

FULL PAPER

# Inducible nitric oxide synthase gene (NOS2A) haplotypes and the outcome of hepatitis C virus infection

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Inducible nitric oxide synthase (iNOS) is an important molecule involved in the host defense against infectious agents. iNOS is encoded by the NOS2A gene and well-defined haplotypes exist with respect to this gene. We examined whether these haplotypes were associated with the outcome of hepatitis C virus (HCV) infection in 619 Caucasian patients from seven European liver centres. We observed five major haplotypes: (–277A) + (–1026G) + (–1659C): haplotype 1; (–277G) + (–1026T) + (–1659C): haplotype 2; (–277G) + (–1026G) + (–1659C): haplotype 3; (–277G) + (–1026T) + (–1659T): haplotype 4; and (–277A) + (–1026T) + (–1659C): haplotype 5. Distributions of these haplotypes are comparable with those of previous studies. Homozygotes for haplotype 2 or those with haplotypes 2/4 were more likely than those with the 1/1 (wild type) combination to have self-limiting infections (odds ratios (OR) = 3.43; 95% confidence intervals (95% CI): 1.10–8.0;  $P = 0.0206$  and OR = 5.15; 95% CI: 1.32–14.32;  $P = 0.0018$ , respectively). Conversely, carriage of haplotype 1 was associated with the lack of self-limiting disease (OR = 0.48; 95% CI: 0.27–0.83;  $P = 0.009$ ). The effect was mainly among males (OR = 0.41; 95% CI: 0.182–0.942;  $P = 0.031$  for males, and OR = 0.55; 95% CI: 0.24–1.37;  $P = 0.136$  for women). Carriage of haplotype 1 was not associated with initial response ( $P = 0.268$ ) or sustained response ( $P > 0.171$ ). Combinations of haplotypes 1/4 were more likely to respond to interferon monotherapy in comparison of initial responders to nonresponders (OR = 2.25; 95% CI: 1.05–5.68;  $P = 0.0275$ ).

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## Introduction

Nitric oxide (NO) is an important signalling molecule that is also involved in combating microbial infections. It is formed by the oxidative deamination of the amino-acid L-arginine to L-citrulline by nitric oxide synthases (NOS).<sup>1</sup> NO possess potent antimicrobial effects, including the ability to inhibit the growth of many infectious organisms *in vitro*.<sup>2</sup> Three isoforms of NOS enzymes are currently known to exist: neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS) also referred to as NOS2). Of these three isoforms, iNOS is absent in resting cells, but is capable of being rapidly expressed in response to proinflammatory stimuli such as cytokines, including interferon. When present, iNOS is capable of synthesising 100–1000 times more NO than the other

forms over a prolonged period of time.<sup>1</sup> These properties make iNOS an important part of the host response to infectious agents. Experiments employing iNOS inhibitors and iNOS knockouts have demonstrated *in vivo*, the important role of iNOS in the host defense to infectious agents.<sup>3–6</sup> Although animal data suggest a central role for NO in infectious diseases, discovering its role in human infection has proved more difficult.<sup>1</sup> This is partly due to difficulties in accurately measuring organ-specific NO *in vivo*. A genetic epidemiologic approach, exploring common promoter variants in the transcriptionally regulated NOS2A gene have been associated with a number of human diseases.<sup>7</sup> iNOS is encoded by the NOS2A gene and polymorphisms have been described in the NOS2A gene at positions –277, –1026, and –1659 numbered relative to the transcription initiation site. Strong linkage disequilibrium exist between these loci and several conserved, well-defined haplotypes have been observed in Caucasian populations (Table 1).<sup>8</sup>

Hepatitis C virus (HCV) infection is characterised by a diversity of outcomes. Some individuals may naturally clear the virus (self-limiting infection), while the majority of individuals develop chronic (persistent) infections. Interferon- $\alpha$  has been an important agent used in anti-HCV therapy, first as monotherapy<sup>9,10</sup> and, more

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**Table 1** Major Caucasian *NOS2A* haplotypes and their designations

Haplotype designation (nomenclature in Burgner <i>et al</i> ) <sup>a</sup>	-277 (A/G)	-1026 (G/T)	-1659 (C/T)
Haplotype 1 (Haplotype 1)	A	G	C
Haplotype 2 (Haplotype 8)	G	T	C
Haplotype 3 (Haplotype 4)	G	G	C
Haplotype 4 (Haplotype 6)	G	T	T
Haplotype 5 (Haplotype not observed)	A	T	C

<sup>a</sup>In the study by Burgner *et al*,<sup>8</sup> several other polymorphic loci were examined. Consequently, additional haplotypes were observed that are not found in the present study. For purposes of clarity, we have numbered the haplotypes sequentially in the present study. For each of the haplotypes we report, the corresponding nomenclature used in the Burgner study is provided within parenthesis.

recently, in combination with ribavirin.<sup>11,12</sup> The addition of a polyethylene glycol moiety to interferon- $\alpha$  has also been developed and has been administered alone or in combination with ribavirin as well.<sup>13,14</sup> During the first 3 months of therapy, HCV viral loads usually fall to undetectable levels in response to interferon. However, in a small proportion of patients (nonresponders), HCV viral loads persist at or near pretreatment levels. Among those patients with an initial response (initial responders) to treatment, up to 50% will relapse after treatment is discontinued (relapsed responders), whereas the remainder will have a sustained response as determined by the absence of detectable viraemia 6 months after treatment has stopped. Given the potent antimicrobial properties of NO, we examined whether these previously described haplotypes of the *NOS2A* gene<sup>8</sup> were associated with the outcome of HCV infection.

## Results

### Cohort characteristics

The present cohort ( $N=619$ ) consisted of 55.7% males and 44.3% females. All patients were Caucasian. The average age at HCV acquisition was  $43.5 \pm 15.8$  years s.d. Viral genotyping was available on 177 patients in the present study, and 66.7% of these individuals had viral genotype-1 infections, while 33.3% had non-1 genotype infections. Among the 619 individuals included in the study, 76 had self-limiting HCV infection and 543 chronic (persistent) HCV infection. In all, 314 individuals were treated with interferon monotherapy, of these 112 achieved sustained response and 102 were relapsed responders (214 initial responders), while 100 were nonresponders.

### *NOS2A* haplotypes and HCV outcome

We observed five major *NOS2A* haplotypes with the following frequencies: haplotype 1, 60.9%; haplotype 2, 16.2%; haplotype 3, 8.7%; haplotype 4, 12.8%; and haplotype 5, 1.4%, and is comparable with frequencies reported in other Caucasians.<sup>8</sup> Stratification of observed haplotype frequencies by study centre did not yield any significant differences. Homozygosity for haplotype 2 (Table 2) was associated with a 3.4-fold higher likelihood of self-limiting infection (odds ratios (OR)=3.43; 95%

**Table 2** Observed effects of *NOS2A* haplotypes among 619 Caucasian individuals in the HENCORE Study

Haplotype	Self-limiting	Persistent	OR	95% CI
1/1 <sup>a</sup>	24	206	—	—
1/2	14	112	1.07	0.43–1.64
1/3	5	53	0.81	0.20–1.70
1/4	12	90	1.14	0.45–1.86
1/5	0	8	ND	—
2/2	6	15	3.43	1.10–8.0
2/3	3	12	2.14	0.32–6.95
2/4	6	10	5.15	1.32–14.32
2/5	0	2	ND	—
3/3	0	9	ND	—
3/4	4	13	2.64	0.52–7.58
4/4	1	8	1.07	0.02–6.81
4/5	1	4	2.15	0.04–18.45
5/5	0	1	ND	—

<sup>a</sup>Measured effects of each haplotype combination were calculated using haplotype 1/1 as the referent as it was the most commonly observed haplotype combination in each of the calculations.

ND = not determinable due to 0 in one of the cells.

In all, 76 individuals had self-limiting HCV infection and 543 individuals had persistent HCV infection.

confidence intervals (95% CI): 1.10–8.0) than those with haplotype 1/1 (the most frequent haplotype (wild type)), while possession of the combination of haplotypes 2 and 4 was associated with a 5.15-fold higher likelihood of self-limiting infection than those with haplotype 1/1 (OR = 5.15; 95% CI: 1.32–14.32).

The combination of haplotypes 1 and 4 were associated with a greater than two-fold higher likelihood of initial response to interferon therapy than possession of the 1/1 haplotype combination (OR = 2.25; 95% CI: 1.05–5.68). Marginal associations with nonresponse were observed with homozygosity for haplotype 3 (OR = 0.30; 95% CI: 0.04–1.43), as those with the 3/3 haplotype were 70% less likely to respond. No significant associations were observed between *NOS2A* haplotypes and sustained response to interferon therapy.

The relative infrequency of these haplotypes precluded multivariable analysis. As an alternative, we stratified the observed haplotypic effects by patient gender and viral genotype—both important potential confounding factors. Stratification of the association between haplotype 2 homozygosity and self-limiting infection yielded a stronger effect among men than women (OR = 3.72; 95% CI: 0.95–14.60 for men, and OR = 1.30; 95% CI: 0.26–6.39 for women). Stratification of the haplotype 2/4 combination by gender yielded similar strengths of association for both males and females (OR = 3.2; 95% CI: 0.32–31.34 for men, and OR = 3.4; 95% CI: 1.05–11.06 for women), although the association was statistically significant only among women. Stratification of the association between the combination of haplotype 1/4 and initial response to therapy yielded a stronger effect among women than men (OR = 0.99; 95% CI: 0.39–2.54 for men, and OR = 7.6; 95% CI: 1.74–33.27 for women). Stratification of the association of the combination of haplotypes 1/4 and initial response by viral genotype yielded a stronger effect among those with genotype-1 virus infections (OR = 6.79; 95% CI: 1.51–

30.52 for genotype-1 infections, and OR = 1.8; 95% CI: 0.21–15.45 for non-1 genotypes).

Carriers of haplotype 1 were less likely to have self-limiting disease (OR = 0.48; 95% CI: 0.27–0.83;  $P = 0.009$ ). Stratification by gender suggested that the effect was mainly among males (OR = 0.41; 95% CI: 0.182–0.942;  $P = 0.031$  for males, and OR = 0.55; 95% CI: 0.24–1.37;  $P = 0.136$  for women). Carriage of haplotype 1 was not associated with initial response ( $P = 0.268$ ) or sustained response ( $P > 0.171$ ).

## Discussion

Our observations show an association between *NOS2A* haplotypes and the outcome of HCV infection. Homozygosity for haplotype 2 and the combination of haplotypes 2/4 were associated with self-limiting infection. Haplotype 2, counted as an allele, was also associated with self-limiting infection (OR = 1.7; 95% CI: 1.1–2.6) (data not shown), suggesting that there is a gene dosage effect with haplotype 2 and self-limiting HCV infection. Stratification by gender suggested that the effect of the homozygous haplotype 2 was stronger among males than females. These differences by gender are not surprising, as the natural history of HCV has been observed to differ with respect to gender. Specifically, with respect to self-limiting infection, some studies suggest that females are more likely to clear HCV viraemia naturally than men.<sup>15,16</sup> It is plausible that *NOS2A* polymorphisms exert a differential biologic effect in males and females, or that the iNOS protein has a differential functional effect by gender. Different associations by gender for putative genetic markers and HCV outcome have been observed before.<sup>17</sup> Future studies are needed to understand the biological mechanisms behind these epidemiologic observations. Stratification by viral genotype showed an effect of genotype-1 on initial responses for individuals with haplotype 1/4.

We also observed an association of the homozygous haplotype 3 with nonresponse (in comparison with initial responders). Unfortunately, the frequency of haplotype 3 homozygotes is low (three with IR and five with NR). Consequently stratified analysis was precluded. The combination of haplotypes 1 and 4 was associated with initial response to interferon along with haplotypes 1 and 5. The relatively low numbers of individuals with these haplotypes also precluded meaningful stratified analyses. Carriage of haplotype 1 was also associated with a lower likelihood of having self-limited infection. Whether this observed effect is largely due to a detrimental effect of haplotype 1 or a more beneficial effect of haplotypes 2 or 4 must await further functional studies.

Determination of whether the effect underlying our observed associations are due to the haplotype as a whole or the individual loci must await future functional studies. It is also plausible that our observed associations are due to linkage disequilibrium with another as yet unidentified marker(s), and that the observed haplotypes reflect linkage with another locus. Previous studies have clearly shown the importance of haplotypic analyses in detecting disease associations where the functional variant is unknown.<sup>7</sup> *NOS2A* is a transcriptionally regulated gene, and as a result promoter variants may

directly affect the disease outcome(s), although little functional data currently exist with respect to *NOS2A* variants. A study by Burgner *et al*<sup>8</sup> observed that the variants at the –1659 position exhibited altered nuclear protein binding. Given the strong linkage disequilibrium between the various polymorphic sites in the *NOS2A* gene,<sup>7,8</sup> it is important for future studies to examine whether it is an individual single-nucleotide polymorphism (SNP) or the haplotype as a whole that confers functional effects. In addition, several studies suggest that *NOS2A* expression may be complicated, demonstrating cell- and stimulus-specific differences in *NOS2A* expression.<sup>18,19</sup>

While more functional data more closely examining the biologic effects of polymorphic variants or the different *NOS2A* haplotypes are needed, a number of association studies have highlighted the potential importance of *NOS2A* variants in the outcome of infectious as well as noncommunicable diseases.<sup>1</sup> Future studies should also address the functionality of *NOS2A* haplotypes. At present, there are little functional data on *NOS2A* variants. In addition, future investigations are needed to replicate these findings and it is important that these studies also be sufficiently powered to not only accommodate simultaneous adjustment for potential confounding variables through multivariable analyses, but also the analysis of host–pathogen interactions, such as the possible interaction of gender with host genetics.

## Patients and methods

### Patients

We studied 619 Caucasian patients enrolled in the HENCORE (Hepatitis C European Network for Collaborative Research) study. The details of this study have been described before.<sup>20</sup> Briefly, this is a multicentre European study of HCV that enrolled individuals from nine centres between October 1995 and June 2001, and the cohort includes those with both self-limiting and persistent HCV infections. A portion have also been treated with interferon monotherapy. Patients were recruited randomly or sequentially in each centre in order to minimise selection bias. Each patient gave informed consent and ethical approval was obtained from the local research ethics committee at each centre.

Patients were classified into the following groups as follows: (1) *self-limiting HCV infection*: individuals with antibodies to HCV who have no evidence of viraemia on at least two occasions and who have persistently normal liver transaminase levels; (2) *persistent HCV infection*: individuals who have evidence of viraemia for at least 6 months; (3) *sustained treatment response*: patients with persistent HCV infection treated with interferon- $\alpha$  alone who had normal liver transaminases and no evidence of viraemia 6 months after the end of treatment; (4) *response relapse*: patients with persistent HCV infection treated with interferon- $\alpha$  who had normal liver transaminases and no evidence of viraemia at the end of treatment, but in whom viraemia returned during the follow-up period; (5) *nonresponse to treatment*: patients with persistent HCV infection treated with interferon- $\alpha$  who never lost viraemia during treatment. Individuals considered ‘initial responders’ to interferon were those who experienced a loss of HCV viraemia during the first 12 weeks of

**Table 3** Primers used for genotyping the three SNP in the promoter region of the NOS2A gene

SNP	Primer specificity	Primer sequence
NOS2A -277 (A/G)	Forward common primer A-specific reverse primer G-specific reverse primer	5'-CTGGCTCCGTGGTGCC-3' 5'-CAGGGTGGCTGCTAAGAT-3' 5'-CAGGGTGGCTGCTAAGAC-3'
NOS2A -1026 (G/T)	Forward common primer G-specific reverse primer T-specific reverse primer	5'-GGCATTATAAGGAATGAAATTATAGGCC-3' 5'-GATTACAAGGGTTAGCCACC-3' 5'-GATTACAAGGGTTAGCCACA-3'
NOS2A -1659 (C/T)	Reverse common primer T-specific forward primer C-specific forward primer	5'-GGGATGGTATGGTGCTGATG-3' 5'-CCTTGAACAAGGCAGAACT-3' 5'-CCTTGAACAAGGCAGAACC-3'

treatment, regardless of whether they ultimately achieved sustained response (sustained responders + relapse responders).

### HCV antibodies and viraemia

Antibodies to HCV antigens were detected with both an enzyme-linked immunoassay or a recombinant immunoblot assay containing four HCV antigens on a cellulose acetate strip used according to the manufacturer's instructions (Abbott Laboratories, North Chicago, IL, USA). The presence or absence of viral particles in the serum was determined by reverse transcription polymerase chain reaction (PCR) using a commercially available assay (Amplicor, Roche), with a sensitivity of approximately 200 genomes/ml.

### HCV genotypes

Treatment for HCV with interferon monotherapy was conducted irrespective of viral genotypes and as a result the patients in this cohort were not assessed for viral genotypes at the time of enrolment.<sup>9,10</sup> As subsequent studies identified viral genotypes as a major factor affecting the therapeutic outcome,<sup>11,12</sup> we retrospectively genotyped patient samples for viral genotype using stored sera collected at the time of patient enrolment. HCV genotyping was conducted using the Inno LiPA HCV Kit (Innogenetics, Ghent, Belgium). Genotypes were dichotomised into genotype-1 and non-1 to facilitate categorical analyses.

### Genotyping of allelic variants

Three SNPs were targeted in the present study: an A to G substitution at position -277, a G to T substitution at position -1026, and a C to G substitution at position -1659 of the NOS2A gene were genotyped by allele-specific real-time PCR.<sup>21</sup> The primers used for this procedure are provided in Table 3. Each reaction was comprised of 0.2 µM of each of the specific primers (Table 3); 2.5 U of Stoffel Gold Polymerase (David Birch, RMS); 1 × Stoffel Gold buffer (10 mM Tris-HCl, 10 mM KCl at pH 8.0); an additional 30 mM KCl for a final concentration of 40 mM; 3 mM MgCl<sub>2</sub>; 50 µM each dATP, dCTP, and dGTP; 25 mM TTP; 75 mM dUTP; 2 U of UNG (PE), 0.2 × SybrGreen I (Roche Molecular Probes); 2 µM ROX (Roche Molecular Probes); 5% DMSO; and 2.5% glycerol. Kinetic PCR reactions were performed on a GeneAmp 5700 Sequence detection System (PE Applied Biosystems). An initial incubation step of 2 min at 50°C (to allow UNG-mediated elimination of carryover PCR product contamination) and an enzyme heat activation

step of 12 min at 95°C were followed by 40 two-step amplification cycles of 20 s at 95°C for denaturation and 20 s for 58°C for annealing and extension, and a final 5 min extension at 72°C. All PCR reactions were performed using 5–50 ng genomic DNA in a total volume of 20 µl. Assignment of NOS2A haplotypes was according to previously published data employing UK Caucasians.<sup>8</sup>

### Statistical analyses

Standard univariable analyses were conducted using contingency tables. OR and 95% CI were calculated along with maximum likelihood and Fisher's exact *P*-values, as appropriate. The SAS<sup>®</sup> (Cary, NC, USA) and STATA<sup>®</sup> (College Station, TX, USA) statistical software packages were used.

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