agreements and royalty sharing. The main progress was:

- A cell line has been established suitable for GMP production of human anti-HCV antibody G8 – production yields are around 0.3-0.5mg/ml.
- The protein produced by the cell line is stable and very little degradation is observed.
- The cell line has been transferred to Univercells, who have developed an appropriate cGMP process.
- A cGMP Master Cell Bank (MCB) for the anti-E2 antibody production has been generated.

Objective 1.2 Pre-clinical development of a lead monoclonal antibody targeting the HCV entry receptor Scavenger Receptor Class B Type I (SR-BI)

The main task undertaken by CEINGE was the generation of a high-producing stable cell line for the anti-SRB1 mAb c1671, suitable for GMP production, with a minimal productivity of 500 mg/liter.
The objectives were:

- to furtherly improve productivity of the previously generated suspension CHO dhFr stable pool, producing about 140 mg/liter (see previous report)
- to finalize the process development of the mAb (this step will be carried out by our collaborator Univercells) in order to begin preclinical studies
- to define an antibody formulation to ensure antibody stability and suitable for *in vivo* injection
- to design preclinical studies in NHP

As indicated in the previous report (HepaMAb Partner's Activity Report 1), we generated two stable cell lines by two different approaches:

1. an HEK 293 stable clone by the mammalian Sleeping Beauty transposon/transposase system, with a productivity of 10 mg/lt;
2. an adherent CHO dhfr- cell clone by DHFR (dihydrofolate reductase)/MTX (methotrexate) amplification system, producing about 40 mg/lt of the mAb.

As the productivity of these cell lines was significantly lower than the target one, we devised some new strategies to improve productivity. The basis of these strategies is the same as the one used for the generation of the CHO cell line mentioned above (dihydrofolate reductase/methotrexate amplification system), but some modifications to the expression cassette were made.
The main differences between the old and the new expression systems are the following:

Strategy 1: expression plasmid

- Replacement of the EF1α promoter with the stronger CMV promoter (Figure 1). Higher efficiency of expression by the CMV-c1671 plasmid (about 10 fold) was demonstrated by Western blot on culture media collected from CHO dhFr- cells, after transient transfection of these two plasmids (Figure 2).

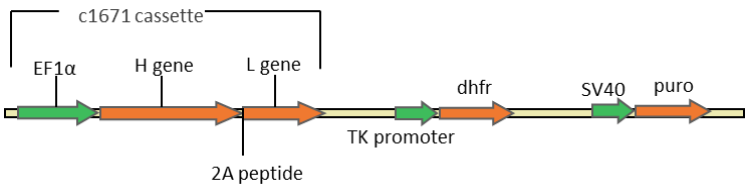


Figure 1. Schematic representation of pjc13-CMV-c1671 plasmid, expressing c1671 cassette under the control CMV promoter.

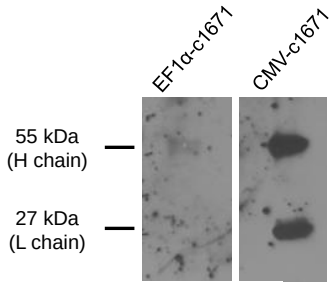


Figure 2. Western blot on culture media from CHO dhFr- cells transfected with plasmid pjc13- EF1α c1671 (EF1α-c1671) and plasmid pjc13-CMV-c1671 (CMV-c1671) expressing c1671 cassette under the control of EF1α and CMV promoters.

Strategy 2: expression plasmid

- Replacement of the EF1α promoter with the stronger CMV promoter.
- Replacement of heavy and light chain genes with heavy and light chain cDNAs.
- Introduction of a point mutation (Ser228Pro) in the hinge region of the antibody, to increase its stability in vivo by preventing fab arm exchange (Figure 3).

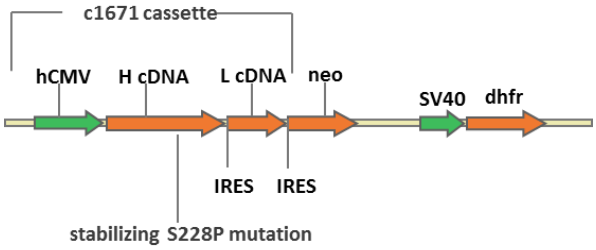


Figure 3. Scheme of the expression cassette used to transfect suspension CHO DG44 cells.

Host cell lines: adherent CHO dhFr- cells (strategy 1) and suspension DG44 CHO cells (strategy 2) were used as a substrate for transfection. Both these cell lines carry mutations in dhFr genes, that prevent them to grow in absence of hypoxantine and thymidine.

Generation of adherent CMV-CHO clone:

The steps followed to generate this stable cell line are summarized below:

- transfection (liposome methods) of adherent CHO dhFr- cells with the linearized plasmid described in figure 1;
- 14-day culture in the presence of puromycin and in absence of hypoxanthine and thymidine;
- at this step, we performed two parallel experiments:

    1. picking of the resistant cell pools and drug-induced gene amplification and selection on the best producer cell pool through a stepwise increase in (MTX) concentration.
    2. single cell cloning of the whole population of resistant cells, followed by MTX selection on the clones.

Culture media from each step of selection were analyzed by quantitative ELISA assays using SR-B1 positive and negative cells (HepG2 and CHO dhfr-, respectively), in order to identify the time-point with the highest productivity.

Generation of suspension CMV-CHO cell line:

For the establishment of a suspension CHO cell line, we used the following protocol:

- Transfection of CHO DG44 suspension cells with the plasmid described above;
- selection in absence of hypoxanthine and thymidine for 1 month;
- drug-induced gene amplification by a stepwise increase in MTX concentration (100, 300, 500, 1000, and 2000 nM, every 4 weeks;
- a cloning step on the best producer cell pool (500 nM pool) by two different approaches, 1 cell/well and 10 cells/well cloning.

1. **After antibiotic selection on transfected cells, we** screened some resistant pools for antibody expression in the culture media by ELISA assays. The best producer cell pool subsequently underwent MTX selection (5, 10, 20 nM concentrations). We stopped selection at 20 nM because we observed no increase from 5 nM to 20 nM. Besides, the cell pools at these 3 timepoints had a very low productivity (8 mg/lt) compared with the EF1a CHO cell clone we had established before (40 mg/lt)

Productivity was determined by simulating end-of-production conditions:

- Seed **1.0 x 10^6 cells in a 150 mm dish**
- After 4 days (**17.0 x 10^6 cells)**, replace the culture medium with a **FBS-deprived medium**
- After 4 days, collect the medium (96 h) and test it by ELISA.

2. To clone the entire pool of resistant cells before MTX selection, we labelled the cells to detect the secreted antibody that remains attached to cell surface and we made single cell cloning of the top

fluorescent cells. This strategy was not successful; in fact we screened the 20 clones obtained but all of them showed undetectable levels of mAb in their culture media.

Suspension CMV-DG44 cell line:

Antibody titers were measured by quantitative ELISA on culture media collected in end-of-production conditions:

- Seed 3**.0 x 10^5 cells/ml** in 30 ml of complete medium, in a 125 ml shaking flask;
- After 7 days (**about 8,0 x 10^6/ml**), collect the medium and test it by ELISA.

A standard curve with purified c1671, produced in EBNA 293 cells by transient transfection of two plasmids encoding the light and heavy chain, was used for quantification.

Among the MTX-selected pools, the 500 nM pool was the best producer and so he was chosen for subsequent cloning. Among the 100 clones screened, 9 of them produced the mAb at high levels; however, their productivity was below the one of the pool they derived from and it was not stable over the time. One possible reason for loss of titer was that these populations were not true clones and came from more than one cell, and low expressers over grew the (relatively) high expressers. Therefore, the best clones were recloned (subcloned) before expanding them to shake flasks, to obtain true clones. Despite this attempt, productivity did not improve.

So the best population obtained was a cell pool, but not a clone, with a productivity of 140mg/lt.

We also demonstrated that the c1671 antibody produced by this new plasmid had comparable affinity for SR-B1 receptor with the reference antibody produced in EBNA cells in our lab.

SAMPLE	Kd (nM)
Reference c1671 (from EBNA)	0,60
Suspension CHO pool	0,13

Considering the low productivity of the CHO cell pool (140 mg/L), at the end of 2015 CEINGE purchased from Amplycell a boosted cell suspension pool which was then used for single cell cloning. The protocol was based on a proprietary technology, called “Cell Fitness”, involving physico-chemical stimulations that are applied to cell lines so that they become more resistant, stable and productive while nothing changes with regards to culture properties. Briefly, the process consisted in 4 steps:

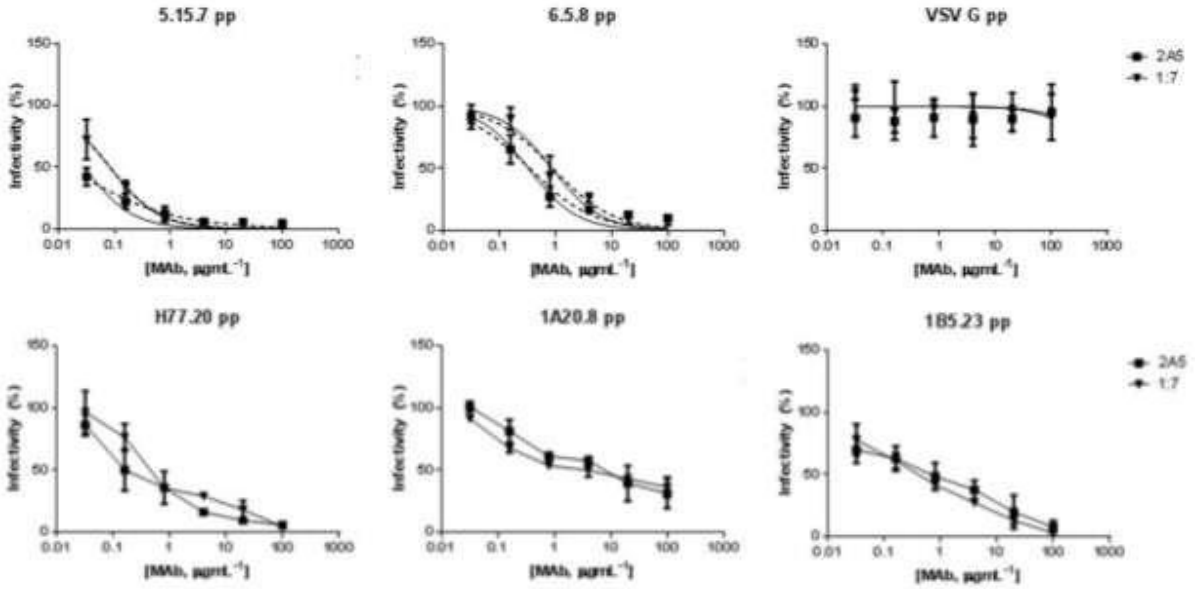
1. ‘’Diagnosis’’ phase on the cell line provided by CEINGE: research for mycoplasma, control of antibody secretion, sub-cloning in order to determine the percentage of antibody producing cells;
2. ‘’boosting’’ phase: application of boost treatments;
3. ‘’recovery’’ phase: screening by ELISA to select cells that provided the best response to the boost treatments (that means a better productivity compared to the original cells). This led to the selection of about 30 clones;
4. ‘’study’’ phase: identification of cell surface markers that demonstrated for the stability and the productivity of the cells. Measure of productivity and growth rate compared with the original cells. At the end of this final phase, the best five clones were retained.

CEINGE tested the best five clones and one of them was chosen to start the process development by Univercells. Univercells finalized the process development of this mAb and shipped it to CEINGE. With our collaborator, we defined a buffer formulation for an antibody concentration of 40-50mg/ml, suitable for in vivo injection, in order to perform tox studies in non-human primates (cyno monkeys).

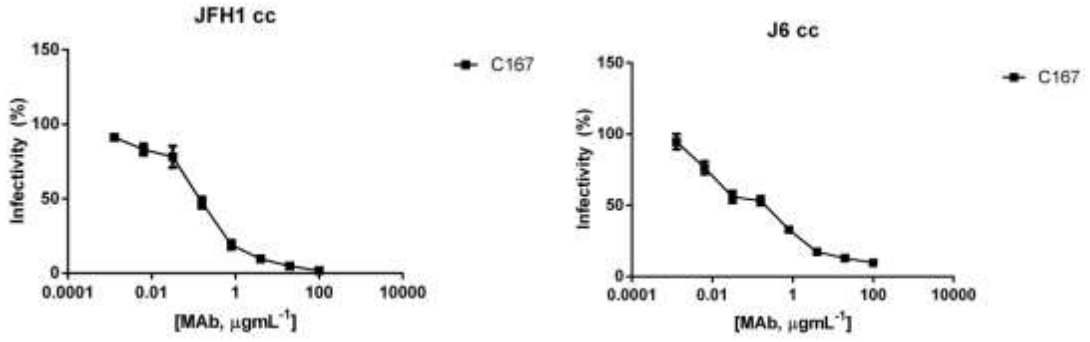
We evaluated the stability and affinity of the mAb in the buffer formulation after various stresses (immediately post one cycle of freezing-thawing (t0); 48h post thawing; 24h post thawing and a subsequent over-night incubation at 37°C; 1 month of storage at +4°C) compared to our reference mAb (c1671 produced by transient transfection and stored in PBS at +4°C). Results of stability studies demonstrated that the limited stress did not alter the integrity and the ability of the antibody to bind to its molecular target SR-B1, so its structure and function are maintained in this formulation and in the limited stress conditions indicated above, which mimic what will be done in toxicology studies.

Objective 1.3 In vitro efficacy testing of lead and back-up antibodies

Cross-reactivity and effective in vitro dose: GLP produced Mabs were tested for neutralising potency using a panel of HCVpp that represent isolates from different genotypes and differing neutralisation sensitivities, as well as a more limited panel of cell culture infectious HCV chimeras. Infectivity of HCVpp supplemented with various E1E2 glycoproteins (or control VSV) in the presence of Mab 1:7 a 1:7 variant (2A5) are presented below:



Against the larger pp panel (representing 12 different isolates and all 6 major genotypes) Mab 1:7 had IC50 values ranging from 0.4-40 ug/ml. In HCVpp, the anti-SRBI Mab C1671 was unable to neutralise. This very surprising finding was reproducible against various HCVpp, despite its proven ability to bind to soluble E2. In contrast this antibody was able to neutralise HCVcc (as indeed was mAb 1:7).



Generation of high throughput systems for screening patient-derived virus sensitivity to lead and back-up antibodies – Chimeric HCVcc development.

The genetic diversity of viruses often demands representation of many isolates in experimental systems to comprehensively phenotype an organism and to probe efficacy of prophylactic and therapeutic intervention (Burton *et al.*, 2012; Das *et al.*, 2013; deCamp *et al.*, 2014; Imhof & Simmonds, 2010; 2011; Pietschmann *et al.*, 2006; Urbanowicz *et al.*, 2015). Reverse genetics systems developed to probe host-pathogen interaction are typically constructed by Polymerase Chain Reaction (PCR) amplification and gene or genome cloning into plasmid vectors (Das *et al.*, 2013; deCamp *et al.*, 2014; Dutta *et al.*, 2013; Imhof & Simmonds, 2010; 2011; Lindenbach *et al.*, 2005; Pietschmann *et al.*, 2006). This involves a protracted multi-step process, utilising iterative rounds of restriction endonuclease (RE) digestion, stitch/fusion PCR and subsequent enzymatic modifications(Edmonds *et al.*, 2010; Imhof & Simmonds, 2010; Lindenbach *et al.*, 2005). Each amplification and ligation reaction can be error-prone, frequently requiring corrective back-mutation steps. Such workflows rely on either naturally occurring RE sites, which are often sub-optimally located, or on the generation of novel RE sites, which can impact on important biological properties such as RNA structure (You *et al.*, 2004) even when mutations are synonymous. Reliance on RE-based cloning can also be adversely affected by the presence of internal RE sites in the target gene, due to natural genetic variation of the virus. These experimental difficulties have limited the range of reference phenotyping systems despite their crucial importance in vaccine and treatment development and monitoring (Burton *et al.*, 2012; deCamp *et al.*, 2014; Imhof & Simmonds, 2011).

Hepatitis C virus (HCV) displays extensive genetic heterogeneity, particularly in the envelope genes (E1 and E2). Studies of the impact of natural variation on antiviral sensitivity(Imhof & Simmonds, 2010) or on neutralisation sensitivity to monoclonal antibodies(Ball *et al.*, 2014) or vaccine-sera(Chmielewska *et al.*, 2014), have suffered significantly from the drawbacks described above and the current lack of culture systems for wild-type patient-derived isolates. This has resulted in the development of drug sensitivity phenotyping methods that require significant genetic manipulation (Imhof & Simmonds, 2010; 2011) or the use of very small panels of infectious virus that are unrepresentative of patient-derived virus for antibody studies(Keck *et al.*, 2013).

DNA assembly technology (Gibson *et al.*, 2009; Irwin *et al.*, 2012) was therefore employed to develop a novel strategy for generating chimeric molecular cloning cassettes. Using this method a panel of 49 functional full-length HCV genomes containing patient-derived E1E2 has been derived. This has revealed novel insights into their function hitherto impossible using existing phenotyping resources, such as pseudoparticle assays. Importantly this approach can be widely used in reverse genetics studies of any genetically variable virus, or indeed other organisms, employing plasmid constructs.

Sequence files of parental HCV genotype 1 Bi-Gluc-H77C(1a)/JFH (T2700C, A4080T)(Reyes-del Valle *et al.*, 2012) and genotype 2 J6/JFH-1 (Lindenbach *et al.*, 2005) chimeric clones were screened for RE sites in the online NEBcutter 2.0 tool (http://nc2.neb.com/NEBcutter2/, New England Biolabs [NEB]). FseI was identified in both parental clones as a non-cutting RE site with 3' overhangs and therefore leaving the smallest footprint in standard In-Fusion cloning, retaining only 2 bases (5' GG, 3' CC) of the RE site post-cloning at each chimeric junction. The desired chimeric junction points at the 5' end of the signal peptide of E1 and the 3' C-terminus of E2, were scanned for the retained GG and CC dinucleotide motifs respectively, and candidate sites located (Fig. 1a). In the absence of convenient dinucleotide motifs, enzymatic blunting of 5' overhangs could be performed to extend choice of cloning sites to single bases. The reliance on the presence of only the double stranded remnant at the RE site thus increases potential chimeric junction options by several orders of magnitude.

Site directed mutagenesis (SDM) primers were then designed using the online NEB BaseChanger tool (http://nebasechanger.neb.com/) to simultaneously knock-out the parental E1E2 sequence between the GG and CC junction points and introduce the 4bp motif CCGG to create a complete FseI RE site (GGCCGGCC, Fig. 1aii; primers 1, 2, 14 and 15, Table 1). All primers were synthesised by Eurofins. SDM and transformation into *E.coli* with ampicillin selection was carried out using the Q5 SDM kit (NEB) according to the manufacturer's protocol, with resulting transformants screened by colony-PCR utilising HotStarTaq (Qiagen) as per the manufacturer's instructions and primers 3, 4, 16, 17 (Table 1). Colony PCR products of correct size were diluted 1 in 5 or 1 in 10 with ultrapure water and sent without purification for Sanger

sequencing confirmation (Source BioScience, Nottingham UK) with appropriate PCR primers. Transformants containing correctly formed ΔE1E2FseI E1E2 cassettes were selected for overnight growth in LB broth containing 100µg/ml ampicillin from which plasmid DNA was purified using a GenElute miniprep kit (Sigma Aldrich). ΔE1E2FseI E1E2 cassettes were RE digested overnight with FseI (NEB, Fig. 1aiii), digestion confirmed by agarose gel electrophoresis and then column purified using a QIAquick PCR purification kit (Qiagen). Purified digested plasmid cassettes were quantified using a NanoDrop 1000 spectrophotometer (Thermofisher Scientific).

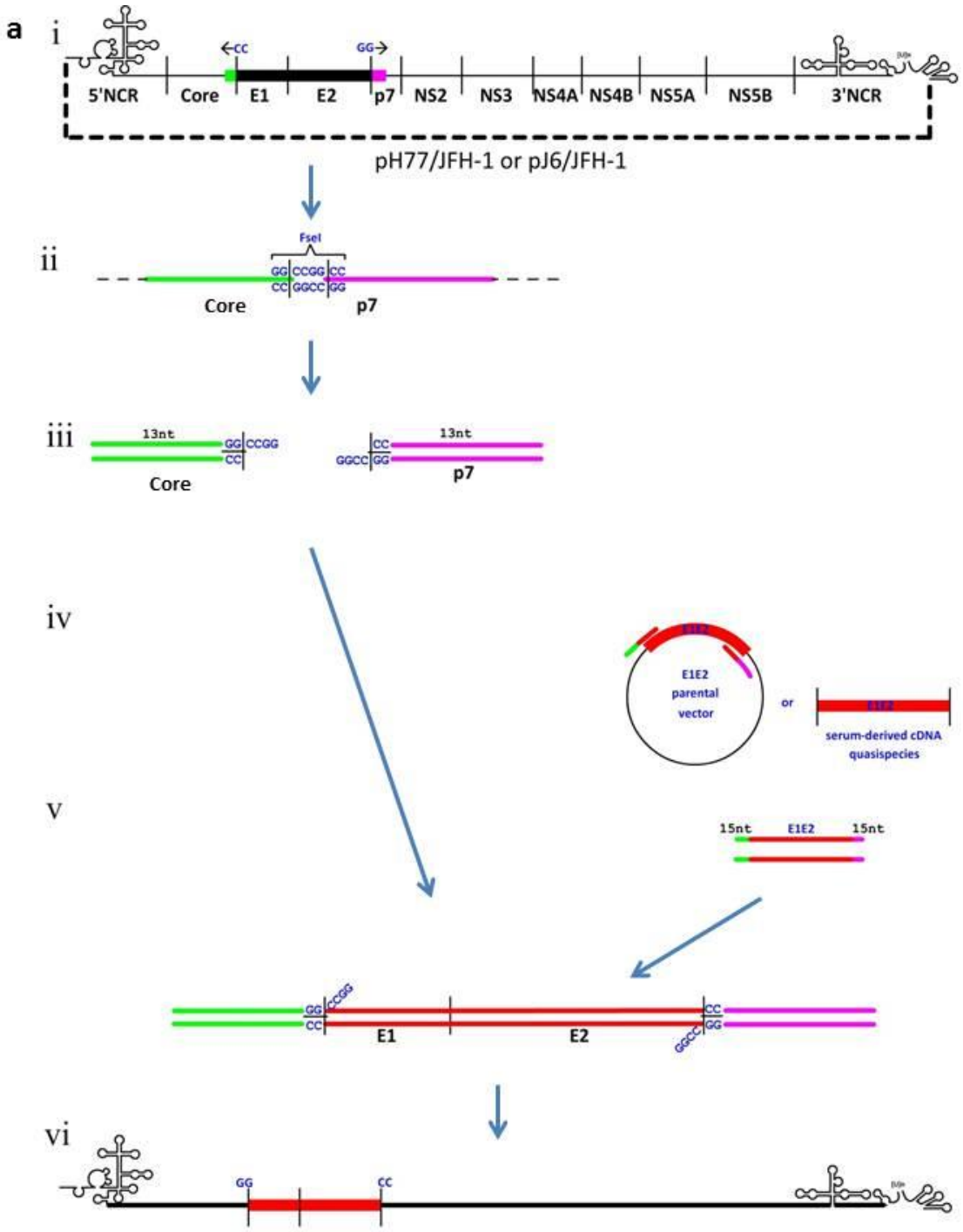


Figure 1 a) ΔE1E2FseI HCV cassette and chimera construction schematic. (i) The E1E2 region of the full genomic parental clone is deleted between the naturally occurring non-overhanging termini of a RE site (FseI here) not found in the parental clone, and replaced with the complete novel RE site by a single SDM reaction (i). The ΔE1E2FseI plasmid cassette (ii) is RE digested at the novel FseI RE site (iii) and assembled by In-Fusion cloning (v) with patient derived E1E2 amplified with 15bp terminal homology to the digested cassette (iv). The resulting chimera contains only wild type sequence from both parental full length genome and patient-derived E1E2 isolates.

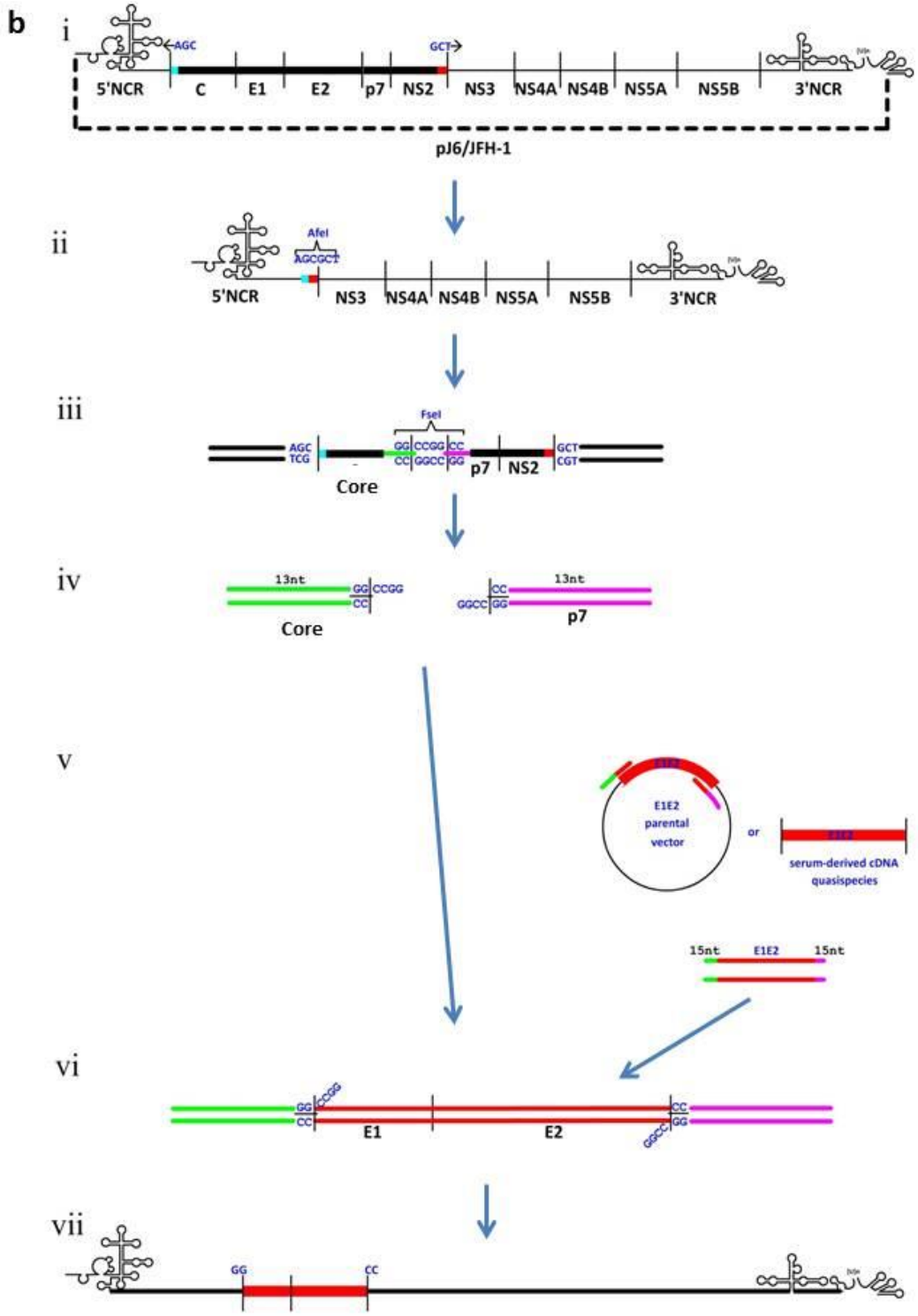


Figure 1b ΔCore-NS2 AfeI Inter-genotypic HCV cassette and chimera construction schematic. The Core-NS2 region of the full genomic parental clone is deleted between the naturally occurring non-overhanging termini of a RE site (AfeI here) absent in the parental clone, and replaced with the complete novel RE site by a single SDM reaction (i). The ΔCore-NS2 AfeI plasmid cassette (ii) is RE digested at the novel AfeI site and assembled by In-Fusion cloning with *in vitro* synthesized Core-NS2 with 15bp terminal homology to the digested clone and E1E2 region replaced with a unique RE

site (FseI here, iii) . The resulting ΔE1E2FseI cassette can then receive patient derived E1E2 of the same genotype as the inserted core and NS2 genes to create functional inter-genotypic chimeras (iv – vii).

A ΔCore-NS2AfeI cassette was also created to further test the strategy and facilitate the construction of chimeras with patient-derived genotype 3 E1E2. J6/JFH-1(Lindenbach *et al.*, 2005) was screened in NEBcutter 2.0 for non-cutting RE sites and the component termini of AfeI were identified as naturally-occurring at the desired chimeric junction points at the 5' start of core (AGC) and the 3' end of NS2 (GCT, Fig. 1bi). Whilst the chosen parental HCV genome presented a fortuitous mutagenesis option, the RE digest mapping described above could simply be applied to select a novel non-cutting site, even the same FseI as used in the E1E2 region as this would be lost in the first DNA assembly cloning reaction. SDM primers 33 and 34 (Table 1) were then designed in NEB BaseChanger to remove the Core-NS2 sequence between the AGC and GCT junction points to create a complete AfeI RE site (AGCGCT). SDM and clone verification was performed as above (with insert screening primers 35 and 36, Table 1) to create a ΔCore-NS2 AfeI JFH-1 plasmid cassette (Fig. 1biii). This process essentially removed the J6 component present in the parental chimera. The nascent ΔCore-NS2 AfeI cassette was RE digested with AfeI (Thermofisher Scientific) and reverted to its parental wild type using J6/JFH-1 core-NS2 amplified with PCR primers 37 and 38 (Table 1) by In-Fusion cloning (see below), to confirm unaltered phenotype. To create a novel HCV genotype 3 ΔE1E2FseI cassette, a Core-NS2 genotype 3 sequence (Genbank GU814263.1) was synthesized (Gene Strings, Invitrogen) already containing the above described ΔE1E2FseI modification and 15bp terminal homology to the AfeI RE-digested ΔCore-NS2 AfeI cassette and cloned by In-Fusion (see below) into the ΔCore-NS2 AfeI JFH-1 plasmid cassette (Fig. 1biii). This ΔE1E2FseI S52/JFH-1 cassette was verified using primers 35 and 36, then RE digested with FseI and prepared for In-Fusion cloning as above (Fig. 1biv).

54 HCV E1E2 isolates were derived from patients as previously described(Urbanowicz *et al.*, 2015), and clones UKN1A2.3, UKN1A2.4, UKN1A2.10, UKN1A2.12, UKN1A3.10, UKN1A5.18, UKN1A5.20, UKN1A5.22, UKN1A5.24, UKN1A6.1, UKN1A6.32, UKN1A7.50, UKN1A9.46, UKN1A11.1, UKN1A14.7, UKN1A14.38, UKN1A14.42, UKN1A16.7, UKN1A19.3, UKN1A20.8, UKN1A30.380, UKN1A56.126, UKN1A56.211, UKN1A59.508, UKN1A60.3, UKN1A70.2, UKN1A83.7, UKN1B12.6, UKN1B47.44, UKN1B48.8, UKN1B48.9, UKN1B48.14, UKN2A1.2, UKN2A1.5, UKN2A2.4, UKN2A3.12, UKN2B1.1, UKN2B2.8, UKN2C4.3, UKN2C4.6, UKN2C4.7, UKN2C4.8, UKN2C4.15, UKN2C4.19, UKN3A1.9, UKN3A1.28C, UKN3A13.6, UKN3A13.7, UKN3A13.15, UKN4.11.1, UKN4.21.16, UKN5.14.4, UKN5.15.7, UKN6.5.8 (Sequences in Genbank submission) were used in this study. DNA clone sequences were aligned using ClustalW, as implemented in the MEGA version 6 software(Tamura *et al.*, 2013), and complementary primers were designed to amplify the region between the aligned GG/CC junction points identified above (primers 5-13, 18-32 and 41-43, Table 1). Primers were also tagged with a 15 base region complementary to the appropriate end of the digested knock-out cassette, ending in the GG dinucleotide motif. 1 – 10 ng of plasmid clones were amplified using the Q5 high-fidelity DNA polymerase (NEB) as per the manufacturer's instructions (Fig. 1aiv and Fig. 1bv, with yield and specificity verified by agarose gel electrophoresis. 7.5µl of each PCR product was incubated with Cloning Enhancer (Clontech Laboratories Inc) as per the manufacturer's instructions. PCR products were then inserted without purification into the cassette by In-Fusion cloning (Fig. 1av and Fig. 1bvi).

In-Fusion reaction transformants were screened for presence of inserted DNA by colony PCR using cassette-specific primers (3 and 4, 16 and 17 and 39 and 40 for genotypes 1, 2 and 3 respectively, Table 1). Putative chimeric colony PCR product sequences were verified against parental ΔE1E2FseI E1E2 plasmid and patient derived E1E2 sequences in MEGA6. Confirmed chimeric plasmids (Fig. 1avi and Fig. 1bvii) were prepared by overnight *E.coli* culture as above. Cloned HCV plasmid constructs were linearised overnight with XbaI (Thermofisher Scientific), purified by the QIAquick kit and used as a template to generate HCV RNA transcripts with a MEGAscript T7 kit (Thermofisher Scientific).

Primer Number	Primer name	Purpose	Target region	Sequence (5' to 3')
1	H77_ΔFse_SDMf	SDM	H77 E1E2	GGCCAAGCGGAGGCGGCTTT
2	H77_ΔFse_SDMr	SDM	H77 core	GGCCAGGAAGGTTCCCTGTTGC
3	H77_E1E2_SCRf	E1E2 Screening	H77 core	GCGCAATTTGGGTAAGGTCA
4	H77_E1E2_SCRr	E1E2 Screening	H77 NS2	ACCCTTCAGATACCACGCAA
5	G1E1E2>H77Fa	E1E2 In-Fusion	H77 / 1a E1E2	AGGGAACCTTCCTGGTTGCTCTTTCTCTATC
6	G1E1E2>H77Fb	E1E2 In-Fusion	Gt1 E1E2	AGGGAACCTTCCTGGTTGCTCTTTCTCTATCTTCCTTC
7	G1E1E2>H77Ra	E1E2 In-Fusion	Gt1 E1E2	GCCGCCTCCGCTTGGGATATGAGTAACATCATCCA
8	G1E1E2>H77Rb	E1E2 In-Fusion	Gt1 E1E2	GCCGCCTCCGCTTGGGCTATGAGTAACACCATCCA
9	G1E1E2>H77Rc	E1E2 In-Fusion	Gt1 E1E2	GCCGCCTCCGCTTGGGCTACCAGCAGCATCATCCA
10	G1E1E2>H77Rd	E1E2 In-Fusion	Gt1 E1E2	GCCGCCTCCGCTTGGGCTATCAGCAGCATCATCCA
11	G1E1E2>H77Re	E1E2 In-Fusion	Gt1 E1E2	GCCGCCTCCGCTTGGGCTATGAGTAGCATCATCCA
12	G1E1E2>H77Rf	E1E2 In-Fusion	Gt1 E1E2	GCCGCCTCCGCTTGGGATATGAGTAGCATCATCCA
13	G1E1E2>H77Rg	E1E2 In-Fusion	Gt1 E1E2	GCCGCCTCCGCTTGGGCTATGAGTAACATCATCCA
14	J6_ΔFse_SDMf	SDM	J6 E1E2	CCGGCCGAAGCAGCACTA
15	J6_ΔFse_SDMr	SDM	J6 core	CCGGGTAAGTTCCCTGTTGC
16	J6_E1E2_SCRf	Screening	J6 core	GCTTTGCCGACCTCATG
17	J6_E1E2_SCRr	Screening	J6 NS2	GTACCAAGCAGCCACGAAAA
18	G2E1E2>J6Fa	E1E2 In-Fusion	Gt2 E1E2	AGGGAACTTACCCGGTTGCTCTTTTTCTATC
19	G2E1E2>J6Fb	E1E2 In-Fusion	J6 E1E2	AGGGAACTTACCCGGTTGCTCCTTTTCTATC
20	G2E1E2>J6Fc	E1E2 In-Fusion	JFH-1 E1E2	AGGGAACTTACCCGGTTTCCCCTTTTCTATC
21	G2E1E2>J6Ra	E1E2 In-Fusion	Gt2 E1E2	CTAGTGCTGCTTCGGCTTGGCCCA
22	G2E1E2>J6Rb	E1E2 In-Fusion	J6 / JFH-1 E1E2	CTAGTGCTGCTTCGGCCTGGCCCA
23	G1E1E2>J6Ra	E1E2 In-Fusion	Gt1 E1E2	CTAGTGCTGCTTCGGCCTGGGATATGAGTAACAT
24	G34E1E2>J6Fa	E1E2 In-Fusion	Gt3/4 E1E2	AGGGAACTTACCCGGTTGCTCTTTTTCTATC
25	G1356E1E2>J6Fb	E1E2 In-Fusion	Gt1/3/5/6 E1E2	AGGGAACTTACCCGGTTGCTCTTTCTCTATC
26	G3E1E2>J6Ra	E1E2 In-Fusion	Gt3 E1E2	CTAGTGCTGCTTCGGCCTGTGAAATCATCAGCAT
27	G3E1E2>J6Rb	E1E2 In-Fusion	Gt3 E1E2	CTAGTGCTGCTTCGGCCTGTGATATCATCAGCAT
28	G4E1E2>J6Ra	E1E2 In-Fusion	Geno4 E1E2	CTAGTGCTGCTTCGGCTTGACTTACCATGAACAT
29	G4E1E2>J6Rb	E1E2 In-Fusion	Gt4 E1E2	CTAGTGCTGCTTCGGCTTGACTTACCATAAACAT
30	G5E1E2>J6Ra	E1E2 In-Fusion	Gt5 E1E2	CTAGTGCTGCTTCGGCCTGACAGACTAGGAGCAT
31	G5E1E2>J6Rb	E1E2 In-Fusion	Gt5 E1E2	CTAGTGCTGCTTCGGCCTGGCAAACTAGGAGCAT
32	G6E1E2>J6Ra	E1E2 In-Fusion	Gt6 E1E2	CTAGTGCTGCTTCGGCGTTGGTTATGAGCAGCAT
33	J6_ΔAfeI_SDMf	SDM	J6 Core	GCTCCCATCACTGCTTATGCCCAGCAAACAC
34	J6_ΔAfeI_SDMr	SDM	J6 NS2	GCTCATGGTGCACGGTCT
35	JFH_C-NS2_SCRf	Screening	JFH-1 5'UTR	ACTCTATGCCCGGCCATTT
36	JFH_C-NS2_SCRr	Screening	JFH-1 NS3	GGCCTGTTCTGTCCTGTCA
37	J6_C-NS2>JFHf	CoreNS2 In-Fusion	J6 Core-NS2	CCGTGCACCATGAGCACAAATCCTAAACCTCAAAGAA
38	J6_C-NS2>JFHr	CoreNS2 In-Fusion	J6 Core-NS2	AGCAGTGATGGGAGCGAGAAGACTCCACCCCTT
39	G3_E1E2_SCRf	Screening	Gt3 E1E2	CGTAGGAGGCGTCGCAAG
40	G3_E1E2_SCRr	Screening	Gt3 E1E2	AGCCAATACCATGTGTCCCA
41	G3E1E2>G3Fa	E1E2 In-Fusion	Gt3 E1E2	AGGGAACTTGCCCGGTTGCTCCTTTTCTATCTTCC
42	G3E1E2>G3Fb	E1E2 In-Fusion	Gt3 E1E2	AGGGAACTTGCCCGGTTGCTCTTTCTCTATCTTC
43	G3E1E2>G3R	E1E2 In-Fusion	Gt3 E1E2	AGCATCAGCCAAAGGGCAACGCACACGC

Table 1: Primers for PCR amplification. Primer numbers, names, target gene, breadth of target and sequence are detailed. HCV genotype is abbreviated to 'Gt' and 15 base 5' homology tags are indicated in grey.

Initially 21 patient-derived (10, 6 and 5 clones from genotype 1, 2 and 3 respectively) and 3 reference E1E2 isolates (H77, J6 and S52 of genotype 1, 2 and 3 respectively) were selected based on their functionality in the pseudoparticle assay (Lavillette *et al.*, 2005; Tarr *et al.*, 2011; Urbanowicz *et al.*, 2015). All of these isolates

were able to replicate and produced infectious virus at all three sampled time points (24, 96 and 192 hr post electroporation). The analysis was then extended to include E1E2 isolates that have been classed as non-functional in the pseudoparticle assay (below the limit of detection, data not shown). Again, all 28 patient-derived E1E2 isolates (22 genotype 1 and 6 genotype 6) were both replication competent and produced infectious virus at the three sampled time points (Fig. 2a-c). The inclusion of five genotype 3 isolates is a leap forward and begins to reduce the paucity of isolates from this genotype. Previous studies have shown that patients harbour a high frequency of entry-deficient E1E2 (Lavillette *et al.*, 2005). Crucially, the data presented here shows that the HCVpp system is not a good predictor of E1E2-mediated cell entry (Fig. 2d). This technique has thus generated a panel of infectious HCV chimeras that is significantly larger than all the isolates produced to date; expanding this panel even further has now become readily achievable.
An additional panel of 10 chimeras was generated using selected non-genotype 2 E1E2s (HCV E1E2 UKN 1A20.8, 1A14.42, 3A13.6, 3A13.7, 3A13.15, 4.11.1, 4.21.16, 5.14.4, 5.15.7 and 6.5.8) in the J6/JFH-1 ΔE1E2FseI genotype 2 cassette. Although these were all positive in the pseudoparticle system and replication competent they did not yield infectious virus (data not shown). This confirms that infectious chimera construction relies on the presence of genotype matched core-NS2 genome segments, as has been described previously (Lindenbach *et al.*, 2005; Pietschmann *et al.*, 2006).

In summary, our method facilitates flexible and rapid creation of diverse, functional and seamless molecular chimeras for development and testing of novel therapeutics. This method can also be applied to personalised medicine by pre-testing patient-specific viruses for treatment sensitivity (Angus *et al.*, 2003; Imhof & Simmonds, 2011). Importantly, location of the cloning site is only limited by the need to identify a RE site not contained in the parental cassette backbone and, given that any RE digest overhang is removed during the cloning step, targeting to single nucleotide positions is possible. This gives a degree of flexibility and ease hitherto unheard of for a single-step chimeric reverse genetics procedure.

We previously reported the generation of 52 chimeric HCVcc clones (including 3 reference clones and 49 patient-derived E1E2 clones; 32 from genotype 1, 12 from genotype 2 and 5 from genotype 3). All of these isolates were able to replicate and produced infectious virus at all three sampled time points (24, 96 and 192 hr post electroporation). The inclusion of five genotype 3 isolates is a leap forward and begins to reduce the paucity of isolates from this genotype. However, these genotype 3 clones gave low virus titres, hampering their use in antibody neutralisation experiments.

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We introduced an additional 13 patient-derived E1E2 clones to the Gt3 cassette, however they gave very poor titres or not infectious virus.

Therefore, it was deemed necessary to improve the chimeric HCVcc in several aspects.

1) Increase virus titre of genotype 3 clones to enable further characterisation
2) Assess the viability of a reporter-based system to facilitate high-throughput use of these chimeric viruses
3) Expand the number of genotype 3 clones
4) Expand the chimeric HCVcc system to include genotype 4, 5 and 6
5) Establish the chimeric HCVcc system as a resource for other researchers

Increase virus titre of genotype 3 clones to enable further characterisation:

In the original work describing the generation of a viable chimeric genotype 3 virus including the core-NS2 genes from the S52 isolate, 2 cell culture adaptions were identified which conferred viability without the requirement for additional mutations. These were I793S in the p7 protein and K1404Q in NS3. In the generation of the genotype 3 cassette we included the p7 mutation I793S into the fabric of the cassette. In an attempt to increase virus titre of the genotype 3 clones we introduced the NS3 K1404Q mutation into the JFH-1 region of cassette by site-directed mutagenesis. Comparison of the reference clone S52 E1E2 genes in the original and K1404Q backbones showed significantly increased virus titres in the K1404Q setting.

Therefore we cloned 18 patient-derived clones into the K1404Q cassette and compared the virus titres generated in the two backbones. High titre virus preparations were achieved for 13 of 19 genotype 3 clones from different patients (Table 1). This represents a huge advance in the diversity of genotype 3 clone infectious clones available for antibody neutralisation studies.

		FFU/ml			
		wt Gt3 bb		K1404Q Gt3 bb	
		24h P.E.	96h P.E.	24h P.E.	96h P.E.
Genotype 3 E1E2 clone name	**S52**	0	0	1,621	22,813
	3A1.4	0	0	0	0
	3A1.9	3	0	29	3,042
	3A1.16	0	0	363	2,084
	3A1.21	0	0	1,120	12,625
	3A1.22	3	0	0	3
	3A1.23	0	0	260	4,188
	3A1.24	0	0	0	0
	3A1.28	115	145	1,242	38,906
	3A1.29	N/T	N/T	0	0
	3A1.30	3	0	190	2,625
	3A1.31	0	0	1,696	66,250
	3A1.32	0	0	769	5,979
	3A11.2	0	0	0	0
	3A11.4	0	0	14	6,438
	3A11.5	0	0	0	30
	3A13.6	3	75	4,958	102,344
	3A13.7	28	345	269	112,500
	3A13.15	15	110	68	35,208

Table 1. Comparison of genotype 3 Chimeric HCVcc clone titres at 24 and 96 hours post-electroporation in the original (wt) and mutant cassette backbone (K1404Q). Genotype 3 E1E2 clones isolated from 3 patients (3A1, 3A11 and 3A13) and reference clone (S52). Introduction of the single K1404Q mutation into NS3 increased titres ~300 fold at 96h P.E. compared to the wt cassette backbone. P.E. Post-electroporation, N/T Not tested, bb backbone, FFU/ml focus-forming units per millilitre.

Assess the viability of a reporter-based system to facilitate high-throughput use of these chimeric viruses

The original genotype 1 H77/JFH chimera was obtained as a bi-cistronic molecule containing 2 IRES structures, one controls expression of the HCV polyprotein and another controlling expression of the secreted reporter molecule Guassia Luciferase. We investigated the potential of using Guassia luciferase as a surrogate for virus infectivity by cloning the second IRES and G.Luc gene into the genotype 2 cassette. Consistent with the original reports on bi-cistronic viruses, the infectious titres achieved were 0.5 – 2 logs lower than the mono-cistronic counterpart. This was clearly shown when analysing wt JFH-1 E1E2 and 7 point mutant clones (S1-7) in the mono- and bi-cistronic genotype 2 cassettes (Figure 2).

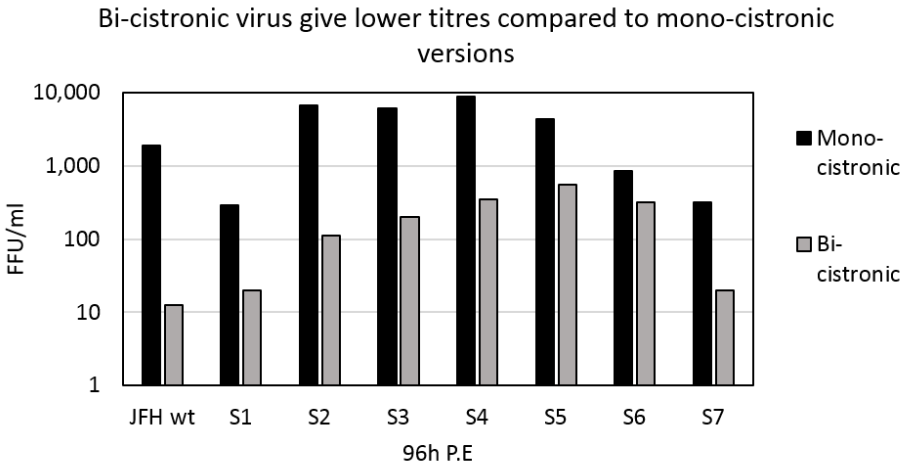


Figure 2. Comparison of virus titres produced by JFH-1 E1E2 clones at 96 hours post-electroporation

Further analysis was performed using the Gt4, Gt5 and Gt6 reference strains (ED43, SA13 and HK6A, respectively) in the corresponding genotype-matched mono- and bi-cistronic cassettes showed that the monocistronic versions yielded infectious virus whereas the bi-cistronic versions did not (Table 2). However, the low titres achieved for genotype 4, 5 and 6 even in the mono-cistronic cassettes suggests that additional genotype-specific mutations in the JFH-1 backbone of the cassettes will be required to generate sufficient virus for analysis. It is note-worthy that the genotype 5 SA13 reference strain chimera never produced any signs of active replication following electroporation (Figure 3) whereas the other reference strain HCVcc chimeras did.

	96h P.E. FFU/ml	
	Mono-cistronic	Bi-cistronic
JFH-1	408.8	42.5
S52	2.5	0
ED43	7.5	0
SA13	0.0	0
HK6A	65.0	0

Table 2

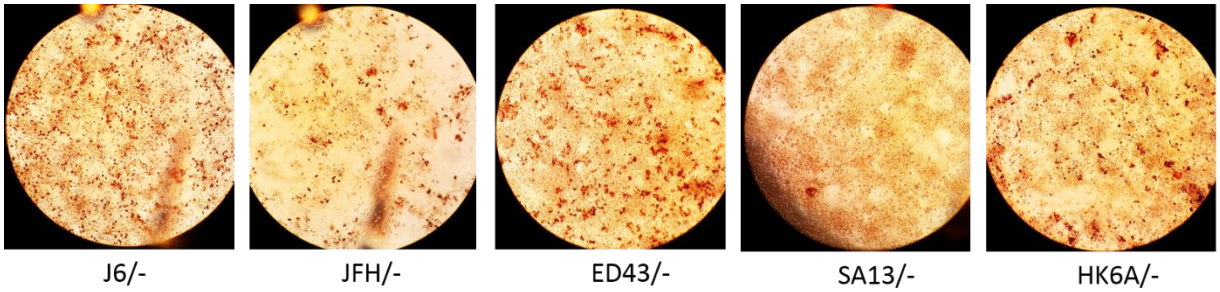


Figure 3

Expand the chimeric HCVcc system to include genotype 4, 5 and 6 clones

The decision was taken not to pursue further development of the Genotype 4, 5 and 6 cassettes for generating HCVcc chimeras containing patient-derived E1E2 clones. The following reasons informed this decision:

1) Poor virus titres were achieved in the wt mono-cistronic cassettes for genotypes 4 and 6, despite high levels of active replication following electroporation, indicating that each cassette would require modification with at least 1 (possibly multiple) characterised culture adaptations in order to produce sufficient yields for subsequent analysis. This would be a time-consuming task, possibly involving numerous steps of SDM, sub-cloning and HCVcc characterisation to achieve a viable cassette.
2) Active replication was not observed for the genotype 5 SA13 reference strain chimera, suggesting a fundamental issue with the chimeric virus clone that could require significant investigation.
3) Since the introduction of the highly effective DAAs, genotypes 4, 5 and 6 have become very readily treatable and so there is a less pressing need for effective culture systems for these specific genotypes.
4) Genotypes 4, 5 and 6 are globally less of a burden than genotypes 1, 2 and 3 and so a greater focus on these genotypes would represent a better cost-benefit outcome.

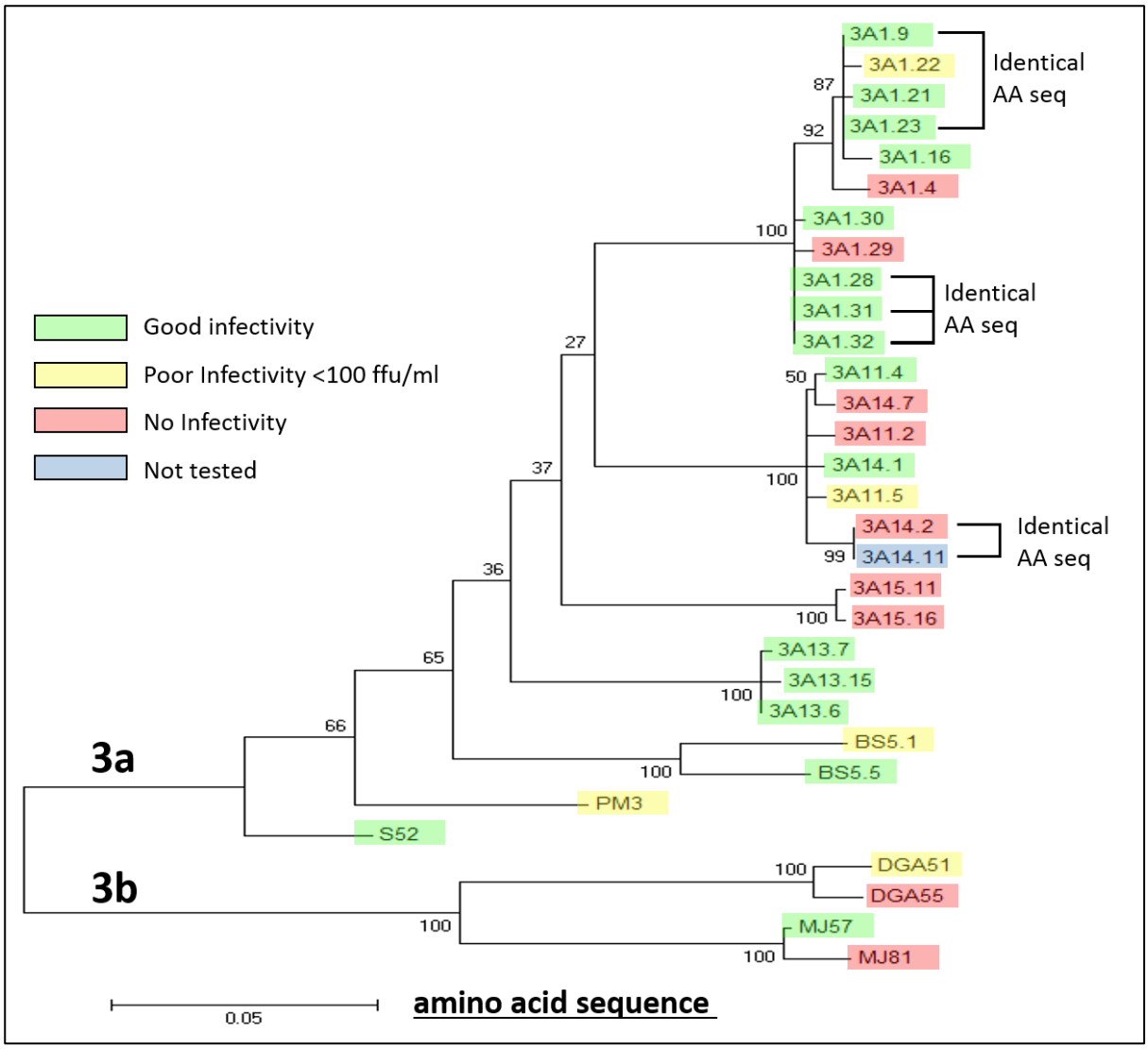
Increase virus titre and genetic diversity of genotype 3 HCVcc clones to enable further characterisation

In the original work describing the generation of a viable chimeric genotype 3 virus including the core-NS2 genes from the S52 isolate, 2 cell culture adaptions were identified which conferred viability without the requirement for additional mutations. These were I793S in the p7 protein and K1404Q in NS3. In the generation of the genotype 3 cassette we included the p7 mutation I793S into the fabric of the cassette. In an attempt to increase virus titre of the genotype 3 clones we introduced the NS3 K1404Q mutation into the JFH-1 region of cassette by site-directed mutagenesis. Comparison of the reference clone S52 E1E2 genes in the original and K1404Q backbones showed significantly increased virus titres in the K1404Q setting.

In the previous reporting period we had cloned 18 patient-derived E1E2 genes into the K1404Q cassette and compared the virus titres generated in the two backbones. High titre virus preparations were achieved for 13 of 19 genotype 3 clones from different patients (Table 1). This represented a huge advance in the diversity of genotype 3 clone infectious clones available for antibody neutralisation studies.

Further to this, we cloned an additional 12 patient-derived E1E2 genes into the K1404Q cassette and assessed the ability of these HCVcc clones to generate infectious virus. These clones represent a great genetic diversity; 4 of these clones are of genotype 3B subtype whereas the previous 18 were all genotype 3A.

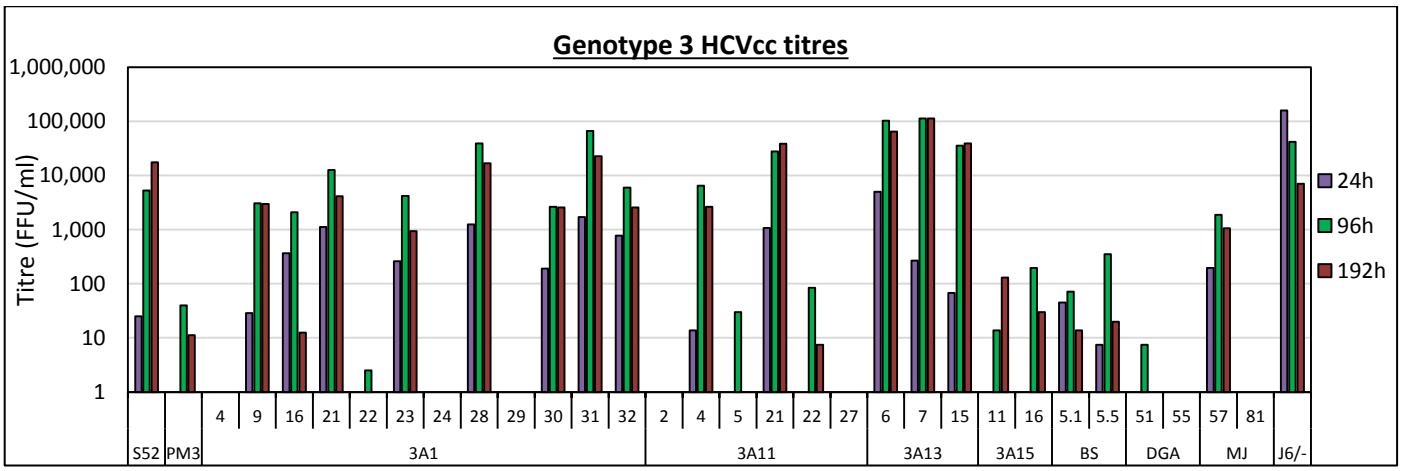
This greater genetic diversity of clones will help to scrutinise whether there is any differential usage of cell receptors, specifically receptor subtype usage that will have relevance to development and analysis host receptor antibodies.



Following phylogenetic analysis, some of the Gt3 patient-derived HCVcc clones were re-named. It was apparent the clones from samples identified as 3A11 and 3A14 were very likely to be from the same patient (both were described as being from a patient with acute angina), but received from a national repository for testing at different dates leading to inconsistent naming.

Therefore, clones labelled as 3A14.1, .2, .7 and .11 be renamed and appended to the 3A11 set. The clone number will be increased by 20 for each of them to prevent any numbering duplication.

Separately, several clones, although they have unique nucleic acid sequences, have identical amino acid sequences. It is encouraging to note that identical HCVcc clones, generated from separate DNA cloning, RNA transcription and electroporation, produce similar virus production profiles (see 3A1.28/31/32).



In vitro resistance investigation completed

Due to GMP material not being produced on time and limited amounts of pre-GMP material, the experiments have been completed using a different antibody, D03. This antibody has been shown to target the same binding region and residues as mAb 1:7, so is an adequate substitute for these experiments.

JFH-1 was passaged nine times in the presence of D03, with increasing concentrations at later passages. (Table 1)

Passage No.	D03 Concentration (μgmL^{-1})	IC value
1-3	10	IC50
4-6	30	IC90
7-9	50	>IC95

Table 1: Concentration of D03 used for each passage

After each passage, the supernatant containing the virus was concentrated (3mL to 0.5mL). Half was kept for analysis, the other half used for the following passage. Each passage was for 3 days. After nine passages, the virus was concentrated and placed in RNAlater. The bulk product was sent for sequencing and 5 mutations were found: one in the E1 protein and four in the E2 protein (Table 2).

Position	Substitution	Codon change	Amino acid change
348 (E1)	T -> C	GTT -> GCT	Valine (V) -> Alanine (A)
402 (E2)	T -> C	GTG -> GCG	Valine (V) -> Alanine (A)
451 (E2*)	G -> A	GGG -> AGG	Glycine (G) -> Arginine (R)
488 (E2)	C -> G	CAC -> GAC	Histidine (H) -> Aspartic acid (D)
497 (E2)	G -> A	GTC -> ATC	Valine (V) -> Isoleucine (I)

Table 2: Position and type of non-synonymous substitution identified after 9 passages in the presence of D03. * denotes change also identified in the control (no antibody) virus.

G451R also appeared in the negative control. This was expected as it is a documented JFH-1 cell culture adaptation that occurs with no selection pressure applied, and is a fitness gain for the virus in cell culture. It has not been found *in vivo* and was excluded from further experiments.

Due to the nature of the sequencing, it is unknown whether all substitutions occurred together or on different transcripts. To test this, 7 mutants were made by site directed mutagenesis (SDM) in

the original JFH-1 background. Three individuals; A402, D488 and I497, three doubles; A402+D488, A402+I497 and D488+I497 and a triple A402+D488+I497.

Significant results:

Establishing the UKNP HCVpp E1E2 plasmid clones as a resource for other researchers

Nearly 80 plasmid clones were characterised, validated, published and submitted to the Addgene international plasmid repository.

Establishing the chimeric HCVcc system as a resource for other researchers

The power of the chimeric HCVcc cassettes mean that collaborators need only provide us with the E1E2 genes in a plasmid and provide the sequence electronically. Using a panel of genotype-specific InFusion PCR primers we are able to amplify the E1E2 genes and clone them rapidly and efficiently. Following requests from collaborators, we have generated ~50 chimeric HCVcc clones (mostly genotype 1) for a range of projects and distributed them for analysis. However, due to restrictions imposed by third party IPR, we are not able to make the chimeric HCVcc clones or the cassettes available through an international repository.

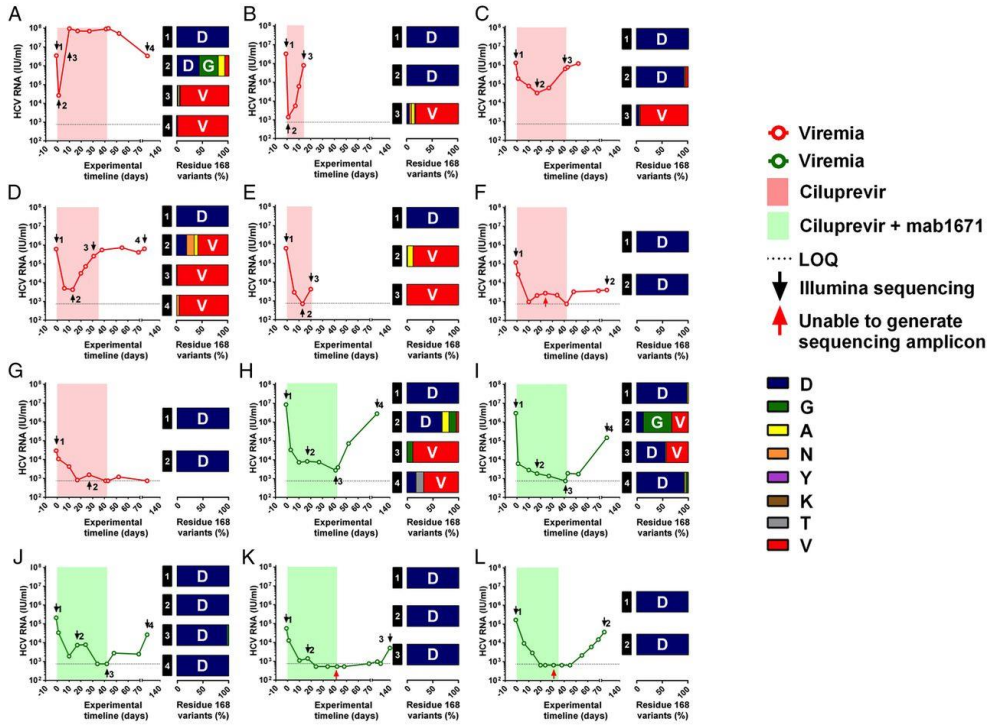
Objective 1.4 In vivo efficacy testing of lead and back-up antibodies

We have previously shown that the anti-SR-B1 antibody mAb1671 is highly capable of preventing an HCV infection of different genotypes, both in a prophylactic and post-exposure setting. Since this antibody inhibits direct cell-to-cell transmission we evaluated whether a mAb1671 therapy would be able to prevent the spread of drug-resistant viruses. By potently inhibiting the spread of resistant variants selected during DAA therapy, anti-SR-BI mAbs may be able to prevent on-therapy breakthrough of resistance-associated variants (RAVs) and improve overall SVR rates in hard-to-treat patient groups. As a proof-of-concept study, we investigated the effect of adding the SR-BI-targeting mAb1671 to PI monotherapy (ciluprevir) in human-liver mice chronically infected with HCV gt1b. Chimeric human-liver mice represent the only small animal model where chronic HCV infection in humans can be mimicked. A productive viremia consisting of a cloud of related viral variants (quasispecies) was achieved with clinically relevant non-laboratory strains and the emergence of RAVs monitored at high resolution using next-generation sequencing.

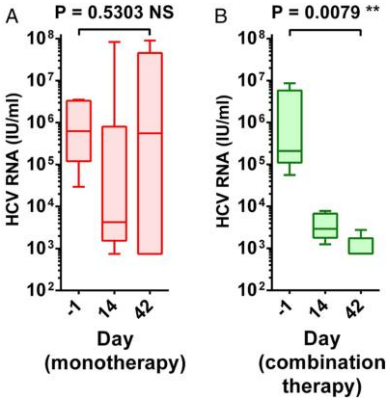
In vivo virological response during mono and combination therapy.

During PI monotherapy, five out of seven mice experienced viral breakthrough (defined by a >10-fold increase in HCV RNA over nadir, or when viral RNA becomes quantifiable after having been below the limit of quantification (LOQ)) (Figure panel A–G). End-of-treatment (EOT) response (HCV RNA below LOQ at day 42) was observed in only two mice (Figure panel F, G). In the combination therapy arm, no viral breakthrough was observed, with EOT responses in four out of five mice (Figure panel I–L).

The mono and combination therapy arms had similar median viral loads at baseline ($p>0.99$) and comparable reductions in median viraemia during the first two weeks of treatment ($-2.17\times\log10$ and $-1.86\times\log10$, respectively) (Figure below). However, the median viraemia diverged markedly between these treatment groups from day 14 to EOT (day 42) ($+2.12\times\log10$ and $-0.59\times\log10$, respectively) and also when considering the complete treatment period ($-0.05\times\log10$ and $-2.45\times\log10$, respectively). In contrast to the monotherapy group, the median viraemia at EOT was significantly reduced in the combination therapy group ($p=0.0079$).

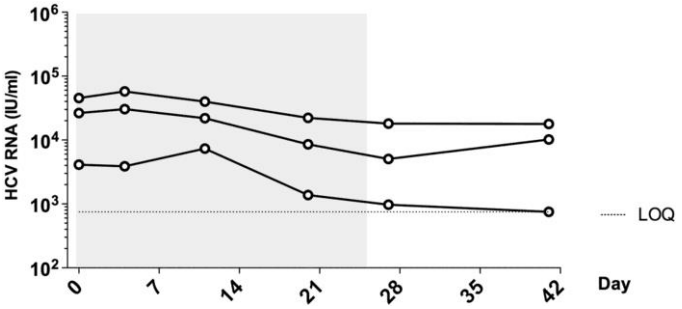


HCV viraemia and protease population variant composition at residue 168 prior to, during and following ciluprevir therapy with and without the addition of an entry inhibitor (the scavenger receptor class B type I-specific antibody mAb1671) in human-liver mice. *Twelve gt1b-infected mice were divided over two study groups, each panel represents a single animal. Seven received ciluprevir monotherapy (A–G, shown in red) and five received the combination of ciluprevir with mAb1671 (H–L, shown in green). The shaded area indicates the duration of therapy. Each data point represents the plasma HCV RNA level (IU/mL) of an individual subject at a given time. The limit of quantification (LOQ) equals 750 IU/mL. Illumina deep-sequencing analysis of the HCV protease domain in NS3 was performed at indicated time points (black and red arrows indicate successful and unsuccessful generation of sequencing amplicons, respectively). The amino acid population frequency at position 168 is presented as horizontal bars accompanying the graphs. The wild-type amino acid, aspartic acid (D), is represented in blue, whereas mutants have been assigned other colours.*



Comparison of median viraemia between day −1, 14 and 42 in ciluprevir monotherapy (A) and ciluprevir/mAb1671 combination (B) therapy groups. *Day –1 represents baseline viraemia prior to start of therapy. End-of-therapy (day 42) viraemias are available for five mice in each group. The Whiskers contain all values. p Values compare median viraemias between baseline and end of therapy in one treatment group using the unpaired non-parametric two-tailed Mann–Whitney test.*

In order to determine whether mAb1671 exerts an antiviral effect as such during chronic infection, three gt1b infected chimeric mice received mAb1671 monotherapy over a 4-week period (Figure 5). Although a subtle downwards trend might be perceivable, the HCV RNA levels in the plasma of these mice did not fluctuate more than what is routinely observed in non-treated infected animals. In addition, a 2-week follow-up did not reveal a general rebound after cessation of therapy, underscoring the absence of a pronounced antiviral effect of mAb1671 during chronic infection.



Evaluation of the antiviral effect of the scavenger receptor class B type I-specific antibody mAb1671 alone in humanized mice chronically infected with gt1b. *Three gt1b-infected mice received mAb1671 three times weekly during four weeks. Each data point represents the plasma HCV RNA level (IU/mL) of an individual chimeric mouse at a given time point. The limit of quantification (LOQ) equals 750 IU/mL.*

Quasispecies analysis during therapy.

The observed differences in EOT outcome were primarily due to the occurrence of on-therapy viral rebound in the monotherapy animals. To investigate whether these breakthrough viruses represented drug-resistant variants, we tracked the evolution of the protease region of the viral genome via Illumina deep sequencing technology. Nucleotide coverage across the protease domain was consistent between samples and independent of study phase or treatment regimen. Viral genomic input into sequencing amplicons was determined empirically and ranged between 1×10^3 and 3×10^6 genomes, representing orders of magnitude greater population sampling than conventional clonal sequencing.

As previously described, the vast majority of variation in the NS3 protease region was detected at silent sites. Besides a minor fraction (<2.2%) of D168A mutants in the viral population of one mouse (Figure panel I), PI-RAVs were not detectable at baseline in the remaining 11 animals. However, in all mice of the monotherapy cohort that experienced a viral breakthrough, PI-RAVs consistently became dominant during viral rebound (Figure panel A–E). The most frequently identified mutation was the D168V substitution, a mutation known to confer resistance to ciluprevir and other PIs. The detection of D168V in monotherapy-treated animals correlated with viral breakthrough in all cases. In some animals, transient low-level R155Q and A156V/T RAVs were detectable at nadir, but these became undetectable following viral rebound (data not shown). This observation is not surprising since R155Q and A156V/T mutations reduce the replicative capacity of the mutated virus to 10 and 18/30% of that of the wild type, while the fitness of the D168V mutant remains nearly unaffected. In mice achieving EOT response upon PI monotherapy, exclusively wild-type sequences were detected (Figure panel F, G). In the combination therapy cohort, however, treatment response did not correlate with the absence of detectable PI-RAVs. Indeed, although no on-therapy viral rebounds were observed, two mice presented with RAVs during therapy (Figure panel H, I) and one developed a minor D168G population (3.1%) at EOT (Figure panel J). Therefore, the occurrence of RAV D168V did not lead to viral breakthrough in the dual therapy arm, indicating the viremia remained controlled, independent of PI-RAVs development, as long as the entry inhibitor was co-administered.

This shows that SR-BI blockade via mAb1671 administration limits breakthrough of PI-RAVs during PI therapy. These data thus demonstrate the effectiveness of an entry inhibitor targeting a host cell receptor, administered in combination with DAAs, at controlling HCV viremia in a preclinical small animal model. Clinical safety and efficacy studies are needed to evaluate whether addition of an entry inhibitor to a cocktail comprised of solely DAAs would suffice to reach SVR, without the need of IFN and RBV.

To elucidate the minimal dosing regimen needed to achieve full protection we decreased the treatment dose to 100 μg/dose. Start of therapy (day -1) and dosing schedule (day -1, 1, 5, 8 and 12) was not changed. As a start, one humanized mouse (ID K1036R) that received this dosing regimen was inoculated with HCV of strain H77 (infectious dose 10^4 IU). Although HCV RNA remained negative (limit of detection = 750 IU/ml) until week 2, a viral load of $7{,}05\text{x}10^5$ IU/ml could be detected at week 3 post inoculation. This indicates that a 4-fold decrease of the treatment dose is no longer effective.

Activity of mAb1671 against virus isolates with reduced SR-BI dependency: The data above shows that prophylactic and post-exposure treatment of mAb 1671 can completely prevent and block HCV infection. However, HCV variants with reduced SR-BI dependency have been described in the literature, which could potentially limit the use of SR-BI targeting therapy. Therefore we wanted to investigate whether these mutated viruses would remain sensitive to our mAb therapy in vivo.

We first confirmed that several viral isolates, Jc1DHVR1, Jc1_G451R, Jc1_mtCD81 and J6/JFH1_clone 2, are indeed less dependent on SR-BI for *in vitro* infection and cell-to-cell spread (Figure 13).

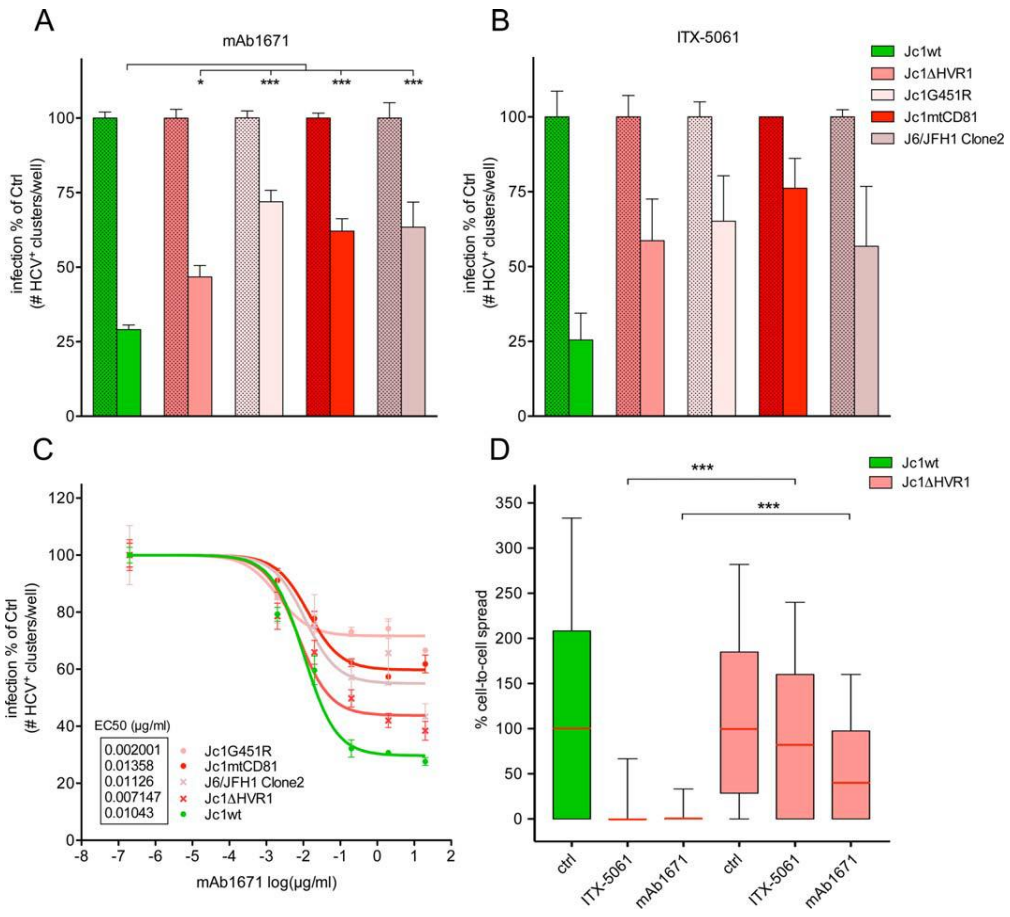


Figure 13: In vitro neutralization assay. *Huh7.5 cells were pretreated with 20 lg/mL mAb1671 (A) and 2 lM ITX-5061 (small molecule SR-BI antagonist) (B) before infection with Jc1wt, Jc1DHVR1, Jc1G451R, Jc1mtCD81, and J6/JFH1 Clone2. After 2 days the number of HCV-positive clusters was counted and normalized to control. The effect of mAb1671 on the infectivity of Jc1wt, DHVR1, and mtCD81 was evaluated in 10 separate wells over four different experiments, while the effect on Jc1G451R and J6/JFH1 Clone2 was assessed over eight separate wells in three different experiments. The data of these experiments were merged and the means are shown. The asterisks (*P<0.05; and ***P<0.001) indicate that the effect of mAb1671 on Jc1DHVR1, Jc1G451R, Jc1mtCD81, and J6/JFH1 Clone2 differs significantly from its effect on Jc1wt infectivity. The effect of ITX-5061 was assessed in one experiment and the means of duplicates are shown (this limited sample size did not allow statistical analysis). (C) HCVcc infectivity under increasing concentrations of mAb1671. All conditions were tested in quadruplicate and the mean values are shown. (D) Box-and-whisker presentation of cell-to-cell spread. While mAb1671 (20 ug/mL) and ITX-5061 (2uM) efficiently inhibit direct cell-to-cell transmission of Jc1wt, only a minor effect can be observed against Jc1DHVR1 (***P<0.001). For each condition, the amount of infected target cells per cluster was determined in at least 100 clusters and normalized to the median of the control. The box extends from the 25th to the 75th percentile, while the whiskers indicate the 10th and 90th percentile. The red horizontal line indicates the median. Error bars in (A-C) represent the standard error of the mean.*

Next we infected humanized mice with these in vitro-resistant viral variants and subsequently started a 2-week therapy with the anti-SR-BI specific antibody mAb1671. Weekly analysis of the mouse plasma showed that these in vitro-resistant variants remain sensitive to our antibody therapy in vivo (Figure 14).

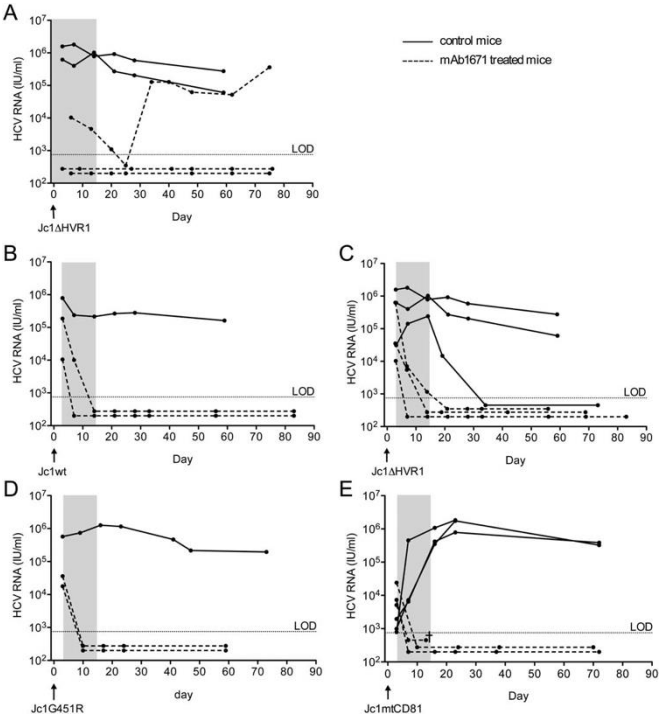


Figure 14: Efficacy of the SR-BI-specific antibody mAb1671 in blocking HCV infection in humanized mice. *Within a 2-week period (indicated by the gray area) the animals received six intraperitoneal injections, each containing 400 lg of the antibody. The antibody was tested in two different settings: (A) a prevention experiment where the first antibody dose was administered one day before viral challenge; and (B-E) a postexposure setup where the anti-SR-BI therapy was initiated 3 days postviral challenge. Antibody-treated mice are indicated with a dotted line, whereas nontreated control animals are represented by solid lines. Chimeric mice were challenged at day 0 with Jc1wt (B), Jc1DHVR1 (A,C), Jc1G451R (D), or Jc1mtCD81 (E). Each data point represents the plasma HCV RNA level (IU/mL) of an individual chimeric mouse at a given timepoint. The limit of detection (LOD) equals 750 IU/ml.*

We found no evidence supporting increased SR-BI-receptor dependency of viral particles isolated from humanized mice compared to cell culture-produced virus. However, we observed that, unlike wild-type virus, the in vitro infectivity of the resistant variants was inhibited by both human high density lipoprotein (HDL) and very low density lipoprotein (VLDL). The combination of mAb1671 with these lipoproteins further increased the antiviral effect. Since these variants are also more susceptible to neutralization by anti-HCV envelope antibodies, their chance of emerging during anti-SR-BI therapy is severely limited.

Objective 1.5 GMP Scale-up and production

The aim of Univercells, in close collaboration with CEINGE, MoMAb and GenIbet was development of a production and purification process suitable for GMP manufacture of the anti-HCV and anti-SRBI mABs. Other key aims were evaluation of the regulatory requirements for the manufacture of antibodies to be used in clinical trials (WP8); follow up of the preclinical *in vivo* studies of lead and back-up antibodies (WP6) and preparation and maintenance of GenIbet's production units to perform the needed GMP productions (WP7).

To develop a process that would produce antibody at the levels required for clinical trial the specialist subcontractor Univercells were recruited. There remit was to:

- Focus on 10L process development
- Manage USP internally
- Work with Merck for the DSP at small scale
- Support the implementation of the process at Genibet, as well as its scaling up to 50L

Throughout the project, GenIbet worked closely with others partners to ensure a straightforward and

successful transfer technology process that could be easily and efficiently implemented, under GMP, at GenIbet. Initially there were major difficulties in obtaining high-producer cell lines, but these were overcome for both the anti-HCV and anti-SRBI mAbs, as described above. To ensure efficient manufacture, a sub-contract with Univercells was established. The sub-contractor developed appropriate processes for both mAbs. Working with Univercells, GenIbet prepared SOP (Standard Operational Procedures) documentation of the GMP Production, based on the processes developed and optimised by the other partners in non-GMP. This included preparation of the Batch Production Record (BPR) for the manufacture and testing of the CEINGE and MoMAb Master Cell Banks., set-up and writing of procedures and SOPs for the in-house testing of the MCB – cell count, cell culture viability and morphology, as well as set-up and approval of the Study Plans for the sub-contracted tests.

GenIBet successfully manufactured and tested 2 CHO Master Cell Banks: CHO DG44 1C5 Anti-SRB1 Master Cell Bank and 23 – CHO - S pC072 - 2129 Master Cell Bank.

Objective 1.6 Clinical Trial of one anti-viral and one anti-receptor monoclonal antibody

The overall goal of the HepaMAb project is to test the clinical efficacy of new human monoclonal antibodies against Hepatitis C virus (HCV) or its receptor SR-BI to prevent liver infection. The clinical trial is dependent on:

- Adequate supply of GMP produced antibody (in sufficient quantity to satisfy needs of dosing regimen and number of individuals enrolled on the trial
- Identification of appropriate cohorts for treatment

Initial discussions focussed on phase IIa study (without the need for a phase I safety study). It was proposed that a Phase IIa study should be performed with enrolment of targeted patient population: open label study on LT patients with HCV viral load higher than 100.000 IU/mL at the moment of liver transplantation (LT) to prevent graft HCV infection. Patients (N=5) will receive 7 doses at 3 days intervals of the anti-E2 mAb or the anti-SRBI. Patients will receive the first dose during the anhepatic phase. Thereafter, they will receive 6 more doses every 3 days . Clinical parameters will be measured during treatment and the follow-up period for the record of adverse effects. Clinical efficacy will be defined by either undetectable HCV-RNA at any time after LT. If absolute clearance is not achieved then there is 80 percent chance that the study will detect a treatment difference at a two-sided 0.05 significance level, if the treatments result in at least a 0.953 log reduction in viral load, assuming that the standard deviation of the viral loads is 0.25.

However, since the project started there have been major advances in the treatment and cure or HCV infection, even in previously difficult to treat individuals, such as those with advanced liver disease.

That said, there is increasing evidence (including evidence from UNOTT's own studies) of naturally occurring high level antiviral resistance in some cohorts of HCV infected individuals. This means that universal treatment is unlikely to be achieved, therefore future need for therapeutic mAbs cannot be ruled out and, given the progress on the HepaMAb project, the consortium will be well placed to meet this need.

Objective 2.1 Development and in vitro characterisation of an anti-viral back-up antibody

The neutralizing activity of human monoclonal antibody 2A5 was assessed against a panel of 6 patient-derived gt1b HCVpp (P09_VA, P09_VB, P09_779, P10_VA, P12_VA and P12_1091). Antibody AP33 was used as reference and prototype HCVpp of H77C, JFH1 and S52 were included. Viral entry was inhibited in a dose-dependent manner and for most viral strains the neutralizing potential of mAb 2A5 was superior to

that of AP33 (P=0.164) (Figure 1). Similar to the E2-binding data, viral strain S52 (gt3a) was difficult to neutralize and viral strain P12_1091 (gt1b) was very efficiently neutralized.

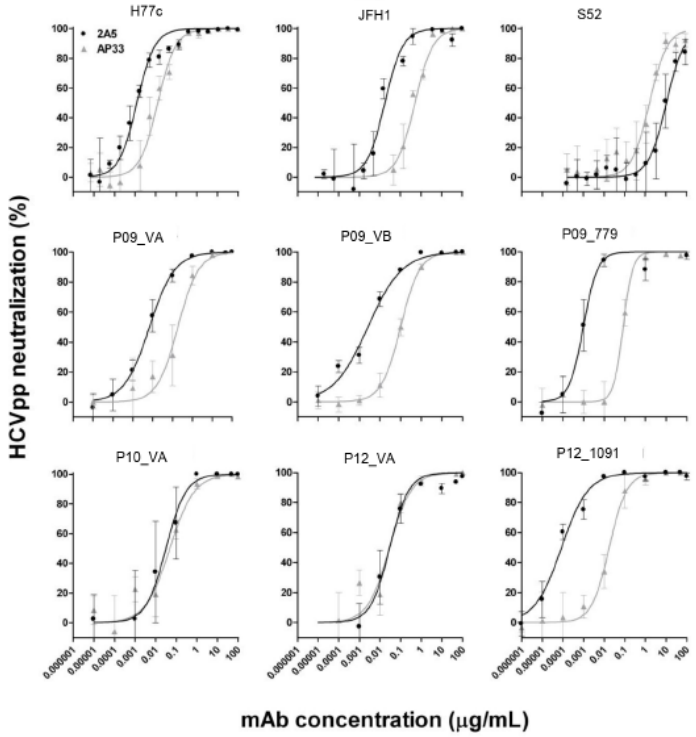


Fig. 1. Neutralization of HCVpp by mAbs 2A5 and AP33. *HCVpp expressing E1E2 derived from prototype isolates (H77c, JFH1, S52) and gt1b patient-derived viral isolates (P09_VA, P09_VB, P09_779, P10_VA, P12_VA and P12_1091) were incubated for 1 hour at 37°C with serial dilutions of mAbs 2A5 or AP33 and added to Hep3B cells. HCVpp entry was analyzed by luciferase reporter gene expression and normalized to isotype controls. Neutralization is expressed as % neutralization (mean±standard deviation (error bars)).*

To further corroborate the neutralizing potency of mAb 2A5 we utilized the HCVcc system covering multiple HCV strains: gt1a (H77c/JFH1 and full length TNcc), gt1b (J4/JFH1), gt2a (JC1), gt3a (S52/JFH1; UKN3A1.28c; and UKN3A13.15), gt4a (ED43/JFH1), gt5a (SA13/JFH1), gt6a (HK6a/JFH1) and gt7a (QC69/JFH1). Overall, results demonstrate that mAb 2A5 efficiently neutralizes most HCV strains, with half maximal inhibitory concentration (IC50) values comparable or superior to the neutralization observed with AP33 (P=0.0078) (Figure 2). The gt6a strain HK6a/JFH1, which could not be neutralized by AP33, was very efficiently neutralized by 2A5 (IC50 = 0.007 μg/mL). Strain S52/JFH1 was barely neutralized, in line with the E1E2 binding and HCVpp neutralization results.

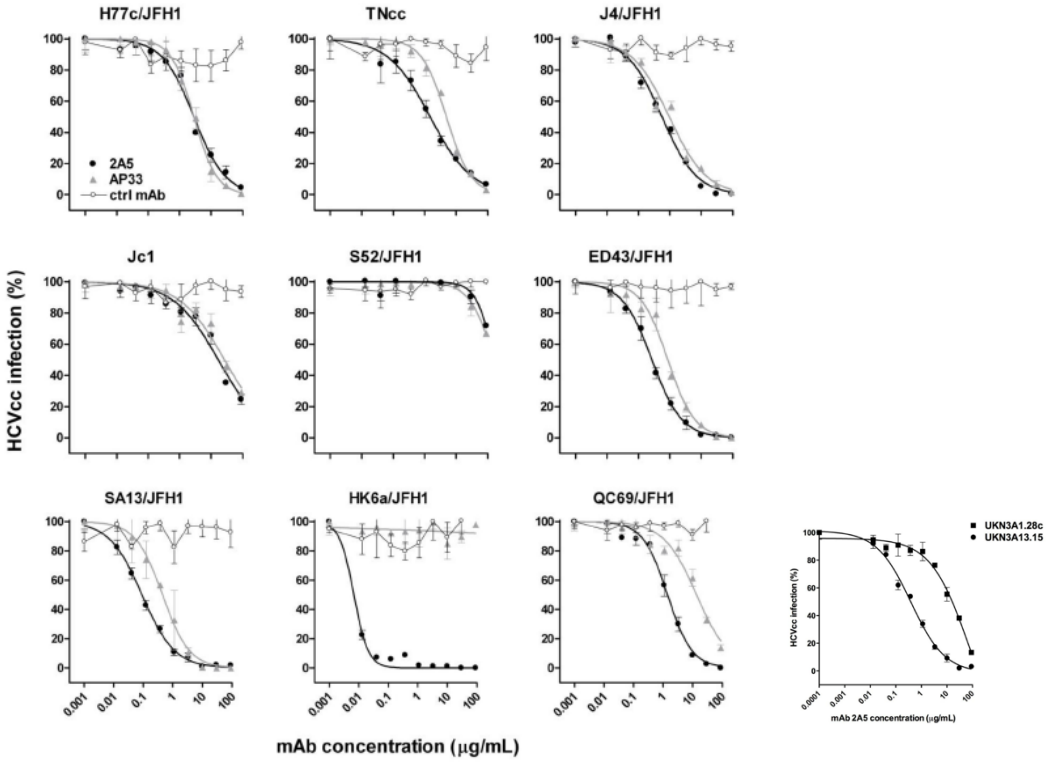


Fig. 2. Neutralization of HCVcc by mAbs 2A5 and AP33. *HCVcc expressing the structural proteins of genotype 1a, 1b, 2a, 3a, 4a, 5a, 6a and 7a isolates were pre-incubated with three-fold serial dilutions of mAbs 2A5, AP33 or a control Ab. The mixture was transferred to Huh7.5.RFP cells and incubated for 4 hours before washing. Two days later, HCV-infected foci were visualized using an NS5A-specific antibody and counted. Results are expressed as percentage of infectivity (mean±standard deviation (error bars)).*

The involvement of this region in 2A5-binding was confirmed and further scrutinized using an in-house enzyme EIA with peptides spanning AA433-443 derived from different gt1a and gt1b natural viral isolates (AA sequence see Fig. 3a). From these binding experiments we could conclude that 2A5-binding is unchanged when (i) position AA440 is occupied by amino acid G or A, or (ii) position AA437 is occupied by amino acid W or F. At position AA434, N confers better binding than Q and at position AA438, L is much more favorable for binding than F (Fig. 3b). To elaborate on the anchor residues, mutant peptides were generated wherein residues at position AA433 and AA443 were replaced by alanine (L433A and Y443A) (Fig. 3c). Results demonstrate that Y at position AA443 is mandatory for optimal binding. In addition, comparison of 2A5-binding to the peptide AA433-443 with that to a peptide covering the region AA433-452, showed enhanced affinity towards the longer peptide indicating additional contact residues within the region adjacent and downstream from AA443 (Fig. 3d). To further elaborate on 2A5-anchor residues within region AA433-452, the binding efficiency was compared between the peptide representing the prototype isolate H77c and its alanine-substituted counterparts (Fig. 3e and f). Our data indicate that AA434, AA438, AA441, AA442, AA443 and AA446 are important for 2A5 binding, with AA442 and AA443 being major anchor residues.

To confirm these results, our peptide binding assays were repeated in the context of full-length E1E2 protein. 2A5-binding was tested for binding to wild-type H77c E1E2 protein and its mutants in which the AA spanning regions 419-447, 522-536 and 612-617 were alanine-substituted (Fig. 3g). Based on the reduction in mAb binding, our analyses revealed several regions within E2 that are critical for 2A5-binding: AA424-428, AA437-443, AA446, AA530 and AA612-617. Results obtained for AA437-446 are largely comparable with EIA-peptide data and are shown in Figure 3f. The finding that several non-adjacent regions within E2 are involved in binding and that mutation at position C429 (forming a disulfide bond) completely abrogates binding, suggests that mAb 2A5 recognizes a conformational epitope.

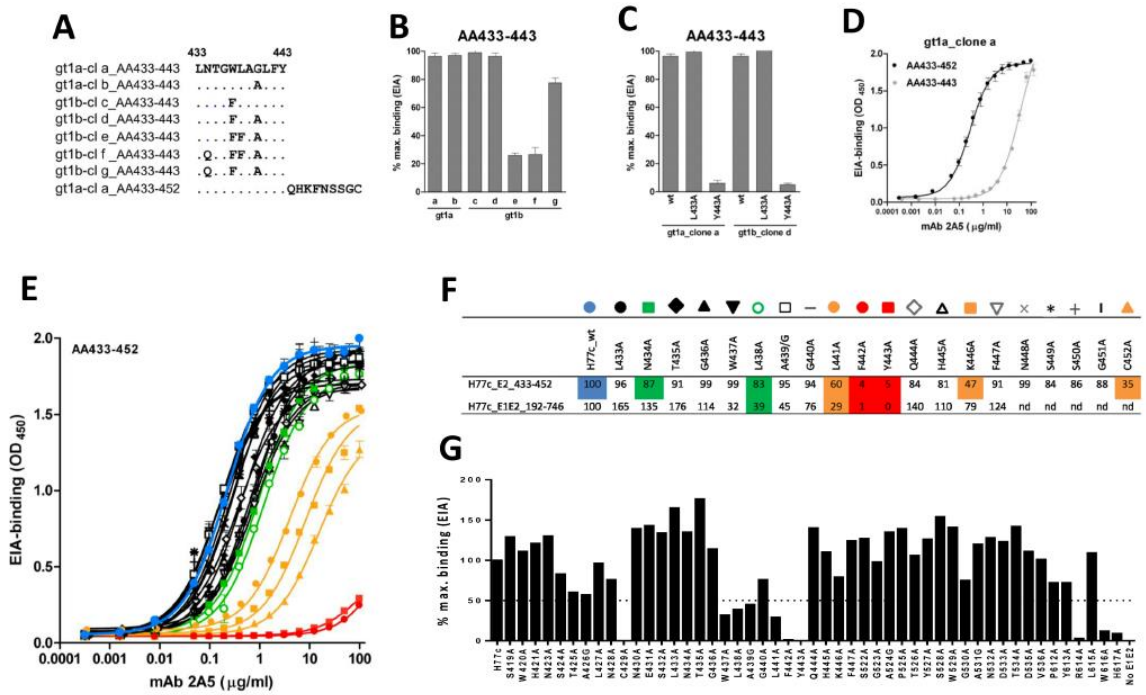


Fig. 3. Epitope mapping of mAb 2A5. *The binding pattern of mAb 2A5 was analyzed using gt1a- and gt1b-derived sequences, of which the E2 region spanning amino acids (AA)433-443 of the different clones (cl) is shown* ***(A)****. After incubation with surface-attached peptides, bound antibodies were detected with an HRP-conjugated anti-human secondary antibody. The 2A5-binding pattern was analyzed using mAb (50 µg/mL) and peptides spanning AA-region 433-443, derived from naturally occurring gt1a (clone a-b) and gt1b (clone c-d) isolates* ***(B)****, and A-substituted peptides (L433A, Y443A) from gt1a_clone a and gt1b_clone d* ***(C)****. Binding was normalized to the best binding peptide (100% binding).* ***(D)*** *To elaborate on the length of the epitope, the efficiency of 2A5-binding was compared between peptides covering region AA433-443 and peptides including an additional upstream sequence (AA433-452), all derived from gt1a_clone a.* ***(E)*** *To further elaborate on 2A5-anchor residues within the region of interest (AA433-452), the binding efficiency was compared between peptides derived from the prototype isolate H77c and their A-substituted counterparts. The corresponding color-codes, representing the different A-substituted sequences, and the % binding relative to native H77c at 10 µg mAb/mL are shown in* ***(F)****.* ***(G)*** *To confirm these results and to reveal additional contact residues for mAb binding, epitope mapping was done using E1E2 proteins of the H77c isolate with A-substitutions in 3 regions (AA419-447, AA522-536 and AA612-617). 2A5-binding to full-length native and mutated E1E2 cell lysates was analyzed. Results represent the % binding relative to E1E2_H77c at 10 µg mAb/mL* ***(F, G)****.*

Alignment of the E2-region spanning AA420-452 of the different HCV strains used in the binding and neutralization studies is shown in Figure 4a. Residues found critical for 2A5-binding in that region are highlighted and, for each strain, the simplified AA-motif is shown on the right. Based on aforementioned experiments, a neutralization-hierarchy is deduced for the different motifs that, for most motifs, can largely predict the neutralization outcome (Fig. 4b). However, two motifs are linked to opposing neutralization data: (i) motif N-I-LFY-K is present in strains TNcc (IC50: 1.49 µg/mL) and S52 (IC50: >90 µg/mL), and (ii) motif N-L-LFY-R is present in strains JFH1 (IC50: 0.02 µg/mL), P10_VA (IC50: 0.03 µg/mL), UKN1B5.23 (IC50: 0.57 µg/mL) and UKN4.11.1 (IC50: 38 µg/mL). Furthermore, the motif Q-L-LFY-K, which was very efficiently neutralized in vitro, could barely be neutralized in vivo (mP05).

A

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                  420       430       440       450
                  |....|....|....|....|....|....|..
                  WHINSTALNCNESLNTGWLAGLFYQHKFNSSGC    N-L-LFY-K
gt1a-H77c
    -UKN1A20.8    ....R......D..T...I...L.H........    T-I-LLY-K
    -TN           ..........D......I.....H........    N-I-LFY-K
gt1b-J4           ....R......D..Q..FF.A...A........    Q-F-LFY-K
    -UKN1B5.23    ....R......D.....F..A...T.R......    N-L-LFY-R
    -UG-P05-VF    ....R......D..Q..F..A...T....A...    Q-L-LFY-K
    -UG-P12-VA    ....R......D..Q..F..A...T.R..A...    Q-L-LFY-R
    -UG-P12-1091  ....R......D..Q..F..A...T....A...    Q-L-LFY-K
    -UG-P10-VA    ....R......D.....F..A...ARR......    N-L-LFY-R
    -UG-P09-VA    ....R......D.....F..S...VS.......    N-L-LFY-K
    -UG-P09-779   ....R......D.....F.TS...VS.......    N-L-LFY-K
    -UG-P09-VB    ..V.R......D.....F..S...VS...A...    N-L-LFY-K
gt2a-JFH-1        ....R......D.....F..A...TNR......    N-L-LFY-R
    -J6           ....R......D..H..FI.S...T.S......    H-I-LFY-S
    -JC1          ....R......D..H..FI.S...T.S......    H-I-LFY-S
    -UKN2A1.2     ....R......D..Q..FI.S...TTS..A...    Q-I-LFY-S
gt2b-UKN2B2.8     ....R......D..Q..F..S...VKN......    Q-L-LFY-N
gt3a-S52          ............I...FI.....Y.....T..    N-I-LFY-K
    -UKN3A1.28c   ....R........I...FI.....Y.....T..    N-L-LFY-K
    -UKN3A13.6    ....R........I...F......Y.....T..    N-I-LFY-K
    -UKN3A13.15   ....R........I...F......Y.....T..    N-L-LFY-K
gt4a-ED43         ....R......D.....F..S...T........    N-L-LFY-K
    -UKN4.11.1    ....R......D.....F.T....VSR......    N-L-LFY-R
gt5a-SA13         ....R......D..Q..FV...L.Y.....T..    Q-V-LLY-K
    -UKN5.15.7    ....R.....QD..Q..FI...L.FN.......    Q-I-LLY-K
gt6a-HK6a         ....R......D..Q..FITS...AKNV.....    Q-I-LFY-N
    -UKN6.5.8     ....R......D..Q..FI.S...FN.......    Q-I-LFY-K
gt7a-QC69         ....R......D..Q..FI.A...T.R......    Q-I-LFY-R
```

B

Fig. 4. Main binding motif recognized by mAb 2A5. (A) *Alignment of the E2-region spanning AA420-452 of the different HCV strains used in the binding and neutralization studies. Residues found critical for mAb 2A5-binding (AA433, AA438, AA441, AA442, AA443 and AA446) are highlighted and, for each strain, the simplified AA-motif is shown on the right.* ***(B)*** *A hierarchy in neutralization potential was deduced for the binding motifs. The AA-motif corresponding to the H77c strain is marked with a black box. AA-motifs shown on the left-side of '>' have better neutralization properties compared to AA-motifs shown on the right-side. Motifs from 'difficult to neutralize' strains HCV-S52, JC1 and J6 are highlighted in red boxes. Motifs from HCV-strains that can be efficiently neutralized by 2A5 (P12_1091, HK6a and UKN5.15.7) are indicated in in green boxes. (*) represents identical motifs with different neutralization efficiencies.*

To analyze whether competition occurs between mAb 2A5 and other known mAbs for binding to the HCV envelope, a competition EIA was set-up wherein anti-E2 (AP33, HC33.1, HC84.26, HC-1AM, CBH-7, 1:7 and 3/11) and anti-E1 (A4) mAbs were used as competing antibodies for 2A5-binding to E1E2 of isolate H77c (Fig. 5). Our results demonstrate that, apart from the anti-E1 mAb, all mAbs compete for 2A5-binding. As expected, only mAb 3/11 and HC33.1 compete for AP33-binding. These mAbs were previously shown to recognize similar epitopes.

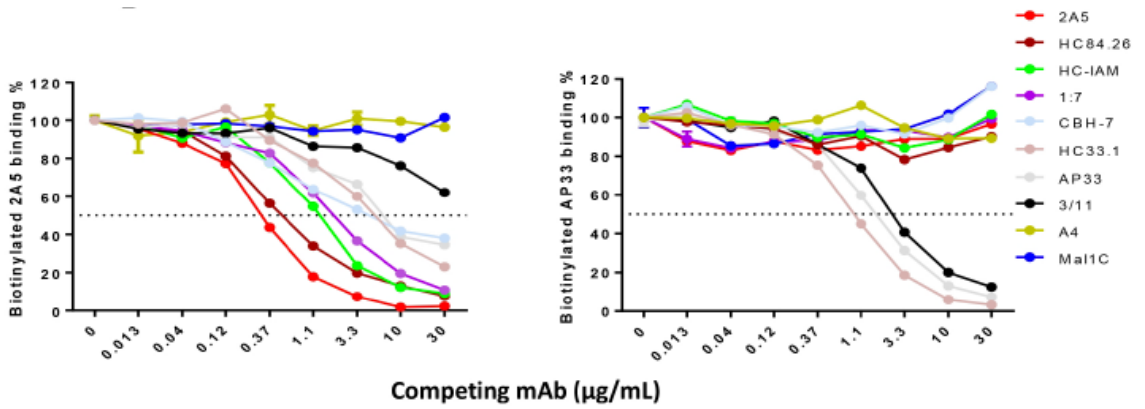


Fig 5. Competition of between 2A5 and other known antibodies. *To analyze whether 2A5 competes with other mAb, a competition EIA was performed wherein anti-E2 (AP33, HC33.1, HC84.26, HC-1AM, CBH-7, 1:7 and 3/11) and anti-E1 (A4) mAbs were used as competing antibodies for binding to the E1E2 protein of the H77c isolate. Three-fold serial dilutions of competing mAbs were used starting at 30 µg/mL. The binding of biotinylated versions of mAb 2A5 (left panel) and AP33 (right panel) was measured using HRP-conjugated streptavidin.*

Cloning and characterization of recombinant mAb 2A5.

Antibodies or Immunoglobulins (Ig) are proteins that consist of four polypeptides– two heavy chains and two light chains joined to form a "Y"-shaped molecule. The amino acid sequence in the tips of the "Y" varies greatly among different antibodies. This variable region, composed of 110-130 amino acids, give the antibody its specificity for antigen binding.

Our strategy was to first identify the sequence of the variable regions of human IgG Heavy (VH) and Light (VL) chain variable genes from an anti-HCV-producing hybridoma celline. Multiple PCR primers are required to amplify entire V-gene repertoires. Here we made use of a set of different V-gene specific primers in combination with Ig-class specific reverse primers, described by Lim et al. (Nature Biotechnology 2010, 27:108-117); see details table. Second, for production purposes the identified variable heavy (V-D-J region) and light (V-D region) chain variable regions were cloned/displayed into mammalian mAb heavy or light expression vectors.

Total RNA was isolated from the anti-HCV producing hybridoma using RNeasy mini kit (Qiagen, Venlo, Netherlands). Random priming of obtained RNA was performed using Superscript III (Invitrogen, Karsruhe, Germany) according to standard procedures. Amplification of the variable regions of human IgG Heavy (VH) and Light (VL) chain was done via standard PCR using a human Ig primerset based on family assigned genes (see table) and Phusion polymerase (New England Biolabs, Frankfurt, Germany). All primers used were obtained from Biolegio (Nijmegen, Netherlands). Amplified fragments of about 650 to 700bp were gel extracted (Qiaquick, Qiagen, Venlo, Netherlands) and ligated into PCR-Blunt® vector (Invitrogen, Karsruhe, Germany) for sequencing. Between 3-6 clones were sequence analysed for each insert. Obtained sequences were analysed using IMGT/V-Quest (http://www.imgt.org/) to assign the variable gene family (Brochet et al., Nucleic Acids Res. 2008,36:W503-508). This analysis indicated that the V, D and J segments of the CEVAC mAb 2A5 could be assigned to the following gene families: HV1-HJ5-HD1: LV2-LJ1.

Next we cloned the variable regions into mammalian expression vectors (pFUSEss-CLIg-hk (light chain) or pFUSEss-CHIg-hIG1 (heavy chain), both obtained from Invivogen), containing the IL2 secretion signal and human IgG1 or Igk constant regions.
Heavy and light chain mammalian expression vectors were transiently expressed in a 2:3 ratio into the mammalian serumfree 293 freestyle celline (Invitrogen *by* Life Technologies) using TransIT-PRO transfection kit (catnr. MIR5700, Mirus Bio LLc). Production was verified by IgG measurements using a nephelometer.

Primerset (Biolegio) to amplify the human variable regions.
The reverse primer is either located at the end of the light chain constant region or placed in the CH1 region of the heavy chain.

Family / Ig Template	Primer name	5' Sequence
Heavy	VH1	CAGGTCCAGCTKGTRCAGTCTGG
	VH157	CAGGTGCAGCTGGTGSARTCTGG
	VH2	CAGRTCACCTTGAAGGAGTCTG
	VH3	GAGGTGCAGCTGKTGGAGWCY
	VH4	CAGGTGCAGCTGCAGGAGTCSG
	VH4-DP63	CAGGTGCAGCTACAGCAGTGGG
	VH6	CAGGTACAGCTGCAGCAGTCA
Light Kappa	Vκ1	GACATCCRGDTGACCCAGTCTCC
	Vκ246	GATATTGTGMTGACBCAGWCTCC
	Vκ3	GAAATTGTRWTGACRCAGTCTCC

	Vκ5	GAAACGACACTCACGCAGTCTC
Light Lambda	Vλ1	CAGTCTGTSBTGACGCAGCCGCC
	Vλ1459	CAGCCTGTGCTGACTCARYC
	Vλ15910	CAGCCWGKGCTGACTCAGCCMCC
	Vλ2	CAGTCTGYYCTGAYTCAGCCT
	Vλ3	TCCTATGWGCTGACWCAGCCAA
	Vλ3-DP?16	TCCTCTGAGCTGASTCAGGASCC
	Vλ3-38	TCCTATGAGCTGAYRCAGCYACC
	Vλ6	AATTTTATGCTGACTCAGCCCC
	Vλ78	CAGDCTGTGGTGACYCAGGAGCC
Constant	Kappa Cκ-rv	ACACTCTCCCCTGTTGAAGCTCTT
	Lambda Cλ1-rv	CTATGAACATTCTGTAGGGGCCACTG
	Lambda Cλ2-rv	CTATGAACATTCCGTAGGGGCAACTG
	IgG CH1-rv	ACTCTCTTGTCCACCTTGGTGTTGC

The activity of the produced recombinant antibody was compared with that of the original hybridoma one using the HCVpp and HCVcc system. The results obtained showed that both antibodies behaved similar (Figure 6).

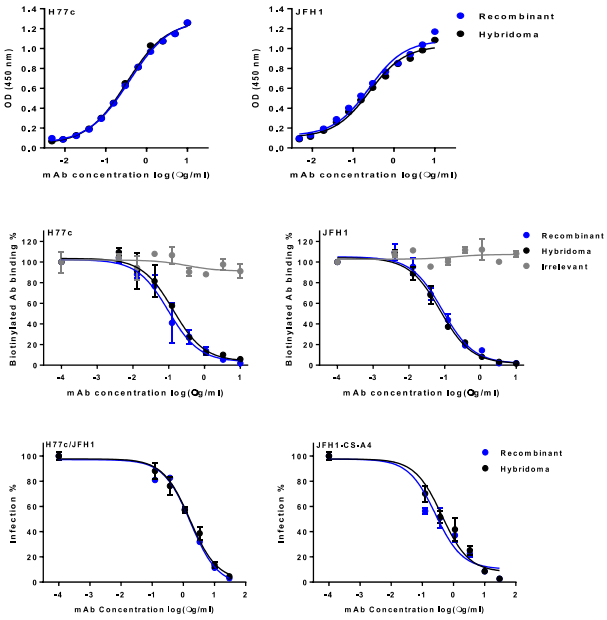


Fig 6. Comparison of recombinant and hybridoma-produced 2A5.

WP5. Development and characterization of a novel human anti-E1 antibody.

Using hybridoma technology we generated a human monoclonal antibody (designated A6) from a chronically infected HCV patient. The specificity of the antibody was assessed by ELISA, using E1E2 protein from multiple isolates (Figure 7). Epitope mapping, using PepperPrint array and a peptide library spanning the entire HCV envelope (E1E2), identified the region spanning amino acid (AA) 225-244 (within the N terminal region of E1) as a critical site for binding. Denaturation ELISA showed that the binding to this epitope is not conformational dependent.

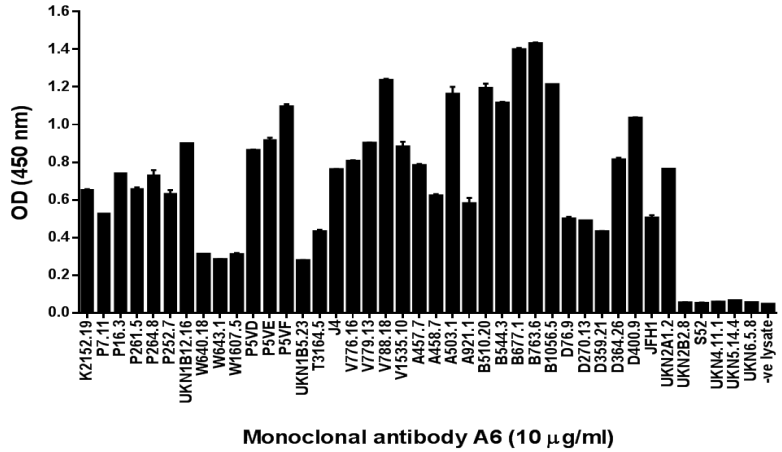


Fig. 7: HCV E1E2 binding assay using novel anti-E1 antibody.

We now showed that the binding of A6 to E1E2 is independent from the glycosylation pattern of E1E2. Western blot analysis using PNGase F-treated, Endo H-treated and non-treated E1E2 cell lysate showed that mAb A6 binds to both glycosylated and deglycosylated E1 (Figure 8).

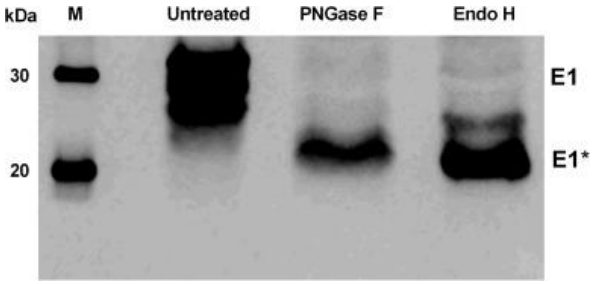


Figure 8: Reactivity of mAb A6 to glycosylated and deglycosylated E1E2 protein. *Lysate of 293T cells expressing H77 E1E2 was treated with PNGase F or Endo H. Following 12% SDS-PAGE, proteins were transferred to a nitrocellulose membrane and revealed using mAb A6 as primary antibody. Positive bands were visible at a molecular weight around 20–22 kDa in case of PNGase F and Endo H-treated cell lysate (lane 3 and 4), which correspond to deglycosylated E1, while the untreated lysate showed reactive bands at around 25–32 kDa (lane 2). The position and molecular weights of protein standard are presented in kDa (lane M). The deglycosylated E1 protein is indicated by an asterisk.*

We also investigated the potential conservation of the A6 epitope among different HCV genotypes. Amino acid sequences of E1 for isolates used in the binding assay were aligned using BioEdit and CLC main workbench. As seen from the sequence alignment and the percentage of conservation, certain amino acids in region 230–239, which is critical for mAb A6 binding, are highly conserved especially among genotypes 1a and 1b (Fig. 9). Amino acids cysteine and tryptophan at sites 238 and 239 respectively are conserved in all 7 genotypes. The residues 234N and 236S are also conserved, except in case of genotype 2b where they are replaced by glycine and leucine, respectively. Likewise for 230V, which is only different for genotype 2. Other positions such as 231, 232 and 237 show high conservation among genotypes 1a, 1b and 2a compared to genotypes 2b, 3a, 4, 5 and 6.

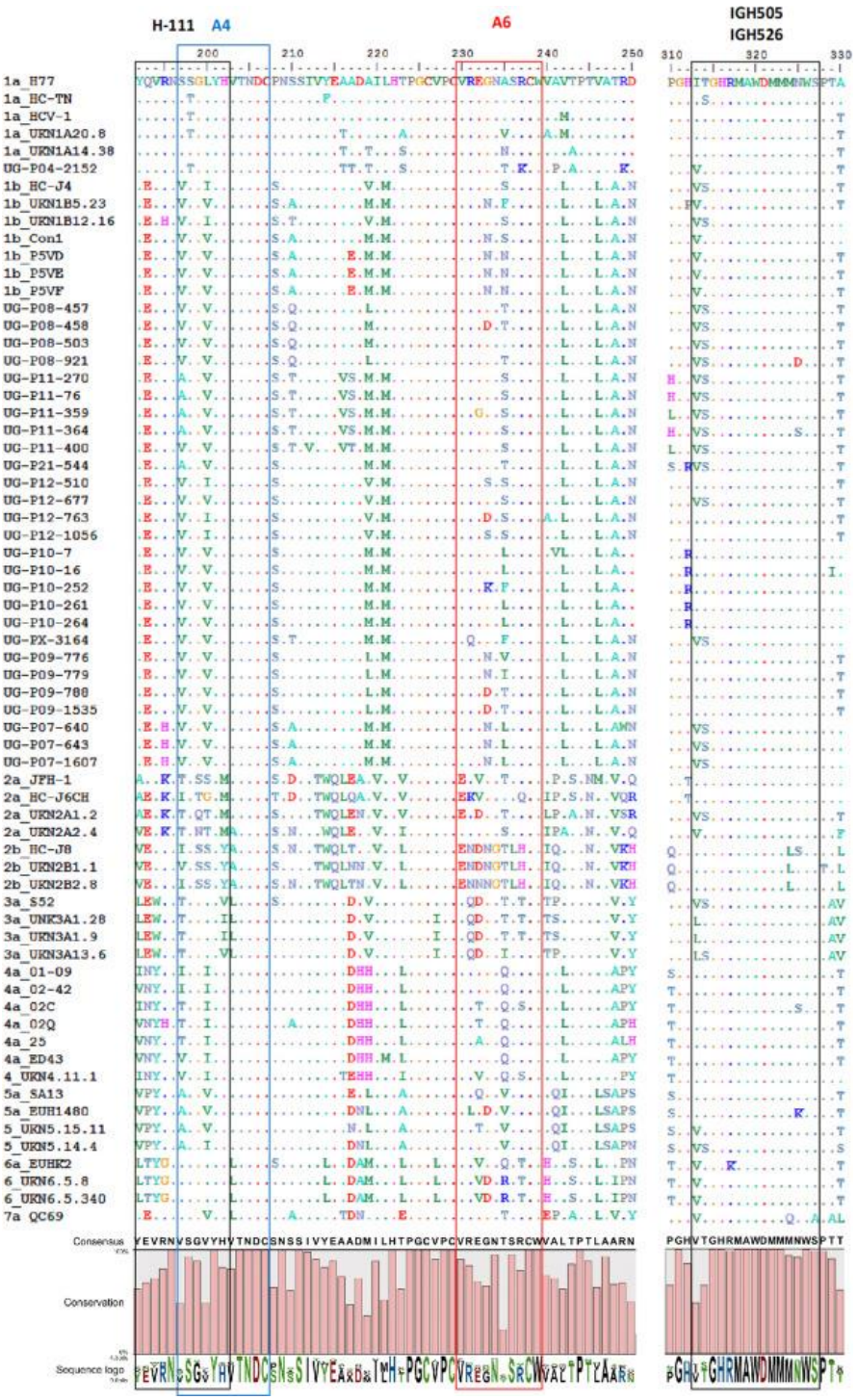


Figure 9: Multiple alignment of the region corresponding to AA 192–250 and AA 310–330 of HCV E1. *The E1 sequence of all HCV isolates used in the current study was retrieved from GenBank and the HCV database and multiple aligned using BioEdit software. The epitopes targeted by antibodies H-111, A4, A6, IGH505 and IGH526 are indicated using colored squares. The dots indicate conservation of the residue relative to the reference prototype H77 (genotype 1a). The lower part of the alignment shows the consensus sequence and the percentage of overall conservation calculated using CLC Main Workbench.*

Neutralization experiments showed non-neutralizing potential of this mAb toward HCV pseudoparticles (HCVpp) and HCV produced in cell culture (HCVcc) of genotypes 1a and 2a. In addition, this antibody did not interfere with the activity of anti-E2 neutralizing antibodies.

Subsequently we showed that staining of H77c/JFH1 infected Huh7.5RFP cells with mAb A6 enabled the visualization of infected cells, which indicates the applicability of mAb A6 for use in immunofluorescence (Fig. 10).

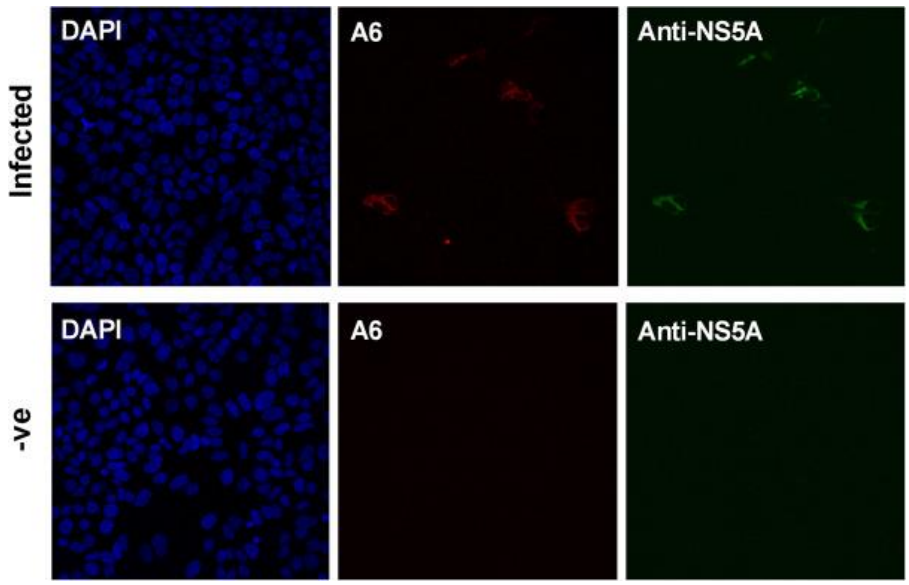


Figure 10: mAb A6-based visualization of infected Huh7.5RFP cells using immunofluorescence. *H77c/JFH1 infected Huh7.5RFP cells (upper panel) and non-infected cells (-ve; lower panel) were incubated overnight with mAb A6 (middle column), after which Alexa 647-conjugated goat-anti-human IgG was added. As a positive control, the same cells were stained with anti-NS5A mAb 9E10 in combination with Alexa 488 conjugated goat-anti-mouse IgG (right column). DAPI was used as counterstain (left column).*

Preclinical in vivo evaluation of mAb 2A5: We have evaluated whether antibody 2A5 was capable of protecting mice with humanized liver from infection with HCV. As challenging virus we used mH77 (gt1a), mP05 (gt1b), mED43 (gt4a) and mHK6a (gt6a). Three days before viral infection all mice were injected with 1 mg of mAb2A5.

As shown in Figure 11, the antibody treatment completely protected humanized mice against a challenge of H77 and ED43 virus, while about half of the mP05 and mHK6a injected mice were protected. Interestingly, most of the mice that were not completely protected experienced a delay in the kinetics of the viral infection, indication that a major part of the injected virus was neutralized. Viral sequencing of the breakthrough virus did not indicate viral escape but rather failure of neutralization.

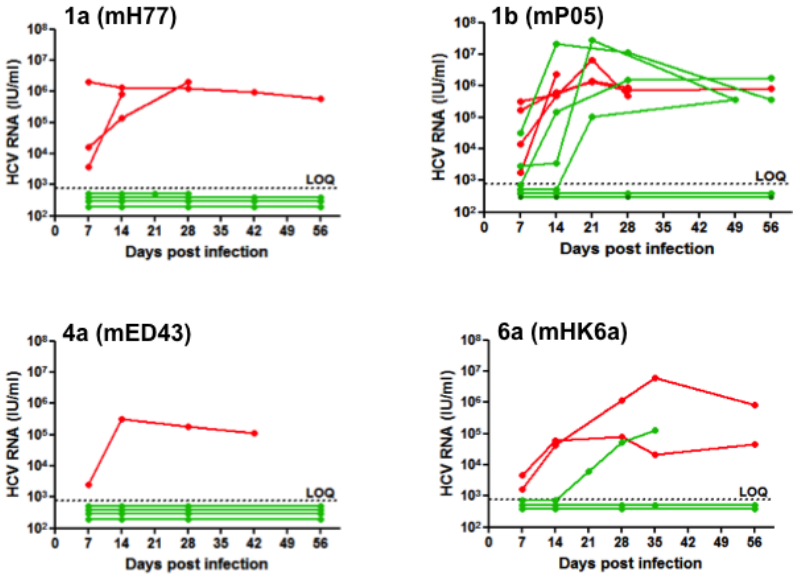
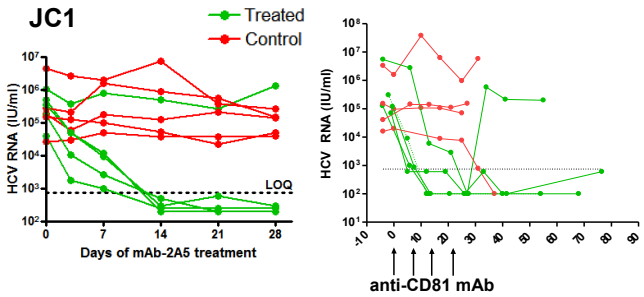


Figure 11: Prophylactic therapy of humanized mice with mAb 2A5.

In a setting of chronic HCV infection we observed that a 4-week mAb2A5 treatment resulted in a rapid decline of viremia in Jc1 infected mice. The viral load decreased to levels below the limit of quantification. A similar result was obtained when chronically infected mice were treated with an antibody that blocks the viral receptor CD81. (Figure 12)

Interestingly, this phenomenon was not observed in mice that were chronically infected with the virus obtained from patient 05 (P05, gt1b). We are currently further investigating the cause of this discrepancy.



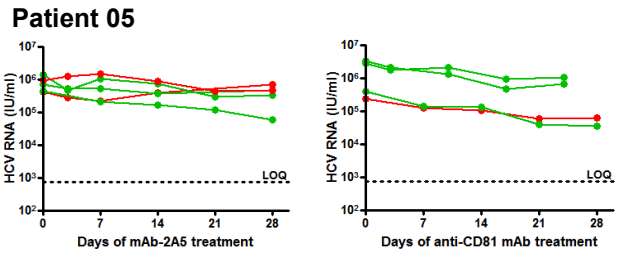


Figure 12:Treatment of HCV-infected humanized mice with mAb 2A5.

We have also analysed the effect of mAb1671 treatment in a setting of chronic HCV infection. Similarly to our observations with antibodies 2A5 and JS81, we did not observe any activity of mAb1671 in mice chronically infected with patient 05-derived virus. Unexpectedly, we also did not observe any (or only very low) activity in mice infected with Jc1 virus. These data are distinct from what we observed with antibodies targeting the viral envelope (2A5) or CD81 (JS81), where we observed a rapid drop in viral load reaching levels below the limit of detection within about 2 weeks.

Objective 2.2 Development and characterization of humanized tight junction-specific mAbs (CLDN-1)

As described in the previous reporting periods, we generated a panel of humanized anti-CLDN1 antibodies and selected the most promising clone (H3L3) for further detailed characterization and humanisation. The following progress was made in achieving those aims.

- Full characterization of a rodent anti-CLDN1 MAb (OM-7D3-B3), using *in vitro* model systems (HCVcc and HCVpp in Huh7.5.1 cells and PHH) and the human liver chimeric uPA-SCID mouse model
    - Cross-neutralization abilities of the anti-CLDN1 MAb characterized in the HCVcc and HCVpp systems
    - Cross-neutralization of anti-CLDN1 MAb validated in PHH using HCVpp
    - *In vivo* efficacy (prevention and treatment) in uPA-SCID mouse model completed
    - Characterization of synergy between the rat anti-CLDN1 MAb and DAAs
    - Study of effect of anti-CLDN1 MAb against DAA-resistant viruses completed

- Humanization of the rodent anti-CLDN1 MAb (OM-7D3-B3) and full characterization of the *in vitro and in vivo* activities of the humanized antibody
    - Successful humanization of the antibody following classical humanization strategies to generate a panel of 9 antibodies with different heavy and light chain combinations
    - The humanized antibodies bound to CLDN1-expressing cells similarly to the parental rat anti-CLDN1 antibody
    - The humanized antibodies potently inhibited HCVcc genotype 2a infection and HCVpp genotype 1a and 1b entry
    - H3L3 was selected for further analysis and was shown to inhibit HCVcc infection of Huh7.5.1 cells and HCVpp entry of all major genotypes into PHH, with similar potency as the parental rat antibody
    - H3L3 inhibited cell-free HCVcc infection of Huh7.5.1 cells and HCV cell-to-cell transmission
    - H3L3 inhibited infection by DAA-resistant HCVcc strains
    - H3L3 demonstrated synergistic activity with two DAAs, sofosbuvir and daclatasvir
    - H3L3 and the anti-E2 human MAb HC84.26.WH.5DL synergistically inhibit HCV infection of Huh7.5.1 cells and PHH
    - H3L3 cured chronic HCV infection in human liver chimeric uPA-SCID mice, as previously shown for the parental rat antibody
    - H3L3 did not induce overt toxic effects in human liver chimeric mice, consistent with previous findings for the rat antibody

- Characterization of a rodent anti-OCLN mAb, using *in vitro* model systems (HCVcc and HCVpp in Huh7.5.1 cells and PHH) and the human liver chimeric uPA-SCID mouse model
    - Mice were immunized, hybridomas were produced and a small panel of anti-OCLN mAbs were generated
    - Neutralization of the anti-OCLN antibodies was characterized in HCVcc and HCVpp assay system
    - Technical problems with large-scale production of the rodent anti-OCLN antibody
    - Given the very promising results with the rodent and humanized anti-CLDN1 antibody, we

focused our efforts on the characterization of the anti-CLDN1 mAb

- Humanization of the rodent anti-OCLN mAb and characterization of its *in vitro* and *in vivo* activities
    - We did not pursue this aim: As described, we experienced technical problems with large-scale production of the rodent anti-OCLN antibody
    - Given the very promising results with the rodent and humanized anti-CLDN1 antibody, we focused our efforts on the characterization of the anti-CLDN1 mAb

Significant results

- The rodent anti-CLDN1 mAb shows potent *in vitro* and *in vivo* efficacy against different HCV strains
- Successful humanization of the rat anti-CLDN1 mAb
- The humanized anti-CLDN1 H3L3 mAb retains the functional activity of the parental rat antibody
- H3L3 pan-genotypically inhibits HCV infection, without detectable resistance or escape in primary human hepatocytes
- H3L3 is active against DAA-resistant HCV strains and synergizes with current DAAs (sofosbuvir and daclatasvir)
- H3L3 cures chronically HCV infected human liver chimeric mice in monotherapy

Major achievements:

- Extensive panel of HCVcc and HCVpp produced, validated and shared with the scientific community
- Improved in vivo models
- Target therapeutic doses of anti-HCV and anti-SRBI mAbs identified using in vitro and in vivo modelling
- Novel anti-viral and anti-receptor mAbs isolated
- Data obtained showing that virus cure using therapeutic mAbs is achievable
- Development of a robust process small- to medium-scale manufacture of therapeutic antibodies
- Establishment of Master Cell Banks for anti-viral and anti-receptor mAbs
- Development and validation of a robust pipeline for anti-HCV and anti-receptor mAbs that have potent and broad activity against HCV

Deviations from Annex I and reasons:

(Also state their impact on other tasks as well as on available resources and planning)

1. Problems with establishing higher producing cell lines (as reported in the previous reports) continued until we were able to purchase suitable lines from two suppliers. These difficulties were overcome in the penultimate reporting period. However, yields would still be insufficient utilising standard manufacturing and purification processes, therefore we set up a subcontract with the Brussels-based CRO Univercells, who have developed a range of proprietary manufacturing processes, to develop a suitable process for the two antibodies. Univercells received suitable cell lines in the 2nd quarter of 2017 and have established a suitable process for the anti-SRBI antibody, and are partway through achieving the same output for the anti-HCV antibody.
2. This has inevitably further delayed production of GLP lots for toxicology studies and also manufactured clinical lots for clinical trial.
3. The all-oral direct acting antivirals, with high safety and efficacy rates, also in patients with advanced cirrhosis, have completely changed the treatment paradigm of patients with chronic HCV infection before and after liver transplantation. In this setting, with most patients being treated (and cured) before LT, we foresee difficulties in using mAb to prevent HCV reinfection in real-life clinical practice.

This has meant that the following deliverables were not achieved:

D2.3 – Validation of GMP lots of antibody
D3.1 – Toxicology report
D6.3 – Confirmation of efficacy of GMP antibody preparations
D7.3 – cGMP manufacturing of anti-SRBI mAb
D7.4 – cGMP manufacturing of anti-HCV mAb
D8.1 – Highest tolerated dose determined
D8.2 – In vivo pK estimates obtained

Reasons for failing to achieve critical objectives and/or not being on schedule:

(Explain the impact on other tasks as well as on available resources and planning)

1. Problems in establishing cell lines with sufficient productivities were reported previously. These have had a major impact on overall progress and have pushed the timelines back considerably.
2. The adverse effects of these delays have been most keenly experienced by GenIbet, who have had to ensure their ability to serve external contracts and commitments.
3. GenIbet had a planned closure of their clean facility over summer 2017 which meant that GLP runs that were needed for toxicology studies could not be completed.

Use of resources:

(Highlighting and explaining deviations between actual and planned person-months)

The overall person/months employed, the amount of consumable expenditure, travel budget and subcontracting costs during the last period was in line with the planned use of resources. Budgets that were underspent were those that had been set aside for GMP activities and clinical trial.

UoN: Expenditure has been as expected
MoMAB: Expenditure has been as expected
CEINGE: Expenditure has been as expected
Genibet: Less than expected because of delay in process development.
UGent: Expenditure has been as expected.
IDIBAPS: Less than expected because clinical trial was not carried out.
UNISTRA: Expenditure has been as expected.

Training and knowledge transfer activities:

- All partners and associated scientists took part in regular HepaMab progress meetings and teleconferences.
- Partners and scientists participated in local, national and international conferences and symposia.
- Scientists also took part in at the bench training, and where appropriate placements and/or short courses.
- All scientists recruited to the project were encouraged to produce a Career Development Plan (examples attached as an Appendix to this report)

Dissemination activities:

- The project website (www.hepamab.eu) was regularly updated with project outputs and major achievements

- The consortium members produced >20 papers in peer reviewed scientific journals
- The participants were involved in general dissemination activities including public understanding of science events, and stakeholder meetings.
- All participants presented in either oral (including keynote talks) or poster format at international meetings

(Detailed description of dissemination activities can be found in the relevant periodic reports.)

Commercialisation activities:

- A patent applications for the G8 anti-HCV and anti-claudin mAbs have been drafted.

Ethics:

1. Clinical samples
    a. The G8 antibody is based on a previously established antibody derived from a human donor in 1993 (as bone marrow). The ethical permit for obtaining that sample is 90:261 (from the Stockholm Regional Ethics Committee in 1990).
    b. Use of patient biological specimens - Left-over liver tumours were isolated after informed consent from patients undergoing liver resection for hepatocellular carcinoma. Use of human liver tissue for research purposes including primary cells was approved by the local ethics committee at the Strasbourg University. The protocol was renewed in 2017 (CODECOH DC-2016-2616; French Ministry approval February 7, 2017). The left-over patient liver resection material was collected *during a medical procedure strictly performed within the frame of the medical treatment of the patient*. Informed consent was provided according to the Declaration of Helsinki.
    c. Patient information and informed consent procedures that were implemented. Patients were given an information sheet which outlines that their clinical and in some cases, personal data and left-over biological material (liver resection) that were collected during the course of their medical treatment, are requested for research purposes by their attending physician. The information sheet outlines the type of biological material and the type of personal information to be requested. The form also states that participation is a strictly personal choice and optional, that patients retain the right to withdraw their consent at any time and that the patient's decline or withdrawal from the study does not in any way compromise their medical care (please see the "Notice d'information du patient MAJEUR" document). Patients are also provided with an informed consent form in order to provide authorization or refuse the use of their biological samples for research purposes including or excluding genetic testing (please see the "Consentement éclairé du patient MAJEUR" document). Patients maintain the right to withdraw their consent at any time and to request the destruction of their biological material which is strictly respected. We comply with the principles enshrined in the Council of Europe Convention on human rights and biomedicine. Our research does not involve under any circumstances children and/or adults unable to give informed consent.
    While there is clinical descriptive data available for the subject, we had no information regarding the identity of the patients. All research records were stored in a locked cabinet, and only the investigators and study staff have access to these records. Each patient had an assigned a Personal ID code. Any electronic information was stored utilizing a code. Only study staff have access. All specimens were coded with a number and stored in a secured area. Participants are not identified by name thereafter in records or in resulting publications. Risks to the subject and adverse events were minimal, and tissue collection was part of clinical care.

    d. Acquiring regulatory approval for clinical trial: The study protocol will be presented and shall be approved by the local ethics committee and the European Medicines Agency. Preliminary discussions on trial design have been carried out as indicated above.

2. *Animal experiments*

a. There were no changes with regards to ethical issues associated with the project. Experiments involving animals were as described in the technical Annex. Small animals were being used in the generation of antibodies (normal in-bred laboratory mice for anti-CLD, SCID mice for back-up anti-HCV) and SCID-uPAR mice were being used in protection and challenge studies. Numbers of animals used for the efficacy studies were carefully calculated based on power calculations, together with estimated level of protection conferred by each mAb.
b. The relevant ethics permission for the studies had been obtained. All animal experimental protocols have been approved by the relevant animal ethics committees:
   Generation and use humanized uPA-SCID mice in antibody protection studies: Animal Ethics protocol AL/01/18/08/12 and renewal of approval 02014120416254981 (APAFIS#72.02); Animal Ethics protocol AL/02/19/08/12 (approval N. 02014120511054408 (APAFIS#74.03), expiry dates 08/03/2020 and 23/02/2020, respectively (Partner 7); and approvals ECD 10-35 and ECD 15-60 (Partner 5)
c. *Brief description of experimental procedures.*
   Generation of humanized uPA-SCID mice
   Human hepatocytes are transplanted into the immunodeficient uPA/SCID mice resulting in repopulation of 70% of the mouse liver with human cells thus rendering the mice susceptible to HCV infection. Briefly, production of uPA/SCID mice with a humanized liver is performed as described by Mercer et al., Nat Med 2001. Following analgesia with an IP injection of Buprecare® 0.1mg/kg and anesthesia by vetflurane the mice are transplanted with primary human hepatocytes (0.5-1x106 cells in 30 µL of PBS) through an intrasplenic injection. The skin is then closed following the injection by suture clips and the mice are allowed to recover under 100% oxygen. The mice are then returned in cages containing hydrated food and monitored daily for signs of distress or pain. If necessary, a second injection of Buprecare® is administered. For the cases were pain is persistent 48h following surgery, the mice are sacrificed. Humanized uPA-SCID mice HCV infection and prevention of infection or treatment with anti-host antibodies. For prevention studies, chimeric uPA-SCID mice were intraperitoneally injected with 500 µg/250 µl (0.25 ml) CLDN1-specific or control mAb at day −1, 1 and 5. For treatment studies, chimeric uPA-SCID mice were chronically infected with HCVcc or mouse passaged infectious serum of genotype 2a or 4. Chronically infected mice received 500 µg CLDN1-specific or control mAb once a week for 4 weeks. Blood was harvested by retro-orbital puncture under general anesthesia to measure serum human albumin levels and HCV RNA levels. A 0.5 ml insulin syringe (29G1/2 or 27G1/2 needle) was used for all injections. Mice were randomly assigned to the different experimental groups by a blinded technician. Experiments were performed in the UGent animal facilities according to Animal welfare laws and procedures to ensure animal welfare and limit animal discomfort. Liver samples were collected after cervical dislocation following exsanguination. The numbers of animals used were the minimum required for statistical purposes to accurately measure and determine differences between the populations being compared.

3. *Use of Genetically Modified Organisms* - Partners 1, 2, 4, 5 and 7 performed experiments involving genetically modified organisms. The committee were satisfied that these partners performed work to the highest standards in terms of fundamental safety issues and good laboratory practices (GLP). All experiments were performed in accordance with UK, French, Belgian, Italian and Swedish guidelines, as appropriate, as well as European guidelines.
   a. Use of GMO
      UNott - Gene cloning and protein expression / E-coli & human and animal cell lines
      MoMAB - Gene cloning and protein expression / E-coli & human and animal cell lines
      CEINGE - Gene cloning and protein expression / E-coli & human and animal cell lines

UGent - Gene cloning, protein expression and chimeric mouse models / E-coli, human and animal cell lines and genetically modified mice

UNISTRA - Gene cloning, protein expression and chimeric mouse models / E-coli, human and animal cell lines and genetically modified mice

There was no environmental impact during the project. The researchers performed all experimentation with GMOs in accordance with national and European guidelines, in particular European directives 90/219/EEC, 93/88/EEC and 98/81/EEC. In the experiments involving the use of vectors, safety of the clinical workforce and the environment from contamination and/or spreading of vectors was ensured and in compliance with the EC Directive on the Protection of Workers from Risks Related to Biological agents at Work (90/679/EEC).

Appendix – Example Career Development Plans

HepaMAb Career Development Plan

Name of Researcher: Biancamaria XXXXXX

Department: CEINGE Biotecnologie Avanzate, Naples, Italy

Name of Supervisor / Mentor: Prof A. Nicosia

Date: 30/11/2018

BRIEF OVERVIEW OF SPECIFIC ROLE IN RESEARCH PROJECT AND MAJOR ACCOMPLISHMENTS EXPECTED (bullet points ½ - 1 side A4 max.):

The CEINGE's main objectives in this project were:

- the generation of a high-producing stable cell line for the anti-SRB1 mAb c1671, suitable for GMP production with a minimal productivity of 500 mg/liter;
- the assessment of the antibody stability in the formulated buffer proper for *in vivo* injection;
- the design of preclinical studies in NHP.

My role in this project was the finalization of the objectives reported above by applying various experimental approaches

LONG-TERM CAREER OBJECTIVES (over 5 years):

1. Goals:

    - establishment of a stable cell line for the anti-SRB1 mAb c1671, which should have a target productivity of at least 500 mg/l;
    - assessment of the stability of an antibody formulation suitable for *in vivo* injection;
    - design of preclinical studies in NHP.

2. What further research activity or other training is needed to attain these goals?

SHORT-TERM OBJECTIVES (1-2 years):

- Research results

    a. I generated some stable cell lines by various approaches:

        - A HEK 293 stable clone by the mammalian Sleeping Beauty transposon/transposase system, with a productivity of 10 mg/lt;

- two CHO cell lines by a drug-induced (dihydrofolate reductase/methotrexate) gene amplification system:
  - an adherent CHO cell clone, with a productivity of 40 mg/lt;
  - a suspension CHO cell line, producing about 140 mg/lt, by modifications of the expression cassette used in the generation of the adherent CHO cell line.

The suspension CHO cell line was a good substrate for a "boosting" protocol (applied by a CRO) which doubled its productivity.
Although the desired productivity of about 500 mg/lt was not reached, the productivity of the boosted clone was enough to enter the GMP production

b. I evaluated the stability and affinity of c1671 mAb in a buffer formulation suitable for in vivo injection after limited stresses (see below), aimed to mimic the handling conditions of a single dose of the mAb before the injection in hypothetical tox studies. I demonstrated that the limited stress did not alter either the structure of the antibody or its affinity for SR-B1 receptor, compared to the reference c1671 mAb, resuspended in a different buffer.

c. I planned toxicology studies in cyno monkeys.

- Anticipated publications:

- Anticipated conference, workshop attendance, courses, and /or seminar presentations:

  - HepaMAb Progress Meeting, London, 26th-27th November 2015
  - HepaMAb Progress Meeting, Barcelona, 19th-20th January 2017

- Research Skills and techniques:
  - Training in specific new areas, or technical expertise etc:

Working on the generation of the cell line, I applied new molecular biology techniques for the manipulation of the mammalian genome. Moreover, as the project also included the characterization of the antibody produced by this cell line, I acquired a lot of experience in biochemistry, particularly about purification methods and binding assays, such as flow cytometry assays (sample preparation, acquisition and data analysis) and quantitative ELISA and cell-ELISA assays.
In addition, I learnt the principles of working in pre-GMP and GMP conditions and I had the opportunity to explore a new investigation field that is preclinical studies, in order to plan toxicology studies in cyno monkeys.

- Research management:
  - Fellowship or other funding applications planned (indicate name of award if known; include fellowships with entire funding periods, grants written/applied for/received, professional society presentation awards or travel awards, etc.)

- Communication skills:

  I developed the ability to show the experimental results in a concise manner thanks to recurrent meetings with my supervisors and to my involvement in teleconferences and some HepaMAb progress meetings with the other European partners of this project.
  I also improved my skills in report writing in order to give the scientific community a clear idea of objectives, achievements and future experiments.

- Other professional training (course work, teaching activity):

- Anticipated networking opportunities

- Other activities (community, etc) with professional relevance:

Date & Signature of fellow: Date & Signature of supervisor/mentor:

Please attach a copy of your CV to this CDP

HepaMAb Career Development Plan

Name of Researcher: Koen XXXXX

Department: Center for vaccinology, Ghent University

Name of Supervisor / Mentor: Philip Meuleman

Date: 06/08/2014

BRIEF OVERVIEW OF SPECIFIC ROLE IN RESEARCH PROJECT AND MAJOR ACCOMPLISHMENTS EXPECTED (bullet points ½ - 1 side A4 max.):

- Preclinical *in vivo* studies of lead anti-SR-BI 'mAb1671'.
- Evaluate *in vivo* anti-HCV efficacy of mAb1671 in the context of resistant mutants.
    - Determine whether mAb1671 remains successful in protecting human-liver mice from infections by HCV variants that have decreased susceptibility to anti-SR-BI therapy *in vitro*.
    - Analyze if addition of mAb1671 to a direct-acting antiviral (DAA) therapy prevents the on-therapy breakthrough of DAA-resistant mutants.
- We expect to demonstrate the applicability of SR-BI inhibition, by mAb1671, at different stages of HCV infections. It could be used to prevent rebound of HCV after liver transplantation or during DAA therapy.

LONG-TERM CAREER OBJECTIVES (over 5 years):

3. Goals: I want to perform and supervise basic research in a medical laboratory setting (clinical microbiology, haematology or chemistry) and manage a small team performing medical laboratory diagnostic tests.

4. What further research activity or other training is needed to attain these goals? I need to complete a three-year specialty program leading to the degree of clinical biologist.

SHORT-TERM OBJECTIVES (1-2 years):

- Research results
    - Anticipated publications:

      "Successful anti-SR-BI mAb therapy in humanized mice after challenge with HCV variants with in vitro resistance to SR-BI-targeting agents."
      Koen Vercauteren, Naomi Van Den Eede, Ahmed Atef Mesalam, Sandrine Belouzard, Maria Teresa Catanese, Dorothea Bankwitz, Flossie Wong-Staal, Riccardo Cortese, Jean Dubuisson, Charles M. Rice, Thomas Pietschmann, Geert Leroux-Roels, Alfredo Nicosia, Philip Meuleman. (*Hepatology*, *in press*)

"An anti-SR-BI mAb prevents HCV virologic breakthrough during a protease inhibitor therapy in humanized mice."
Koen Vercauteren, Ahmed Atef Mesalam, Richard Brown, Juliane Doerrbecker, Naomi Van Den Eede, Ali Farhoudi, Geert Leroux-Roels, Thomas Pietschmann, Alfredo Nicosia, Philip Meuleman. (*in preparation*)

- Anticipated conference, workshop attendance, courses, and /or seminar presentations:

"An anti-SR-BI mAb prevents HCV virologic breakthrough during a protease inhibitor therapy in humanized mice." (*oral presentation, HCV 2014, September, Banff, Canada*)
"An anti-SR-BI mAb prevents HCV virologic breakthrough during a protease inhibitor therapy in humanized mice." (*poster presentation, AASLD 2014, November, Boston, USA*)

- Research Skills and techniques:
  - Training in specific new areas, or technical expertise etc:
    1. Competence in *in vitro* and *in vivo* experimental design
    2. Relevant insights into research methods such as cell culturing, fluorescence microscopy, luminometry, polymerase chain reaction, ELISA, molecular biology, mouse handling, administration of test compounds, collection of mouse samples.
    3. Independent and critical thinking.
    4. Critical analysis and evaluation of my own findings and those of others (support in peer-reviewing articles submitted to different journals)

- Research management:
  - Fellowship or other funding applications planned (indicate name of award if known; include fellowships with entire funding periods, grants written/applied for/received, professional society presentation awards or travel awards, etc.)

    1. BD Pathway fluorescence microscope user meeting, 2011 Heidelberg, Germany, Fluorescent Image contest, awarded 2nd
    2. travel grant for young researchers awarded by the organising committee of The International Symposium on Hepatitis C Virus and Related Viruses (Melbourne, Australia, 2013, 633 EUR)
    3. travel grant for young researchers awarded by the organising committee of the EASL monothematic conference: translational research in chronic viral hepatitis (Lyon, France, 2013, 650 EUR)
    4. travel grant for young researchers awarded by the organising committee of The International Symposium on Hepatitis C Virus and Related Viruses (Banff, Canada, 2014)
    5. A BAEF (Belgian American Educational Foundation) post-doctoral fellowship for the academic year 2014-2015. I have had the particular honor to be named a Cabeaux-Jacobs fellow of the King Baudouin Foundation. The grant covers a stipend of 45,000$. This fellowship supports a one-year research project at the Rockefeller University, New York, at the department of Virology and Infectious Disease in the laboratory of Dr. Charles M. Rice.

- Communication skills:

I have prepared three first-author original research papers, three first-author review papers, and contributed as co-author on multiple papers of collaborating research groups.

I was able to attend, and present my original research data on, the following international conferences:

HCV symposium 2011, Seattle, USA;
ANRS (French national HCV research meeting) 2012, Paris, France (oral presentation);
HCV symposium 2012, Venice, Italy;
Cross-border symposium on HCV 2012, Ghent, Belgium (oral presentation);
ANRS (French national HCV research meeting) 2013, Paris, France;
HCV symposium 2013, Melbourne, Australia (oral presentation);
AASLD 2013, Washington DC, USA (not attended, poster presentation prepared by Koen Vercauteren and presented by Prof. Geert Leroux-Roels);
EASL monothematic 2013, Lyon (poster presentation)

- Other professional training (course work, teaching activity):

I have been involved in supervising and mentoring master students during their thesis preparations.

- Anticipated networking opportunities

I will attend, and present my original research, at the HCV symposium 2014, held in Banff, Canada. This is the most important basic science meeting within our field.

I will attend the AASLD/EASL Special Conference on Hepatitis C in New York, September 2014.

From September 1st of this year, I will start a one-year post-doctoral research project at the Laboratory of Virology and Infectious Disease at the Rockefeller University (RU) in New York, headed by Dr. Charles Rice. This group is one of the leading laboratories in HCV research worldwide. They have established the models needed and possess the instruments and expertise to perform essential experiments for the studies planned. I will communicate and work shoulder-to-shoulder with cutting-edge scientists in my research field which will dramatically boost my intellectual and scientific development. My research facility in Ghent has a long standing and fruitful collaboration with Dr Rice's group at the RU. My work at the RU would continue and reinforce this already established international collaboration. This work will eventually lead to joint publications between the Ghent en Rockefeller universities.

- Other activities (community, etc) with professional relevance:

During the coming year of post-doctoral training I will develop expertise in new research methodologies such as combined flow cytometry-high content image analysis, laser capture microdissection, gene array analysis, etc.

The combination of lab animal expertise available at the Ghent and USA research facilities together with very specifically required equipment, available at the guest research facility, will enable us to study a previously unexplored area in our research field.

For me personally, this post-doctoral training period will surely improve my intellectual and scientific development. I expect that this research project in the USA will increase the possibility of pursuing an academic scientific career.

After this one-year training, I will start a three-year course resulting in the degree of specialist in clinic biology with the ultimate goal to be employed in a university hospital and be involved in research by educating and supervising young researchers.

Date & Signature of fellow: Date & Signature of supervisor/mentor:

Please attach a copy of your CV to this CDP

HepaMAb Career Development Plan

Name of Researcher: Barnabas XXXXX

Department: School of Life Sciences

Name of Supervisor / Mentor: Jonathan Ball

Date: 01/08/2015

BRIEF OVERVIEW OF SPECIFIC ROLE IN RESEARCH PROJECT AND MAJOR ACCOMPLISHMENTS EXPECTED (bullet points ½ - 1 side A4 max.):

Role in Project

- To plan and conduct research using recognised approaches, methodologies and techniques within the research area.
- To analyse and illuminate data, interpret reports, evaluate and criticise texts and bring new insights to research area.
- To write up research work for publication and/or contribute to the dissemination at national/international conferences, resulting in successful research outputs.
- To identify opportunities and assist in writing bids for research grant applications. Prepare proposals and applications to both external and/or internal bodies for funding, contractual or accreditation purposes.
- To build relationships with both internal and external contacts in order to exchange information, to form relationships for future collaborations and identify potential sources of funds and/or opportunities for collaboration.
- To co-ordinate the operational aspect of research networks, for example, arranging meetings and updating web sites etc and contribute to collaborative decision making with colleagues in area of research.
- To provide support, guidance and supervision to other staff, where appropriate in own area of expertise.
- To plan and manage own research activity and resolve problems, if required, in meeting own/team research objectives and deadlines in collaboration with others.

Specific expected accomplishments

- Develop a chimeric HCV clones incorporating patient-derived E1E2 genes for genotype 3.
- Contribute to the analysis of genotype 1 and 2 chimeras
- Develop chimeric HCVcc cloning cassettes for genotypes 4, 5 and 6 and assess viability of reference clones
- Provide chimeric viruses upon request from project partner.
- Develop automated method for evaluating chimeric virus titres and neutralization phenotypes.
- Analyse the neutralisation properties of monoclonal antibodies generated by project partners.
- Contribute to the writing of progress reports

- **LONG-TERM CAREER OBJECTIVES (over 5 years):**

5. Goals: Contribute in a positive manner to the national understanding and conversations in molecular biology and virology.

6. What further research activity or other training is needed to attain these goals?
   Experience of communicating to people of diverse knowledge, cultural and interest backgrounds.
   Active, compassionate listening to those with misconceptions and conflicting opinions
   How to gain 'public trust' in the value of my knowledge and opinion as an expert.

SHORT-TERM OBJECTIVES (1-2 years):

- Research results
  - Anticipated publications:

  Method paper for the generation of HCVcc chimera cassettes
  Characterisation of genotype 3 HCVcc chimeras with patient-derived E1E2 genes
  Comparison of E1E2 entry and neutralisation phenotypes in the context of HCVpp and HCVcc

  - Anticipated conference, workshop attendance, courses, and /or seminar presentations:

  UK Microbiology Society annual meeting
  National HCV annual meeting
  International HCV conference
  European Virology conference

- Research Skills and techniques:
  - Training in specific new areas, or technical expertise etc:

  HCVpp generation and antibody neutralisation assays
  BSL3 training
  Infusion cloning technology

- Research management:
  - Fellowship or other funding applications planned (indicate name of award if known; include fellowships with entire funding periods, grants written/applied for/received, professional society presentation awards or travel awards, etc.)

- Communication skills:

  Oral presentation of work to groups with limited expert knowledge or understanding e.g. undergraduate students, research nurses or non-native English speakers
  Broaden public engagement and understanding of molecular virology in general through a website and/or blog.

- Other professional training (course work, teaching activity):

- Anticipated networking opportunities
  Having worked in 3 different Universities and collaborated with a large number of academics I have many new or more significantly more developed professional connections to the Nottingham Virus Research Group. Having worked on different viruses in the past

and worked closely with other groups specializing in a variety of techniques and viruses, both medical and veterinary, there will likely be opportunity for sharing of reagents, samples and technical expertise.

- Other activities (community, etc) with professional relevance:
  Encourage wider engagement of those academics in the University with an interest in virus research through a unified ‘centre’ or deliberate group organization.

Date & Signature of fellow:

Date & Signature of supervisor/mentor:

Please attach a copy of your CV to this CDP