

SUPPLEMENTARY INFORMATION**Supplementary figures and tables****Supplementary Table S1: Chimeric renilla luciferase reporter virus constructs used in this study.**

Short name	Neutralization cluster	GenBank Accession (reporter virus)	Long name	Publication (reporter virus)	GenBank Accession (E1/E2 sequence)	Publication (E1/E2 sequence)
GT1a (H77)	4	MT955901	H77c/1a/R2a	[1]	NC_038882.1	[2]
GT1b (J4)	4	MT955902	J4/1b/R2a	[1]	AF054247.1	[3]
GT1b (Con1)	1	MT955903	Con1/1b/R2a	[1]	AJ238799.1	[4]
GT2a (J6)	6	MT955904	JcR-2a	[5]	NC_009823.1	[6]
GT2a (2a-3)	3	MT955905	2a-3/2a/R2a	This study	KM361734.1	[7]
GT2b (J8)	1	MT955906	J8/2b/R2a	[1]	JQ745651.1	[8]
GT2b (2b-4)	3	MT955907	2b-4/2b/R2a	This study	KM361730.1	[7]
GT2b (2b-5)	1	MT955908	2b-5/2b/R2a	This study	KM361731.1	[7]
GT2k (2k)	2	MT955909	2k/2k/R2a	This study	AB031663.1	[9]
GT2r (2r)	2	MT955910	2r/2r/R2a	This study	JF735115.1	[10]
GT3a (S52)	1	MT955911	S52/3a/R2a	[1]	GU814264.1	[11]
GT4a (ED43)	4	MT955912	ED43/4a/R2a	[1]	NC_009825.1	[12]
GT5a (SA13)	5	MT955913	SA13/5a/R2a	[1]	MH427311.1	[13]

Fig. S1: Characterization of functional and sequence diversity of HCVcc reporter virus

stocks collected after transient transfection. (A) Culture fluid of Huh-7.5 cells transfected with in vitro transcribed viral RNA was collected at given time points or pooled across these time points (pooled) and used to inoculate naïve Huh-7.5 cells. Seventy two hours later, the inoculated Huh-7.5 cells were lysed and luciferases reporter gene activity was determined as a measure of virus infection. Four technical replicates and mean values are shown. (B) Given reporter virus stocks from (A) were used for neutralization assays with selected pIgs or pIgs from a healthy donor. Data are reported relative to the PBS control as % virus neutralization and with each virus stock denoted as independent dot. (C) Assessment of quasispecies diversity of given reporter viruses as determined by Illumina next generation sequencing.

Fig. S2: Correlation between neutralization susceptibility and virus infectivity, respectively binding of patient-derived pIg. (A) Infectivity of 13 HCVcc reporter viruses is plotted on the X-axis, whereas average neutralization of 104 patient-derived pIg specimen is plotted on the Y-axis. (B) Optical density (OD) of ELISA tests using HCV SA13 (GT5a) E1E2 proteins extracted from virus transfected Huh-7.5 cells is plotted on the X-axis, whereas average neutralization of 104 patient-derived pIg specimen is plotted on the Y-axis. The Pearson's correlations are displayed along with the p-values. The blue lines are the fitted linear models and grey areas represent the 95% confidence intervals for the linear models.

Fig. S3: Correlation between evaluations of cross-neutralization based on 13-virus or 6-virus reference panel. (A) Correlation analysis based on average neutralization data. (B) Correlation analysis based on CNI data. The Spearman's correlation is displayed along with the p-value. The blue line is the fitted linear model and grey area represents the 95% confidence interval for the linear model.

Fig. S4: Correlation between evaluations of cross-neutralization based on 13-virus or

selected single isolates. (A) Correlation analysis based on average neutralization measured with the 13-virus panel versus with only the GT2a (J6) strain. (B) Correlation analysis based on average neutralization measured with the 13-virus panel versus with only the GT2r (2r) strain.

Fig. S5: Analysis if infecting genotype dictates heterologous neutralization preference.

Neutralization data from 104 reference pIg donors analysed with 13-virus panel are plotted. Filled red dots represent ratio of CNI determined against the test viruses from the cognate infecting genotype (for infecting GT1 this is H77C, J4 and Con1) divided by CNI calculated with the remaining test virus from other GTs. A ratio greater than one indicates preferential neutralization of test viruses from the cognate infecting GT. Blue dots denote the same information only for GT2 being the infecting genotype. Note that GT2a (J6), GT2a (2a-3), GT2b (2b-4), GT2b (2b-5), and GT2k (2k) are the cognate GT2 test virus within the 13-virus panel. Crosses and silhouetted line indicate the overall CNI of a given sample against the entire 13-virus panel. Specimens are colour coded and grouped according to the infecting genotype and rank ordered according to increasing global CNI.

Fig. S6: Dependence of cross-neutralization index measured with 6 reference viruses on infecting genotype.

The CNI for each of the 496 patient-derived pIg specimen was determined using the 6-reference virus panel. Data are plotted grouped according to the genotype of the infecting virus. The box covers 50% of the data range i.e. 2nd and 3rd quartile, whiskers indicate the complete data range from 1st to 4th quartile, thick black bar indicates the median and '+' the mean. The points outside the whiskers are outliers.

Fig. S7: Metric multidimensional scaling of neutralization data (104 pIg samples and 13 or 6-reference viruses).

(A) Distance of each pIg specimen to the central point (i.e. centroid)

of the pIg cluster of its cognate genotype. Cognate genotype means the genotype of the infecting virus of the patient that the sample was drawn from. Data are plotted for the MDS with 13 viruses. **(B)** Same as in (A) only that data are plotted for MDS with 6-reference virus only. Note that analysis with the more balanced 6-reference virus panel moves the GT1 pIg closer to the average distance observed for the other GTs. The box shows 50% of the data range i.e. 2nd and 3rd quartile, whiskers indicate the complete data range from 1st to 4th quartile, thick black bar indicates the median and '+' the mean. The points outside the whiskers are the outliers.

Supplementary materials and methods

Cell culture. Huh-7.5 and Huh7.5.1 cells were cultured in Dulbecco's modified Eagle medium (Invitrogen, Darmstadt, Germany) with 10% fetal bovine serum (Capricorn scientific, Ebsdorfergrund, Germany), 1x nonessential amino acids (Invitrogen), 100 µg/ml streptomycin (Invitrogen), 100 IU/ml penicillin (Invitrogen) and 2mmol/L L-glutamine (Invitrogen). This medium is designated DMEM-complete.

Preparation of in vitro transcribed viral RNA for electroporation. Up to 20µg of reporter virus plasmid DNA was linearized using an appropriate restriction enzyme (with a restriction site downstream of the HCV genome). Restricted plasmid DNA was purified using the Qiaprep Spin Miniprep kit according to the vendors' instructions. Subsequently, 2µg of restricted and purified plasmid DNA were used as template for in vitro transcription. Reactions were completed in a total volume of 100µl containing the following components: 80 mM HEPES (pH 7.5), 12 mM MgCl₂, 2 mM spermidine, 40 mM dithiothreitol (DTT), a 3.125 mM concentration of each ribonucleoside triphosphate, 1 U RNase inhibitor (Promega), 0.6 U of T7 RNA polymerase (Promega) per µl. After incubation of the reaction mix for 2h at 37°C, 0.3 U T7 polymerase/µl was added and the reaction continued for 2 additional hours at 37°C. Subsequently, transcription was terminated by addition of 7.5 U of DNase (Promega) and incubation for 30 minutes at 37°C. In vitro transcribed RNA was purified from DNA debris and enzymes by using the NucleoSpin RNA Clean up kit (Macherey & Nagel) according to the instructions of the manufacturer. The quality of in vitro transcripts was checked by agarose gel electrophoresis, and RNA amount was quantified using a Nanodrop (Thermo Fisher Scientific).

Production of HCV reporter viruses by electroporation. To generate renilla luciferase reporter virus particles, we electroporated Huh7.5.1 cells with 5µg *in vitro* transcribed reporter

virus RNA essentially as described elsewhere [14]. Briefly, single-cell suspensions of Huh-7.5.1 cells were prepared by trypsin-treatment of cell monolayers and subsequent resuspension of cells in DMEM-complete. Subsequently, cells were washed with phosphate-buffered saline (PBS), counted and resuspended at a cell density of 10^7 cells per mL of Cytomix. Cytomix is composed of 2mM ATP, 5mM glutathione, 120 mM KCl, 0.15 mM CaCl_2 , 10 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (pH 7.6); 25 mM HEPES, 2 mM EGTA, 5 mM MgCl_2 . The pH of Cytomix was adjusted to pH 7.6 with KOH. The buffer was sterilised by filtration and stored at room temperature until use. ATP and glutathione were added just prior to use. 400 μ l of the cell suspension in Cytomix were gently mixed (pipetting up and down 5-times) with 5 μ g in vitro transcribed RNA, transferred to an electroporation cuvette (gap width 0.4 cm; BioRad), and electroporated with a BioRad Gene-pulser using 975 μ F, and 270 V settings. Electroporated cells were quickly transferred into 10-16 mL DMEM-complete and seeded into cell culture vessels. We collected and pooled supernatants of electroporated cells 48, 72 and 96 h later, filtered them through a 0.45 μ m pore-size filters and stored them at -80°C . Reporter virus constructs H77c/1a/R2a, Con1/1b/R2a, J4/1b/R2a, J8/2b/R2a, S52/3a/R2a, ED43/4a/R2a, and SA13/5a/R2a for production of cell culture-derived HCV particles (HCVcc) were described recently by Haid et al. [1]. These chimeric reporter virus genomes encode the E1/E2 genes of H77c (NC_038882.1) [2], Con1 (AJ238799.1) [4], J4 (AF054247.1) [3], J8 (JQ745651.1) [8], S52 (GU814264.1) [11], ED43 (NC_009825.1) [12], or SA13 (MH427311.1) [13], respectively. The J6-JFH1 renilla-luciferase reporter virus construct JcR-2a was described recently [5], and it encodes the E1-E2 genes of the J6CF isolate [6]. Novel reporter virus constructs 2a-3/2a/R2a, 2b-4/2b/R2a, 2b-5/2b/R2a encode glycoproteins from given viral GTs, which were recently cloned from HCV infected patients [7]. The reporter virus 2r/2r/R2a encodes the E1-E2 genes of the HCV QC283 isolate [10] (NCBI GenBank accession number JF735115.1) representing the GT2r subtype [15]. The reporter virus 2k/2k/R2a encodes the E1-E2 genes of the HCV isolate VAT96 [9](NCBI GenBank accession number AB031663.1)

representing the GT2k subtype [15]. Constructs were created by standard cloning techniques. Sequences of all reporter virus genomes used in this study were submitted to the NCBI GenBank database. Supplementary table S1 lists all reporter virus constructs including their historic names, references and GenBank numbers.

Isolation of immunoglobulin G. For purification of polyclonal human immunoglobulins (pIg) antibody spin trap columns (GE Healthcare) were used. Columns were inverted and centrifuged to remove the storage liquid (30 s at 100 x g). Equilibration was performed by adding 600 μ l binding buffer and subsequent centrifugation (30 s 100 x g). Thereafter, 200 μ l human serum (heat treated at 56°C for 40 min) was mixed with 400 μ l PBS and the mixture was incubated for 4 minutes on the column. After centrifugation (30 s at 100 x g), the column was washed twice with 600 μ l binding buffer. Bound antibody was eluted by adding 400 μ l elution buffer (1M glycine-HCl, pH 2.7) and the eluate was collected in a tube containing 30 μ l of neutralization buffer (1M Tris-HCl, pH 9). In a next step, cytotoxic buffer was washed out by adding a mixture of eluted antibodies and 5 ml phosphate-buffered saline (PBS) onto Amicon Ultra-4 (30kDa, Merck Millipore) and centrifugation at 4000 x g. The washing out procedure was repeated three times and in the final step, antibody concentration was measured using Nanodrop (Peqlab, Germany).

Antibody-mediated HCV neutralization. For inhibition of HCV infection, 100 μ l of a Huh-7.5 cell suspension (10×10^4 cells per ml) was seeded into each well of a 96-well plate 24 h prior to inoculation. Renilla luciferase reporter viruses were mixed with 500 μ g/ml patient derived IgGs and incubated for 30 to 60 minutes. This mixture was used to inoculate cells for 4 h in triplicates. Thereafter, 170 μ l DMEM cpl₁ was added onto the cells. Infection was quantified 48 to 72 h after virus inoculation by measuring luciferase activity. To this end, cells were washed once with PBS and lysed directly on the plate by addition of 35 μ l Milli Q water. After one freeze/thaw cycle, lysates were resuspended and after addition of luciferase substrate (1 μ mol/L colenterazine; P.J.K., Kleinbittersdorf, Germany; in PBS) relative light units (RLUs)

were measured in a plate luminometer (Lumat LB Centro, Berthold, Germany). The neutralization data was analysed using GraphPad Prism V.6.0b and GraphPad Prism V8.1.2 (GraphPad Software, La Jolla, California, USA).

ELISA assays. Binding of pIg to virus E1-E2 proteins was measured as described recently [16]. Cells transfected with viral RNA were extracted with RIPA buffer. Glycoproteins within extracts were captured by using 96-well Nunc-Immuno Maxisorbent plates that had been coated for at least 1h at 37°C with *Galanthus nivalis* lectin (5µg/mL; Sigma-Aldrich). After washing in TBST, the plates were blocked with 2.5% non-fat dry milk and 2.5% normal horse serum in TBST. Glycoproteins from cells transiently expressing GT5a (SA13) or HCV subgenome (SG) were captured overnight at 4°C. Lysates from SG transfected cells served as negative control. Patient IgG were incubated for 2 h at room temperature before bound antibodies were detected using anti-human –HRP and TMB substrate (Sigma Aldrich).

Structure analysis

To investigate whether the described clustering is reflected by variations in the three-dimensional structure of E2, amino acid conservation was plotted on a structural E2 model. For this purpose an alignment was carried out with the Clustal Omega tool [17] using the E2 sequences of isolates 2b-5, 2k, ED43, 2a-3, H77, J8, J6, J4, 2b-4, 2r, S52, SA13, Con1. JalView [18] was used to assign values for the conservation of the chemical acid amino acid properties: 11 (conserved; grey) to 1 (non-conserved; red). Conservation was visualized using PyMOL (The PyMOL Molecular Graphics System, Version 2.2.3, Schrödinger, LLC) to plot the amino acid variations on the structure of a soluble E2 ectodomain (PDB 6MEJ).

E2 contact residues of the bNAb HC84.26 were extracted from the crystal structure in complex with an E2 peptide (PDB 5ERW) and mapped on the truncated E2 ectodomain structure (PDB 6MEJ) to visualise the HC84.26 epitope borders.

Next generation sequencing of reporter virus preparations

Virus stocks of reporter viruses (GT1b(J4), GT2r(2r), and GT3a(S52)) were produced as described above. Virus containing supernatants of GT2r(2r)-transfected cells were harvested 48 hours and 96 hours post infection, and were individually concentrated ~10-fold using Amicon®Ultra-15 Centrifugal Filters 100K (Merck Millipore) by following the manufacturers guidelines. Subsequently, total viral RNA was extracted using the spin column based Quick-RNA™ Viral Kit (Zymo Research). Total extracted RNA was used to generate an RNA sequencing library with CelSeq v2 library preparation kit (Illumina) and sequencing was performed on an Illumina machine with the MiSeq technology using MiSeq Reagent Kits v3 (Illumina). All sequence and variant analyses were performed using the CLC Genomics Workbench 12 software (Qiagen). Generated paired Fastq files were trimmed and mapped against the respective reporter virus sequences (reporter virus sequences are described above and deposited at GenBank).

Supplementary references

- 1 Haid S, Novodomska A, Gentzsch J, Grethe C, Geuenich S, Bankwitz D, *et al.* A plant-derived flavonoid inhibits entry of all HCV genotypes into human hepatocytes. *Gastroenterology* 2012;**143**:213-22 e5.
- 2 Yanagi M, Purcell RH, Emerson SU, Bukh J. Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *Proceedings of the National Academy of Sciences of the United States of America* 1997;**94**:8738-43.
- 3 Yanagi M, St Claire M, Shapiro M, Emerson SU, Purcell RH, Bukh J. Transcripts of a chimeric cDNA clone of hepatitis C virus genotype 1b are infectious in vivo. *Virology* 1998;**244**:161-72.
- 4 Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 1999;**285**:110-3.
- 5 Reiss S, Rebhan I, Backes P, Romero-Brey I, Erfle H, Matula P, *et al.* Recruitment and activation of a lipid kinase by hepatitis C virus NS5A is essential for integrity of the membranous replication compartment. *Cell host & microbe* 2011;**9**:32-45.
- 6 Yanagi M, Purcell RH, Emerson SU, Bukh J. Hepatitis C virus: an infectious molecular clone of a second major genotype (2a) and lack of viability of intertypic 1a and 2a chimeras. *Virology* 1999;**262**:250-63.
- 7 Doerrbecker J, Friesland M, Riebesehl N, Ginkel C, Behrendt P, Brown RJ, *et al.* Incorporation of primary patient-derived glycoproteins into authentic infectious hepatitis C virus particles. *Hepatology* 2014;**60**:508-20.
- 8 Li YP, Ramirez S, Gottwein JM, Scheel TK, Mikkelsen L, Purcell RH, *et al.* Robust full-length hepatitis C virus genotype 2a and 2b infectious cultures using mutations identified by a systematic approach applicable to patient strains. *Proceedings of the National Academy of Sciences of the United States of America* 2012;**109**:E1101-10.
- 9 Samokhvalov EI, Hijikata M, Gylka RI, Lvov DK, Mishiro S. Full-genome nucleotide sequence of a hepatitis C virus variant (isolate name VAT96) representing a new subtype within the genotype 2 (arbitrarily 2k). *Virus Genes* 2000;**20**:183-7.
- 10 Li C, Cao H, Lu L, Murphy D. Full-length sequences of 11 hepatitis C virus genotype 2 isolates representing five subtypes and six unclassified lineages with unique geographical distributions and genetic variation patterns. *The Journal of general virology* 2012;**93**:1173-84.

- 11 Gottwein JM, Scheel TK, Callendret B, Li YP, Eccleston HB, Engle RE, *et al.* Novel infectious cDNA clones of hepatitis C virus genotype 3a (strain S52) and 4a (strain ED43): genetic analyses and in vivo pathogenesis studies. *Journal of virology* 2010;**84**:5277-93.
- 12 Chamberlain RW, Adams N, Saeed AA, Simmonds P, Elliott RM. Complete nucleotide sequence of a type 4 hepatitis C virus variant, the predominant genotype in the Middle East. *The Journal of general virology* 1997;**78** (Pt 6):1341-7.
- 13 Humes D, Ramirez S, Jensen TB, Li YP, Gottwein JM, Bukh J. Recombinant hepatitis C virus genotype 5a infectious cell culture systems expressing minimal JFH1 NS5B sequences permit polymerase inhibitor studies. *Virology* 2018;**522**:177-92.
- 14 Koutsoudakis G, Kaul A, Steinmann E, Kallis S, Lohmann V, Pietschmann T, *et al.* Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. *Journal of virology* 2006;**80**:5308-20.
- 15 Smith DB, Bukh J, Kuiken C, Muerhoff AS, Rice CM, Stapleton JT, *et al.* Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource. *Hepatology* 2014;**59**:318-27.
- 16 Khera T, Behrendt P, Bankwitz D, Brown RJP, Todt D, Doepke M, *et al.* Functional and immunogenic characterization of diverse HCV glycoprotein E2 variants. *Journal of hepatology* 2019;**70**:593-602.
- 17 Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, *et al.* Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular systems biology* 2011;**7**:539.
- 18 Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ. Jalview Version 2--a multiple sequence alignment editor and analysis workbench. *Bioinformatics* 2009;**25**:1189-91.