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The Apolipoprotein ε3 allele is associated with persistent Hepatitis C Virus infection.

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Key words: hepatitis C, genetic susceptibility, case-control association study, chronic infection, acute infection, lipid metabolism

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Abbreviations: HCV, hepatitis C virus; VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; LDLr, low-density lipoprotein receptor; Apo-E, apolipoprotein E; APO-B, apolipoprotein B; H.E.L.P., heparin induced extracorporeal lipoprotein fibrinogen precipitation; LVP, lipo-viral particle; PCR, polymerase chain reaction; IVDU, intravenous drug user.

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Abstract

Background: Host genetic factors may significantly influence the ability to clear hepatitis C virus (HCV) following infection. HCV are associated with very-low-density lipoproteins (VLDL) and low-density-lipoproteins (LDL) in the host’s circulation. Apolipoprotein E (APO-E) is found in VLDL and binds to potential receptors involved in HCV entry into cells, the LDL receptor and the scavenger receptor protein, SR-B1. The APO-E gene is polymorphic with three alleles coding for 3 isoforms: Apo-ε2, Apo-ε3 and Apo-ε4. The aim of this study was to assess if these functional polymorphisms determine disease outcome in HCV infected individuals.

Methods: The APOE genotype was determined in 420 Northern European patients with evidence of exposure to HCV. Genotype and allele distribution were compared with those of 288 healthy controls and progression of liver disease and viral clearance were analysed according to APOE allele status.

Results: The APOE*E2 and APOE*E4 alleles were both associated with a reduced likelihood of chronic infection (OR = 0.39, CI 0.211 – 0.728, p = 0.003 and OR = 0.6, CI 0.38 – 0.96, p = 0.032) and there was a notable absence of the E2E2 genotype in the HCV antibody positive group compared with the control population (p = 0.0067). Overall the genotypes carrying the E2 allele (E2,E3 and E2,E4) were associated with the equivalent of a 3 – 5 fold reduction in risk of chronic HCV infection (GRR = 0.36 and 0.20; respectively).

Conclusion: This study indicates that functional APOE gene polymorphisms may be a determinant of outcome in HCV infection. We hypothesise that the E2 allele may protect against viral persistence via defective binding of HCV lipo-viral particles to the cellular receptors involved in entry of these infectious particles.
Introduction

Hepatitis C virus (HCV) infection is a major global health problem, infecting more than 170 million people worldwide \(^1\). Some newly infected patients recover spontaneously but the majority progress to chronic infection, so that HCV infection is now the leading cause of cirrhosis, hepatocellular cancer and liver transplantation in the Western world \(^2\).

Persisting strong CD8+ T-cell responses are observed in the majority with resolved HCV infection \(^3\) but the role of host factors in viral clearance and disease progression remains unclear. There is some evidence that host genetic factors are implicated in the outcome following HCV infection \(^4\). Specific HLA haplotypes have been associated with a significantly increased likelihood of viral clearance, including the HLA-\(DRB1^*1101-DQB1^*0301\), \(DRB1^*1104-DQB1^*0301\) and \(DRB1^*0401-DQB1^*0301\) haplotypes \(^4-7\). In addition recent data suggests that inherited variation in the killer inhibitory receptor genes may play a significant role in determining the host response to HCV infection \(^8\).

Other host factors associated with disease progression in chronic HCV include male sex, age at infection, excessive alcohol use, total cholesterol level and insulin resistance \(^9\). In addition there is now considerable accumulating evidence to suggest a relationship between hepatitis C virus and lipid metabolism \(^10\). During the early stages of acute HCV infection in chimpanzees host genes involved in lipid metabolism are differentially regulated \(^11\). In patients with chronic HCV serum cholesterol is significantly lower than in appropriately matched controls \(^12\), particularly in HCV genotype 3 infection \(^13\,14\) and this metabolic effect is fully reversible after successful HCV eradication \(^15\) The association of HCV infection with hypocholesterolaemia has been confirmed in HIV-infected patients \(^16\) and in patients with porphyria cutanea tarda \(^17\). In many studies the hypocholesterolaemia has been associated with a decrease in apolipoprotein B (ApoB) in comparison with healthy controls \(^12\,17\,18\) and ApoB levels negatively correlate with hepatic steatosis and viral load \(^18\). Furthermore, HCV viral load can be reduced by H.E.L.P. apheresis, which is an established and approved therapy for hypercholesterolaemia \(^19\).

HCV RNA containing particles are consistently found in the low-density fractions of serum and this fraction has been shown to be highly infectious \(^20\). Further characterisation of these low density HCV RNA containing particles (lipo-viral particles (LVP)) has shown they efficiently bind and enter hepatocyte cell-lines, up-regulation of the low density lipoprotein receptor (LDLr) increases their internalisation and binding of HCV-LVP can be blocked by anti-ApoB and anti-apolipoprotein E (ApoE) \(^21\,22\). This is consistent with other studies showing that endocytosis of HCV can be mediated by LDLr \(^23\,24\). LDLr, a candidate receptor for HCV, normally transports 2 different classes of cholesterol containing lipoprotein particles into cells: LDL, which contains a single copy of ApoB-100 and VLDL, which contains multiple copies of ApoE.

Recent studies with retrovirus/HCV pseudovirus particles \(^25\) suggest that attachment to hepatocytes is via the scavenger receptor protein, SR-B1, with entry into the cell via a co-receptor CD-81 dependent pathway \(^26\,27\). SR-B1, which is expressed primarily in the liver, is a key component in the reverse cholesterol transport pathway, and recognizes a broad variety of lipoprotein ligands, including HDL, LDL, VLDL and oxidised LDL \(^28\,30\).

Apolipoprotein E, a ligand for both LDLr and SR-B1, is a polymorphic protein arising from 3 alleles at a single gene locus. The three major isoforms, Apo-\(\varepsilon2\), Apo-\(\varepsilon3\), and Apo-\(\varepsilon4\), differ from one another by single amino acid substitutions, a change
which has profound functional consequences at both cellular and molecular levels. Apo-ε3 seems to be the wild type isoform with normal function, while Apo-ε2 binds poorly to LDLr and Apo-ε4 induces a down-regulation of LDLr 31. A previous study of APOE gene polymorphisms in HCV infection suggested decreased severity of liver disease in patients with the E4 allele 32. As apolipoprotein E is a ligand for the HCV receptor candidates LDLr and SR-B1 and in view of the considerable evidence suggesting that cholesterol and fatty acid pathways may play a role in HCV replication and infection, the aim of this study was to determine whether the common functional polymorphisms of the APOE gene can influence the clinical outcome of HCV infection.
Patients and Methods

Patients

5 mls of whole blood was obtained from 420 Caucasian HCV antibody positive patients recruited from two centres, the joint hepatitis clinic of the Freeman Hospital, Newcastle-upon-Tyne, England (n=241) and St. James’s Hospital, Dublin, Ireland (n=179). 5 mls of whole blood was also collected from 288 healthy adult volunteers (controls) to assess the apolipoprotein E genotype distribution in the healthy population.

HCV viral RNA was tested in all HCV antibody positive patients using the Amplicor reverse transcriptase polymerase chain reaction (PCR) assay (Roche). 312 patients were HCV RNA positive on at least two occasions indicating a persistent or chronic infection, whereas 108 patients were HCV RNA negative on at least two occasions, indicating spontaneously resolved infection.

Liver biopsies were available on 209 patients with evidence of chronic infection. Each biopsy was scored by a single observer, Prof A.D. Burt, using the Ishak scoring system. Biopsies were graded for necroinflammation on a scale of 0-16 and fibrosis on a scale of 0-6 [fibrosis stage of 5 or 6 indicated severe disease, 2-4 moderate disease and 0 or 1 mild disease]. An estimate of the rate of fibrosis was made on 178 of these patients. A date of infection was either estimated in intravenous drug users (IVDU) as the date when IVDU started, or was calculated as the date of exposure to infected blood or the date of a one off/short period of IVDU. The Ishak stage of fibrosis was divided by the time in years from date of infection to first biopsy to give a calculation of the rate of fibrosis. Rapid progression was identified as patients progressing to cirrhosis within 20yrs (a rate of fibrosis >0.25/yr). In this subgroup of patients, data on alcohol use, age at biopsy, sex and mode of infection were also collected; all data and results were entered into a Microsoft® Access™ database.

Apolipoprotein E genotyping

Genomic DNA was isolated from 2mls of whole blood using QIAamp® midi kits (Quiagen® Ltd, Crawley, UK). A 227bp product of the APOE gene was amplified using a pair of sequence specific primers in a standard PCR reaction. Approximately 100ng of DNA was amplified in a 50µl reaction mix containing 2.5µl of 100% DMSO, 1.5mM MgCl₂, 10mM tris-HCl, 50mM KCL, 0.5µM of each primer, 200µM of each nucleotide and 2.5U of Taq polymerase (ABGENE®, Epsom, Surrey, UK). Amplification conditions were as follows: 2 minutes at 95°C and then 40 cycles of 2 minutes at 95°C, 2 minutes at 65°C and 2 minutes at 72°C, and a final extension for 5 minutes at 72°C. All PCR batches included a water (DNA free) negative control and samples of known genotype. All genotypes were assigned by two independent investigators and any ambiguous genotypes were repeated. The 227-bp product was sub- aliquoted into duplicate reaction tubes and subject to digestion with two different restriction endonucleases *AflIII* and *HaeII*; digestions were performed with 25U of each enzyme at room temperature for a minimum of 3 hours. The resulting restriction fragments were separated on 3% agarose gels and the bands were visualised using ethidium bromide staining on a UV trans-illuminator and documented on an AlphaImager™ 2200 Documentation and Analysis System (AlphaInnotech Corp., USA). *AflIII* digests the PCR amplicons of the APOE*2 and E3 alleles only creating bands of 117bp and 50bp (amplicons of E4 do not digest leaving the 227 bp amplicon intact). *HaeII* digests the E3 and E4 amplicons only resulting in bands of 195bp and 32bp (amplicons of the E2 allele are not digested leaving an intact 227bp amplicon). Thus, by comparing the restriction patterns for the two digests on every sample we can assign composite genotypes for E2,E2; E2,E3; E3,E3; E2,E4; E3,E4 and E4,E4.
Statistical Analysis
Genotype and allele distribution were compared using Stata. As the $E3$ allele is associated with normal APOE activity the risk for the common (wild type) genotype $E3,E3$ was fixed (at 1) and risk for all other genotypes are reported relative to this. In the whole HCV antibody positive cohort ($n=420$) the following comparisons of allele and genotype distribution were made:

- Control group ($n = 288$) versus HCV exposed (HCV antibody positive) population.
- Chronic HCV infection ($n = 312$) versus spontaneously resolved (cleared) infection ($n = 108$).

In the subgroup with chronic HCV infection whose liver biopsies were scored by a single observer the following comparisons of allele and genotype distribution were made:

- Severe liver disease versus mild/moderate chronic hepatitis ($n=209$)
- Slow fibrosis progression versus rapid fibrosis progression ($n=178$)

Risk is reported as Genotype Relative Risk (GRR) for comparison of genotype distribution and Odds Ratio (O.R.) for comparison of allele distribution with 95% Confidence Intervals (C.I.). Multivariate binary logistic regression was used to assess independent variables including sex, alcohol usage and age of infection in relation to $APOE$ status in HCV antibody positive patients. All additional computations were performed using SPSS-10 for windows.
Results
Comparing all patients versus healthy controls there was a significantly lower frequency of the \(E2,E2\) genotype in the diseased population \(\chi^2 = 7.34, p=0.0067\).

Table 1: \textit{APOE} genotype and allele distribution (numbers and percentage) for HCV infected patients and healthy controls.

<table>
<thead>
<tr>
<th>\textit{APOE} genotype</th>
<th>Controls (n=288)</th>
<th>HCV cohort (n=420)</th>
<th>Probability (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E2,E2)</td>
<td>5 (2%)*</td>
<td>0*</td>
<td>0.0067</td>
</tr>
<tr>
<td>(E2,E3)</td>
<td>32 (11%)</td>
<td>39 (9%)</td>
<td>NS</td>
</tr>
<tr>
<td>(E2,E4)</td>
<td>11 (4%)</td>
<td>9 (2%)</td>
<td>NS</td>
</tr>
<tr>
<td>(E3,E3)</td>
<td>167 (58%)</td>
<td>263 (63%)</td>
<td>NS</td>
</tr>
<tr>
<td>(E3,E4)</td>
<td>66 (23%)</td>
<td>105 (25%)</td>
<td>NS</td>
</tr>
<tr>
<td>(E4,E4)</td>
<td>7 (2%)</td>
<td>4 (1%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Comparison of the \textit{APOE} genotypes in the two clinical subgroups (table 2) patients with spontaneously resolved infection (HCV RNA negative cleared infection; \(n = 108\)) versus chronic infection (HCV RNA positive chronic infection; \(n = 312\)) demonstrated that the genotypes \(E2,E3\) and \(E2,E4\) were mostly strongly associated with clearance of HCV \((p = 0.005, \text{GRR} \ E2,E3 = 0.36 \text{ CI} 0.18 – 0.73 \text{ and } p=0.02, \text{GRR} \ E2,E4 = 0.2 \text{ CI} 0.52- 0.78, \text{respectively})\). In addition whilst there was a significant effect of the \(E3,E4\) genotype \((p = 0.022, \text{GRR} = 0.55 \text{ CI} 0.33 – 0.92)\) the \(E4,E4\) genotype was not significant \((p = 0.811)\) due to the low number in the comparator subgroups. Comparing allele distribution the lowest risk of chronic infection was associated with the \(E2\) allele \((\text{OR} = 0.39, \text{C.I.} 0.21 – 0.73)\) compared to the \(E4\) allele \((\text{OR} = 0.59, \text{C.I.} 0.38 – 0.96)\).

Table 2: \textit{APOE} genotype distribution (numbers and percentage) in patients with spontaneously resolved infection (HCV RNA negative cleared infection) versus chronic infection (HCV RNA positive chronic infection)

<table>
<thead>
<tr>
<th>\textit{APOE} genotype</th>
<th>chronic HCV infection (n=312)</th>
<th>cleared HCV infection (n=108)</th>
<th>GRR (95% C.I)</th>
<th>Probability (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E2,E2)</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(E2,E3)</td>
<td>23 (7.5%)</td>
<td>16 (15%)</td>
<td>0.36 (0.18 – 0.73)</td>
<td>0.005</td>
</tr>
<tr>
<td>(E2,E4)</td>
<td>4 (1%)</td>
<td>5 (4%)</td>
<td>0.20 (0.52 – 0.77)</td>
<td>0.020</td>
</tr>
<tr>
<td>(E3,E3)</td>
<td>210 (67.5%)</td>
<td>53 (49%)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>(E3,E4)</td>
<td>72 (23%)</td>
<td>33 (31%)</td>
<td>0.55 (0.33 – 0.92)</td>
<td>0.022</td>
</tr>
<tr>
<td>(E4,E4)</td>
<td>3 (1%)</td>
<td>1 (1%)</td>
<td>0.76 (0.77 – 7.42)</td>
<td>ns</td>
</tr>
</tbody>
</table>
**APOE*2** was the only independent predictor of viral clearance in a multivariate logistic regression model. No other significant associations were found comparing patients and controls, or comparing those with chronic versus those with resolved HCV infection. In addition there was no significant association between **APOE** genotype or **APOE** alleles and the clinical phenotypes defined by severe inflammation (Ishak grade >8, n = 37), severe fibrosis (Ishak stage 5 and 6, n = 45) or fast fibrosis (rate of fibrosis ≥0.25/year, n = 60).

**Discussion**

Apolipoprotein E plays an important role in the transport of cholesterol and other lipids among cells of various tissues. Three major isoforms, encoded by three common alleles at the **APOE** locus, have different binding affinities for the Apo E receptors. The **E2** allele is associated with defective binding to LDLr and the **E4** allele with down regulation of LDLr compared to **E3**. In this study **APOE*2** and **APOE*4** alleles were both associated with an increased likelihood of viral clearance and, interestingly, there were no HCV antibody positive patients with the **APOE E2,E2** genotype, though this is relatively rare in healthy controls (0.5-2%) [37]. A previous investigation of the influence of **APOE** polymorphism and outcome of HCV infection [32] suggested that the **APOE*4** allele protected against severe liver disease. Our results did not confirm this finding but did suggest that this allele may also be associated with a reduced risk of viral persistence. However it is probable that neither this nor the earlier study is adequately powered to look at subtle effects of the Apo-ε4 isoform on disease progression. This latter comment is especially true when other factors associated with disease progression which may vary between cohorts are considered [9].

Another small study has suggested that heterozygosity for the **APOE*4** allele might be associated with better histological outcome in recurrent HCV infection in the liver transplantation setting [38], but this situation is complicated by possible differences in donor/recipient **APOE** alleles. It is also of interest that chronic HCV is associated with cognitive dysfunction and CNS infection [39] and a recent study has demonstrated an association between **APOE*4** and neuro-psychiatric symptoms during interferon alpha treatment for chronic HCV [40].

The association found between the **E2** allele and viral clearance is intriguing as this allele binds poorly to LDLr [31]. It is plausible that such defective binding could result in poor uptake of HCV lipo-viral particles into the hepatocyte with a resultant decrease in replication of the virus, thus altering the balance between virus replication and the immune response and favouring clearance of the virus before chronic infection can be established. This is supported by the lack of **E2,E2** genotype among patients who are HCV antibody positive, suggesting that the **E2,E2** genotype (0.5-2% of healthy controls [37]) may confer relative resistance to establishing HCV infection in exposed individuals. This could be somewhat analogous to the role played by the chemokine receptor-5 delta 32 mutation, which leads to defective uptake of the HIV virion and therefore resistance to HIV/AIDS [41]. In the latter case, homozygotes for the delta 32 mutation exhibit a strong, although incomplete, resistance to HIV infection, whereas heterozygotes display delayed progression to acquired immunodeficiency syndrome. Conversely the results reported here may indicate an association between the **APOE*3** allele and persistence of hepatitis C virus. This would fit a model whereby **APOE*3** binds to receptors with high affinity and is associated with normal serum cholesterol and triglyceride levels.
In conclusion this study suggests that common genetic variations at the APOE locus influence the outcome of HCV infection with the APO*E2 and APO*E4 alleles favouring viral clearance. We hypothesise that ApoE2, which binds poorly with its receptors, may be associated with resistance to HCV infection, via defective uptake of HCV lipo-viral particles by the candidate receptors LDLr and SR-B1. Further investigations are needed, especially in individuals who are likely to have been repeatedly exposed to HCV but remain anti-HCV antibody negative. Although the inability to replicate the results of case-control studies has led to scepticism about their value, our findings are both biologically plausible and statistically significant. If our findings are confirmed in a validation study, the function of Apolipoprotein E in assembly, processing and removal of plasma lipoproteins and its interaction with HCV could be considered in the development of novel therapeutic strategies.

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