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Broad anti-HCV antibody responses are associated with improved clinical disease parameters in chronic HCV infection

Running title: Antibody responses in chronic HCV infection

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Abstract

During hepatitis C virus (HCV) infection broadly neutralizing antibody (bNAb) responses targeting E1E2 envelope glycoproteins are generated in many individuals. It is unclear if these antibodies play a protective or a pathogenic role during chronic infection. In this study, we investigated whether bNAb responses in individuals with chronic infection were associated with differences in clinical
presentation. Patient-derived purified serum IgG was used to assess the breadth of HCV E1E2 binding and neutralization activity of HCV pseudoparticles. Two panels were compared, bearing viral envelope proteins representing either an inter-genotype or an intra-genotype (gt) 1 group. We found that HCV viral load was negatively associated with strong cross-genotypic E1E2 binding ($P=0.03$). Overall we observed only modest correlation between total E1E2 binding and neutralizing ability. The breadth of inter-genotype neutralization did not correlate with any clinical parameters, however, analysis of individuals with gt 1 HCV infection (n=20), using an intra-genotype pseudoparticle panel, found a strong association between neutralization breadth and reduced liver fibrosis ($P=0.006$). Broad bNAb response in our chronic cohort was associated with a single nucleotide polymorphism (SNP) in the HLA-DQB1 gene ($P=0.038$) as previously reported in an acute cohort. Furthermore bNAb responses in chronic gt1 infection are associated with lower rates of fibrosis and host genetics may play a role in the ability to raise such responses.

**Importance**

Globally there are 130-150 million people with chronic HCV infection. Typically the disease is progressive and is a major cause of severe liver cirrhosis and hepatocellular carcinoma. While it is known that neutralizing antibodies have a role in spontaneous clearance during acute infection, little is known about their role in chronic infection. In the present work we investigate the antibody response in a cohort of chronically infected individuals and find that a broad neutralizing antibody response is protective, with reduced levels of liver fibrosis and cirrhosis. We also find an association with SNPs in class II HLA genes and the presence of a broad neutralizing response indicating that antigen presentation may be important for production of HCV neutralizing antibodies.
Hepatitis C virus (HCV) is a significant cause of liver morbidity and mortality worldwide (1). The majority (75%) of those infected proceed to chronic infection (2). Symptomatic acute HCV infection is rare; therefore HCV has the potential to spread undetected within those at risk. In countries with high prevalence, poor healthcare infrastructure and lack of funding make eradication of HCV unlikely through curative therapies alone (1, 3). Thus, effective preventative strategies are needed to achieve global eradication of the virus (4).

Antibodies targeting the HCV envelope glycoproteins E1 and E2 can contribute significantly to viral clearance in HCV infection (5, 6). These proteins are responsible for virus attachment and entry into host cells through interaction with the receptors SR-B1, CD81, Claudin and Occludin (7-10). Previous observational studies have shown rapid onset of anti-HCV antibodies to be associated with higher likelihood of clearance (11). More recently, it has been proposed that developing an antibody profile capable of neutralizing diverse HCV strains (broadly neutralizing) predicts acute clearance in a cohort infected with gt1a HCV (12). Further studies have suggested that broadly neutralizing antibodies (bNAbs) may be able to control levels of virus and contribute to clearance even after infection has become established (13). In one case of a chronically infected patient who spontaneously cleared HCV, a bNAb response was generated, initially with subsequent restoration of T cell activity and resolution of infection (5).

Only a small number of individuals with bNAbs have been studied in detail with little information on the regions of the E1E2 glycoproteins that are preferentially targeted by these antibodies. This has largely involved epitope-mapping of patient-derived monoclonal antibodies (mAbs) (14-16). Human neutralizing and non-neutralizing mAbs have been used to identify distinct immunogenic domains of E1E2 (15, 17-21). However, in vivo, a polyclonal response is generated which may target multiple regions of the envelope proteins. A recent study demonstrated that some HCV-infected individuals largely target one immunogenic domain whereas others produce antibody responses to multiple
domains (21). The importance of interplay of antibodies binding different epitopes has not been fully explored, although there are conflicting reports of some antibodies conferring additive or interfering effects on virus neutralization (22, 23).

While diverse HCV strains are usually categorized by genotype, this does not correlate well with sensitivity to neutralization by mAbs (24, 25). The genetic diversification of HCV, both within and between hosts, introduces the potential to escape from monoclonal bNAbs with several studies reporting naturally occurring, single amino acid (aa) mutations conferring escape (18, 26). The recent E2 crystal structures (27, 28) provide evidence that antibody resistance is complex with mutations distant from the targeted epitope affecting antibody binding, presumably through structural changes (26). Therefore, further studies of the antibody response to a varied range of envelope proteins \textit{in vivo} are required. As animal models of HCV infection and adaptive immunity are suboptimal, we can still gain useful information from studying the humoral responses of chronically infected individuals using \textit{in vitro} models.

Although there is evidence that bNAbs have clinical relevance in acute infection, their role in chronic infection is not clear. Indeed, the immune response to HCV can have pathological consequences as seen in cryoglobulinaemic vasculitis (29). If large scale vaccination was to be considered, it is important to determine that stimulating such a response will not be harmful in the event of an authentic infection. Studying clinical associations in patients with bNAbs can reveal potential adverse outcomes and yield insights into factors associated with NAb production.

In this study, we investigate bNAb responses in chronically infected HCV (CHCV) patients, determine any association with clinical and host factors, and characterize the epitopes targeted by these antibodies. We identify an association between bNAb response and less severe liver disease and show that a bNAb response targets multiple neutralizing E2 epitopes within different immunodomains. We also report an association between bNab response and age, however no
association with estimated duration of infection or age at infection was found. Finally, we confirm that SNP rs2395522 in the HLA-DQ gene is strongly associated with production of a bNAb response.

**Methods**

**Patient characteristics.** Subjects with either gt1 or gt3 CHCV were prospectively recruited from 3 local liver clinics. Individuals with co-existing liver pathologies, body mass index (BMI) ≥ 31 or hepatocellular carcinoma were excluded. Healthy controls with no liver pathologies or significant co-morbidities were also recruited and their samples used as negative controls in subsequent experiments. All subjects completed a symptom questionnaire, clinical details were recorded and baseline biochemistry, virology and Interleukin-28B (rs12979860) profiles were determined. Liver stiffness was measured by transient elastography using a Fibroscan (Echosens). Serum and whole blood samples were obtained and stored at -70°C. Ethical approval was granted for this study by the West of Scotland Research Ethics Committee and all patients gave informed consent.

**Cell lines.** Human hepatoma Huh-7 cells, Huh7-J20 cells (30) and human epithelial kidney 293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 5% nonessential amino acids, Penicillin, Streptomycin and 200 mM L-glutamine. Huh7-J20 cells were supplemented with 2μg/ml puromycin.

**Antibodies.** The anti-E2 human monoclonal antibodies (HmAb) CBH-4B, CBH-7, HC-1, HC-11 and HC-84 (31-33) were a generous gift from Steven Foung. The anti-E2 mouse mAb AP33 has been described previously (34).

**E1E2 binding assay.** The enzyme-linked immunosorbent assay (ELISA) to detect antibody binding to HCV glycoproteins was performed as described (35). Briefly, HCV glycoproteins in lysates of HEK-293T cells transfected with E1E2 expression plasmids were captured onto Galanthus nivalis agglutinin (GNA) coated Immulon II plates (Thermolabsystems). Protein G-purified patient IgG was
isolated from serum using the Nab protein G spin kit (Thermoscientific) and the concentration was quantified by absorbance at 280nm. Purified patient IgG was added at 200 µg/ml in PBS containing 0.05% Tween-20 and 2% Skimmed Milk Powder (PBSTM) and bound antibodies were detected using HRP-conjugated anti-human IgG antibody (Sigma A0170) and TMB (3,3, 5, 5’-tetramethylbenzidine, Sigma) substrate. Two representative healthy control samples were included for quality control purposes. A dilution curve of mAb AP33 that binds to a highly conserved linear epitope was also included to enable comparison between plates. Absorbance values were measured at 450 nm and normalized according to the AP33 curve to give a value representing the concentration of AP33 (in µg/ml) which gave the same absorbance as the test sample.

**Generation of HCV pseudoparticles (HCVpp), infectious virus (HCVcc) and neutralization assays.** HEK-293T cells were co-transfected with plasmids expressing murine moloney leukaemia virus (MLV) Gag-pol, the MLV transfer vector carrying firefly luciferase reporter and plasmid expressing the relevant HCV E1E2. After 72 h, the medium was harvested, filtered through a 0.45 µM membrane and used as a source of HCVpp as described (36). Infectious virus was produced in Huh7 cells by electroporation of RNA encoding full-length JFH-1 or chimeric JFH-1. The intra-genotypic chimera 2B.1.1/JFH1 and the inter-genotypic chimera HQL (1a H77/JFH1) have been described previously (20, 30). For neutralization assays, HCVpp and purified patient IgG at 100 µg/ml (equivalent to serum dilution of 1:100-1:250 based on the known IgG levels in chronic HCV patient sera (37)) were mixed and incubated for 1 h at 37°C, then used to infect Huh7 cells for 4 h. The inoculum was removed and fresh media was added. Cells were lysed 3 days post-infection and infectivity assessed using the GloLysis Luciferase assay (Promega). Similarly, neutralization of HCVcc infection was tested in Huh7-J20 cells and infectivity assessed 3 days post-infection using the Phospha-Light assay system (Thermofisher) to measure secretory alkaline phosphatase (SEAP) levels (30).

**Amplification of viral E1E2 sequences derived from patients.** A cDNA library was created from HCV RNA isolated from patient serum using the QiaAMP Viral RNA mini kit (Qiagen). The
complete E1E2-encoding region was amplified using degenerate nested primers (Gt1 outer sense 5’
GTGAAYTAYGCRAAGGGAA, Gt1 outer antisense 5’GCAAAGCAGAARACACGAG, Gt1
inner sense 5’CACCATGGGTGCTCYTTYTCTATCTTCC, Gt1 inner antisense 5’
AAAGTTTCTAGATTACGCTCYGCTCGGAKA) and cloned into the phCMV expression
vector using the Gateway recombination cloning system (Invitrogen).

DNA isolation and SNP analysis. DNA was extracted from whole blood using the QiAMP DNA
Mini Kit (Qiagen). Custom primers for SNP rs2395522 and reporter dye mix (ThermoFisher) were
included in a qPCR reaction using Taq mastermix on a 7500HT Fast Real-Time PCR system (Applied
Biosystems). Samples were assigned as A/T heterozygotes or AA or TT homozygotes according to
the fluorescence signal.

Epitope targeting. A soluble form of gt1a E2 (H77) protein (sE2) was purified following expression
in High Five insect cells. Immulon II plates were coated with sE2 at 1 µg/ml and incubated with
purified patient IgG at 200 µg/ml in PBSTM. Subsequently, biotinylated antibodies to known epitopes
were added at a concentration close to their half maximal effective concentration (EC50) (14, 17, 18,
38). Streptavidin-HRP was added and binding measured as above. The reduction in relative binding of
each biotinylated antibody (calculated as percentage reduction in absorbance) on addition of patient
IgG compared to PBSTM control was determined.

Analysis. Results of assays and any statistical analysis of association with clinical features were
conducted using GraphPad Prism 6 Software (GraphPad Software, California) and SPSS v. 19.09
(IBM, New York). Statistical comparisons were made using non-parametric tests (Chi Squared for
categorical data, Wilcoxon Rank Sum for ordinal or numeric data) unless otherwise stated.
Results

**Cohort.** Fifty-one HCV-infected patients (27 gt1, 24 gt3) from the CHCV cohort and 8 healthy controls were recruited. Demographics of the cohort are shown (Table 1). Apart from a higher prevalence of Asian ethnicity in the gt3 group ($P=0.04$), there were no significant differences in demographics between subjects infected with either gt1 or gt3 HCV.

**E1E2 HCVpp panels.** To determine reactivity and neutralization of patient-derived IgG across a diverse range of envelope sequences, two panels of HCV pseudoparticles bearing test envelope proteins were used. The first, termed Panel XG, enabled analysis of antibody reactivity with E1E2 proteins of different viral genotypes. This panel comprised E1E2 from 6 subgenotypes (39): gt1a H77c (Accession number: AF011751), gt1b UKN1B5.23 (AY734976); gt2a JFH-1 (AB047639); gt2b, UKN 2B1.1 (AY734982); gt3a UKN3A13.6 (AY894683); gt4 UKN4.11.1 (AY734986). The second, termed Panel Gt1 was created to allow investigation of antibody reactivity within a single genotype as might arise during a natural infection. 103 patient-derived E1E2 sequences from eighteen gt1 HCV-infected patients collected from 3 cohorts across the UK (Trent HCV study group (40), St. Mary’s Acute Hepatitis C Cohort (41), Glasgow chronic HCV cohort (42)) were tested in the HCVpp system for infectivity (data not shown). As found previously in other studies not all sequences were infectious in this system (25, 43). 64% of the E1E2 sequences tested were functional giving a robust luciferase signal >10-fold above background. All the amino acid sequences were aligned in ClustalW, the best protein model with the lowest BIC score (16423.799) was used to generate a phylogenetic tree using the Maximum Likelihood method (Fig.1). The panel selection criteria was based on the overall genetic difference ($\rho$ distance) and representation of amino acid variability found in all gt1 E1E2 sequences registered with the Los Alamos HCV sequence database. Analysis of >3800 gt1 E1E2 sequences in the database identified 400aa residues that were conserved in at least 90% of sequences; of these the majority (306aa) are conserved in 99% of sequences. 154aa were found to be variable in more than 10% of gt1 E1E2 sequences. Eleven infectious sequences including the reference gt1a sequence H77 and ten-patient derived E1E2 sequences from nine HCV-infected
individuals were selected for inclusion in the gt1 panel Accession numbers: AF011751, AY734976, AY734971.1, AY734968.1, EU155192.1 and KU645403 to KU645407. These sequences represent the variability at 145/154 variable residues. In addition, 31aa residues represent minor variants found in fewer than 10% of sequences in the database.

Cross-genotypic antibody reactivity is associated with lower viral load. Patient IgG reactivity to whole E1E2 from Panel XG was tested by ELISA. Relative binding strength based on absorbance readings normalized to an AP33 standard curve were ranked from 1 (highest) to 51 (lowest) for each gt; all gt ranks were combined and a final rank position assigned (Table 2). This gave an overall indication of the relative binding breadth for each patient. The cohort was divided in half by binding rank and the top ranked half compared to the lower half to determine any clinical associations with breadth of antibody binding. Those with broader ELISA reactivity profiles had a significantly lower viral load ($P=0.03$, Fig. 2A). Individuals with a broader breadth of binding were more often infected with gt1 HCV and conversely individuals with a narrower breadth of binding were more often infected with gt3 HCV ($P=0.04$; $P=0.04$; Supporting Table S3). These associations appear to be independent as there was no association between gt of infection and viral load (Supporting Table S3, $P=0.59$).

Neutralization is not associated with viral load. Purified IgGs derived from the CHCV cohort were tested for their ability to neutralize HCVpp bearing envelope proteins from Panel XG and HCVcc bearing envelope proteins from Gt1a (1A-HQL), Gt2a-JFH-1 and Gt2b.1.1 (Table 3). In accordance with Urbanowicz and coworkers we found that HCVpp were more readily neutralized than HCVcc (25). Therefore neutralization was categorized as a reduction in infectivity of 50% in the HCVpp system and 40% in the HCVcc system. In both systems, gt2B.1.1 was particularly resistant to neutralization. The breadth of neutralization of HCVpp Panel XG, defined as broad (neutralization of $>3/6$ genotypes) or narrow ($<4/6$ genotypes), was analyzed for association with clinical factors. Twenty (40%) of the 51 individuals tested had broad cross-genotypic neutralizing IgGs. There were no significant associations of breadth of cross-genotypic neutralization with viral load (Fig. 2B).
Similarly, IgG from 20 CHCV individuals with gt1 infection were tested for neutralizing activity against Panel Gt1 (Table 4) and the number of pseudoparticles neutralized to the 50% level by each individual calculated. Neutralization of >7/11 strains was defined as ‘broad’ while ‘narrow’ neutralization was defined as <8/11 strains. As with Panel XG, there was no association between breadth of neutralization of Panel Gt1 and viral load (Fig. 2B).

Correlation between ELISA binding profiles and neutralization. To determine the level of agreement between the ELISA binding profiles and neutralization activity used to determine antibody ‘breadth’ we calculated non-parametric correlation co-efficients between the assays. For the full cohort there was a modest correlation of cross-genotypic ELISA binding rank with number of genotypes neutralized in Panel XG and Panel Gt1 (Spearman’s Rho correlation co-efficient -0.31, \( P=0.03 \) and -0.49, \( P=0.03 \), respectively) (Figs. 3A, 3B). Neutralization was determined for the full cohort against HCVpp and HCVcc (Table 3). Overall there was a significant correlation between the neutralization rank in both systems (Spearman’s Rho correlation co-efficient = 0.44, \( P=0.001 \), Fig. 3C). Indeed the strongest neutralizing IgGs efficiently neutralized HCVpp and HCVcc. Interestingly, IgG isolated from some individuals neutralized HCVcc more effectively than HCVpp and vice versa, (compare C1021, C1061 with C1046, C1035, Table 3). There was also a significant correlation between the proportion of genotypes neutralized in the two HCVpp panels (Spearman’s Rho correlation co-efficient =0.58 \( P=0.007 \), Fig. 3D) for the gt1-infected subgroup. Neutralization is only one possible mechanism through which HCV-binding antibodies exert potential antiviral selection.

The modest correlation between ELISA binding and neutralization highlights that while an individual with strong HCV-binding antibodies often has strong neutralizing antibodies this is not always the case. This disparity is evident in the lack of association between the breadth of neutralizing antibodies and viral load, despite an association between HCV-binding and viral load.

Association of increased age and liver fibrosis with narrow intra-genotype neutralization. Neutralization breadth for Panel XG was not associated with any clinical features (Fig.4A, 4C, 5A, 5C, 5E). Interestingly, analysis of the subgroup of gt1-infected individuals tested against Panel Gt1
found an association between breadth of neutralization and age since the broadly neutralizing group was significantly younger ($P=0.009$; Fig. 5B). However, there was no association of neutralization breadth with the estimated duration of infection or age of acquisition for the gt1-infected subgroup (Fig. 5D, 5F). Most notably, there was a striking association between breadth of neutralization activity and liver fibrosis for the gt1-infected subgroup. The broadly neutralizing group had significantly lower levels of liver fibrosis as determined by transient elastography ($P=0.006$; Fig. 4B) and fewer cirrhotic individuals ($P=0.02$; Fig. 4D). Importantly, the association between neutralization breadth and Fibroscan readings remained significant when corrected for age ($P=0.025$, Generalized Linear Model, SPSS V. 19.0).

**Role of HLA-DQ polymorphisms in predicting antibody neutralization breadth.** A recent study showed a link between breadth of neutralizing antibodies against acute gt1 HCV infection and a SNP (designated rs2395522) in an MHC Class II gene, HLA DQB1 involved in antigen presentation (12). Therefore we analyzed our cohort for any association between this and the number of HCVpp neutralized in both panels. There was no significant association between presence of these alleles and the number of HCVpp neutralized in Panel XG ($P=0.41$; Fig. 6A). However, the presence of the rs2395522 AA or AT allele was significantly associated with a greater number of HCVpp neutralized in Panel Gt1 ($P=0.038$; Fig. 6C). There was no association between SNP rs2395522 and liver fibrosis for either Panel ($P=0.2$ and $P=0.62$; Figs. 6B, 6D). In addition, we tested another genetically linked HLA-DQA2 SNP rs9275224. Here the GG or AG allele was also significantly associated with increased neutralization breadth in panel Gt1 ($P=0.038$, data not shown as the graphs are identical due to the genetic linkage between the SNPs). SNP rs2395522 is present in the intergenic region of HLA-DQB1; the functional consequences are unknown. However, the linked SNP rs9275224 is associated with autoimmune diseases including systemic sclerosis and rheumatoid arthritis (44, 45).

**E2 epitopes targeted by patient IgG.** In an effort to understand the epitopes targeted by the gt1 patient IgGs used for the intra-genotypic analysis, we determined if they competed for binding to E2 with some well-characterized antibodies. The conformational antibodies selected have been
characterized as binding to specific hypothetical immunodomains of E2, designated A, B or C. Their precise locations on E2 have not been identified to date, however, antibodies that bind immunodomain A including HmAb CBH-4B are non-neutralizing. Antibodies that bind to immunodomain B (HmAb HC-1, HC-11 and HC-84) and C (HmAb CBH-7) are able to neutralize HCV. While immunodomain C has not been characterized, immunodomain B has been shown to contain the CD81 receptor binding site (15). Lastly, the mouse NAb AP33 that binds a linear epitope (aa412-423) was also selected. The majority of the broadly neutralizing samples were able to efficiently compete with 3 or more of the E2 antibodies tested, in contrast most of the narrow neutralizing samples could only compete with 2 or fewer E2 antibodies (Fig. 7A). Interestingly, while 15/20 samples tested competed with HmAb HC-11 at the >50% level, the majority of broadly neutralizing samples inhibited both HmAbs CBH-7 and HC-11 binding, 6/10 that recognize different neutralizing immunodomains of E2 compared to 1/10 of narrow neutralizing samples. There was also a significant association with intra-genotypic 1 neutralization breadth ($P=0.004$, $P=0.002$; Fig. 7B).

Together, these data suggest that individuals with a broadly neutralizing phenotype compete with antibodies at multiple sites on E2. Moreover, they have antibodies that efficiently bind to more than one neutralizing immunodomain whereas those with a narrow neutralizing profile effectively target limited numbers of neutralizing epitopes.

**Discussion**

Our analysis of a clinical cohort chronically infected with HCV has yielded new insights into the importance of the antibody response in disease progression and factors associated with functional breadth of the antibody response. A broad cross-genotype HCV-binding antibody response was significantly associated with gt1 HCV infection and independently with reduced viral loads. Importantly these clinical associations with broad HCV-binding were not evident when analyzing the breadth of neutralization activity, highlighting distinct biological roles for non-neutralizing and neutralizing anti-HCV antibodies. The association of ELISA binding and lower viral loads could reflect the presence of antibodies binding to conserved non-neutralizing regions which would help
clear virus from serum through opsonisation and subsequent phagocytosis as demonstrated by Eren and coworkers or complement dependent lysis but would not directly inhibit hepatocyte cell entry (38). Indeed, there is evidence that binding of virus by non-neutralizing antibodies may prevent neutralization by antibodies targeting epitopes required for cell entry (46).

While no clinical features were significantly associated with broad or narrow neutralization as characterized using Panel XG, we did observe significant associations using a larger intra-genotype 1 panel. Most importantly, we show that individuals infected with HCV gt1 who are better able to neutralize an HCVpp panel incorporating different gt1 E1E2 sequences are less likely to have cirrhosis or significant liver fibrosis. The panel is composed of strains collected in the UK over the past decade from different geographical locations and incorporating changes at the majority of variable aa positions observed within a large sequence database. Therefore this panel represents the most common viable aa substitutions that may occur within host virus.

The association with lower levels of liver fibrosis with bNAbs is insufficient evidence alone to demonstrate a protective effect. However, this adds to case studies which have suggested that individuals with genetic or iatrogenic suppression of the antibody response show more rapid liver disease progression (47, 48). It is biologically plausible that individuals possessing antibodies capable of preventing spread of new variants of their infecting HCV strain may have a degree of protection from liver injury. Interestingly this ‘broadly neutralizing’ group was also significantly younger. This may suggest that the higher levels of liver fibrosis observed in the narrow neutralizing group was simply caused by longer duration of infection. However, we found no difference in duration of infection between the broad and narrow neutralizing groups and importantly the association between neutralization breadth and reduced fibrosis remained significant when corrected for age. There is already evidence that those infected in older age have more rapid disease progression (49) and that B-cell repertoire narrows with age (50). Therefore, this may also reflect an ageing effect on NAb responses.
Although the association of neutralization breadth in Panel Gt1 and host factors was clear, these were not as apparent in Panel XG. It is not clear from our data if the associations are indeed gt1-specific or simply caused by limitations due to smaller numbers of genotypes used to determine ‘breadth’ in the cross-genotypic panel. Although there was a significant correlation between the numbers of HCVpp isolates neutralized in both panels, some individuals who had narrow cross-genotypic neutralization profiles showed broad neutralization activity against Panel Gt1 suggesting that some bNAbs may be gt1-specific.

No association between levels of E1E2 binding and fibrosis was found. Unlike an earlier study (51), we found no relationship between antibody binding to the autologous genotype and clinical outcomes although there was a trend towards cirrhosis in gt1-infected individuals with poor binding to gt1a E1E2 ($P=0.10$, data not shown). Combined with the neutralization panel data, this suggests that if anti-envelope antibodies do have a protective effect, this is most marked where the antibodies target regions necessary for virus entry.

We have demonstrated that a NAb response is not closely correlated with the extent of patient IgGs binding to the whole E1E2 molecule, suggesting that some individuals preferentially target important neutralization epitopes. Our data also suggests that those gt1-infected individuals who mount a broadly neutralizing response effectively direct antibodies at more than one neutralizing domain on E2. In contrast, those with a narrower neutralizing response appear to target only one region, that recognized by the HmAb HC-11. It is possible however, that the narrow neutralizing samples do contain antibodies that target other neutralizing epitopes albeit at a lower concentration or with lower affinity than found in the broadly neutralizing group. Previous studies have shown that different regions of E2 interact to prevent neutralization, therefore it is likely that an antibody response interfering with multiple regions of E2 maybe more effective than a response targeting one epitope alone (52). Furthermore, Carlsen et al recently showed synergy in neutralization using a combination of two antibodies against differing domains (53). Our identification of epitopes targeted was constrained by the panel of monoclonal antibodies used. However, alternative methods such as
peptide and phage display capture have limited ability to detect antibodies directed at discontinuous
epitopes (21) therefore our data are a valuable complement to information from these studies.

While there are many possible explanations for why individuals might preferentially respond to
particular epitopes, we have confirmed that SNP rs2395522 in the HLA-DQB1 gene is associated
with the development of bNAb in gt1-infected patients (12). HLA-DQB1 genotype has already been
identified as one of the host factors known to influence outcome of HCV infection in Caucasian
populations (54). This may be due to restriction in antigen presenting cell presentation of epitopes to
CD4 cells or may involve another mechanism. In contrast, we did not observe an association of this
SNP with neutralization capability across pseudoparticles of other genotypes. In particular, there was
no association of the SNP with the ability of IgG from gt3-infected individuals to neutralize our
standard gt3a HCVpp (P=0.45, data not shown). This may be due to the limitations of testing one gt3
isolate, alternatively it is possible that other HLA genes could be more important for adaptive
responses to other genotype infections. Further studies will be required to distinguish between these
possibilities.

Our study demonstrates that broad anti-HCV neutralizing responses are associated with lower levels
of liver fibrosis, raising the possibility for a protective role in chronic infection. Our data also show
strong indications that potent neutralizing responses target multiple key regions of E2 rather than a
single epitope. This has significant implications for HCV vaccine design suggesting that a successful
vaccine must induce NAbs to different regions of E2. If we aim to produce a universally protective
vaccine for HCV, a deeper understanding of the role of host genotype and presented epitope sequence
in determining breadth of antibody response requires further exploration across a wider range of
isolates from differing genotypes before vaccine candidates are tested on a wider scale.

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Author contributions: AP and JM designed and supervised the project. RS and MR recruited the cohort with assistance from SB and PM. RS, VC, SC and MR performed the experiments. ET provided patient samples. RS, VC, MR and AP prepared the manuscript. All authors approved the manuscript.

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Table 1: Demographics of Cohorts

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<tr>
<td>Age, Median (range)</td>
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<td>Previously exposed to interferon based treatment⁵, (%)</td>
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<td>HCV RNA load pre-treatment  IU/ml, Median (range)</td>
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<td>Cirrhosis, Present (%)</td>
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<td>Transient elastography (kPa), Median (range)</td>
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</tbody>
</table>

*3 subjects not tested  **no information available for 6 subjects. ⁵All individuals had HCV infection at the time of testing, those previously exposed to interferon were either relapsers or null responders. No individuals were on therapy at the time of sampling.

Table 2: ELISA binding of CHCV cohort to cross-genotypic E1E2 panel.

<table>
<thead>
<tr>
<th></th>
<th>Gt 1A (rank)</th>
<th>Gt 1B (rank)</th>
<th>Gt 2A (rank)</th>
<th>Gt 2B (rank)</th>
<th>Gt 3A (rank)</th>
<th>Gt 4 (rank)</th>
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Table 3: Neutralization activity of CHCV cohort.

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Concentration of AP33 (μg/ml) representing the A450nm absorbance in each sample. The E1E2 binding rank, strongest (1) to weakest (51) is shown in brackets.
|    | C1008 | C1009 | C1010 | C1012 | C1013 | C1015 | C1016 | C1018 | C1020 | C1021 | C1022 | C1023 | C1024 | C1029 | C1030 | C1031 | C1032 | C1033 | C1034 | C1035 | C1036 | C1037 | C1038 | C1040 | C1041 | C1042 | C1043 | C1045 | C1046 | C1047 | C1049 | C1050 | C1052 | C1054 | C1055 | C1056 | C1057 | C1060 | C1061 | C1062 | C1063 | C1064 | C1072 | C1074 | C1089 | C1112 | C1128 |
|----|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|    | 54.6  | 50.8  | 72.9  | 77.7  | 85.8  | 68.0  | 79.9  | 76.8  | 56.1  | 62.7  | 66.9  | 62.8  | 55.4  | 46.4  | 47.2  | 51.7  | 72.6  | 23.5  | 47.3  | 80.7  | 67.2  | 84.3  | 38.6  | 75.3  | 66.6  | 67.9  | 44.4  | 64.5  | 79.4  | 78.2  | 58.1  | 84.4  | 62.0  | 13.6  | 61.5  | 73.8  | 41.3  | 67.0  | 65.2  | 46.3  | 27.8  | 24.6  | 72.5  | 61.3  | 28.4  | 71.1  | 81.7  |
Relative neutralization activity (%) is shown, neutralization HCVpp >50%, HCVcc >40% is shown in blue, <20% neutralization is shown in red. The neutralization rank within the cohort, strongest (1) to weakest (51) is shown for neutralization of HCVpp, HCVcc and the final overall rank.

Table 4: Gt 1 specific neutralization activity of gt 1 panel.

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Relative neutralization activity (%) is shown, neutralization >50% is shown in blue, <20% neutralization is shown in red.

Figure 1: Molecular Phylogenetic analysis of gt 1 E1E2 sequences. Maximum Likelihood method based on analysis of E1E2 amino-acid sequences using the JTT matrix-based model (55). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3680)). The tree with the highest log likelihood (-7055.2655) is shown. The tree is drawn to scale with the genetic distance for each branch length indicated by the scale bar. Bootstrap analysis with 1000 replicates was performed. Branches with >70% bootstrap support are labelled. Sequences classified as functional in the HCVpp system are labelled in blue, non-functional.
sequences in red and sequences included in the gt 1 panel in green. Evolutionary analyses were conducted in MEGA6 (56).

**Figure 2: Association of viral load with E1E2 binding and neutralization profiles.** (A) The relative binding of CHCV cohort IgG to E1E2 from 6 subgenotypes of HCV was determined by ELISA. The IgG samples were ranked from 1 to 51 according to their binding signal for each subgenotype. The sum of these ranks was used to order the samples from highest cross-genotypic binding to lowest. The binding of the upper half of the cohort was regarded as “Broad” and that of the lower half as “Narrow”. The viral load of the two groups was compared using the Mann Whitney U test. (B) Neutralization of HCVpp in both panels at the 50% level by purified IgG was determined. The full CHCV cohort was analyzed against Panel XG and 20 gt 1-infected individuals were tested against Panel Gt1. The Mann Whitney U test was used to compare viral load between broad neutralizers, those that neutralized > 3 HCVpp (n=19) in Panel XG and > 7 gt 1 HCVpp (n=10) in Panel Gt1 and narrow neutralizers, that neutralized < 4 genotypes (n=32) in Panel XG and < 8 gt 1 HCVpp (n=10) in Panel Gt1.

**Figure 3: Non-parametric correlation between E1E2 ELISA binding and neutralization.** For each Panel the number of HCVpp neutralized was plotted against ELISA binding rank, Panel XG (A) and Panel Gt1 (B). (C) Neutralization activity of the full cohort was analyzed against Panel XG and 3 viruses and the neutralization rank in the HCVcc and HCVpp systems plotted. (D) For those gt1-infected individuals tested in both Panels, the number of HCVpp isolates neutralized in Panel XG was plotted against the number of HCVpp isolates neutralized in Panel Gt1. The Spearman’s rho correlation coefficient was calculated for all graphs.

**Figure 4: Association of liver fibrosis with neutralization.** Neutralization of Panel XG by the full CHCV cohort IgGs (A, C) or Panel Gt1 by IgGs from the gt1-infected subgroup (B, D) was determined as described in Fig. 2. (A, B) Broad and narrow neutralizing groups were defined as in Fig. 2 and transient elastography (kPa) values measured using Fibroscan® were compared using the
The number of HCVpp isolates neutralized by IgG from individuals with and without cirrhosis was compared using the Mann Whitney U test.

**Figure 5: Association of age with breadth of neutralization.** We compared the age of individuals in the broad and narrow neutralizing groups as characterized in Fig. 2 for the full CHCV cohort with Panel XG (A) and the gt1 subgroup for Panel Gt1 (B) using the Mann Whitney U test. Similarly, the broad and narrow neutralizing groups were compared for duration of infection (estimated) and age at acquisition (estimated) for the full CHCV cohort (C, E) and the gt1 subgroup (D, F) respectively.

Note that, no data was available for 6 broad and 10 narrow neutralizing individuals in the full CHCV cohort and 4 broad and 4 narrow neutralizing individuals in the gt1 subgroup.

**Figure 6: Association of rs2395522 SNP genotype with breadth of neutralization and liver fibrosis.** All individuals in the CHCV cohort were typed for the HLA DQ-B1 SNP rs2395522. The Mann-Whitney U test was used to compare SNP type with the number of HCVpp isolates neutralized in Panel XG (A) and Panel Gt1 (C) panel. Similarly, SNP type was compared to the level of liver fibrosis as measured by transient elastography (kPa) values for the whole CHCV cohort (B) or the gt1 subgroup (D) by the Mann Whitney U test.

**Figure 7. Competition of patient IgG with monoclonal antibodies to known epitopes on E2.** (A) Competition ELISA of gt1 patient IgG with E2 mAbs CBH-4B, HC-84, AP33, CBH-7, HC-1 and HC-11 was performed. The mean percentage of competition (i.e. reduction in mAb binding) from 3 independent experiments is shown. (B) Association of competition with Hmabs CBH-7 and HC-11 for broad (>7 Gt1) and narrow (<8 Gt1) neutralizing samples using the Mann Whitney U test.

**References**

1. WHO 2012, posting date. WHO fact sheet: HCV vaccines number 164. [Online.]


A

![Graph showing viral load (IU/ml)]

- Broad
- Narrow

* $P=0.03$

B

![Graph showing viral load (IU/ml)]

- Broad
- Narrow

- Panel XG
- Panel Gt1

- $P=0.77$
- $P=0.12$
**Figure A**
Spearman's $r = -0.31, P = 0.03^*$

No. XG HCVpp neutralized $>50\%$

ELISA Binding Rank

**Figure B**
Spearman's $r = -0.49, P = 0.03^*$

No. Gt1 HCVpp neutralized $>50\%$

ELISA Binding Rank

**Figure C**
Spearman's $r = 0.44, p = 0.001^{**}$

HCVcc rank

HCVpp rank

**Figure D**
Spearman's $r = 0.58, P = 0.007^{**}$

No. Gt1 HCVpp neutralized $>50\%$

No. XG HCVpp neutralized $>50\%$
### Table

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<th>Immunodomain C</th>
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### Graph

- **P = 0.004**
- **P = 0.002**

**Reduction in binding (%)**

- <30
- 30-50
- 50-70
- >70