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Research article

Paraoxonase gene polymorphisms and haplotype analysis in a stroke population

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Abstract

Background: Paraoxonase (*PON*) has anti-atherogenic activity due to its protective function against low density lipoprotein (LDL) oxidation. Alteration of enzyme activity due to polymorphisms in the *PON* genes may influence the development of atheroma and thus affect stroke risk. Three *PON* genes (*PON1*, *PON2* and *PON3*) have been identified and mapped to chromosome 7.

Methods: We looked at the distribution of paraoxonase polymorphisms and haplotype arrangement in 397 Caucasian ischaemic stroke patients and 405 controls. We investigated 6 different common single nucleotide polymorphisms (SNP) in *PON* genes; two substitutions in *PON1* ["A/G": Gln (Q)/Arg (R)] at codon 192 and ["T/A": Leu (L)/Met (M)] at codon 55, two in *PON2* at codon 311 ["G/A": Cys (C)/Ser (S)] and codon 148 ["C/G": Ala (A)/Gly (G)] and two SNPs, both "A" to "G" substitutions, in *PON3* – intronic rs2074353, which we designated *PON3*-1 and [Ala (A)/ Ala (A)] at codon 99, designated as *PON3*-3. Dynamic Allele Specific Hybridisation (DASH) was used as the genotyping assay. Haplotype analysis was performed using both PHASE and EHPLUS programs.

Results: Genotype and allele frequencies were similar in cases and controls. Lipid profiles were not influenced by *PON* genotype. Haplotype frequencies for the six loci (*PON2*-148, *PON2*-311, *PON3*-3, *PON3*-1, *PON1*-55 and *PON1*-192) were estimated. Comparison of the two programs showed a significant difference in haplotype arrangements with EHPLUS (p-value = 0.005) but not with PHASE Ver.2 (p-value = 0.12). The 112211 (1 = frequent allele, 2 = rare allele) haplotype arrangement was commoner in cases than controls (p = 0.015), and the 111121 haplotype was commoner in controls (p = 0.006).

Conclusion: Our study did not identify a role for individual paraoxonase gene polymorphisms in the pathogenesis of ischaemic stroke. Findings of haplotype differences should be confirmed in large scale studies. The importance of using a well-validated haplotype analysis program is also underlined.

Background

Ischaemic stroke is a complex trait, with genetic influences likely to work through various risk factors for cerebrovascular disease. Hypertension and dyslipidemia are known to influence atherosclerosis and thus predispose to ischaemic stroke. Although lipids are a weaker risk factor for stroke than blood pressure or age, hyperlipidemia contributes to stroke risk [1], while lipid lowering therapy significantly reduces the risk of stroke [2]. Low HDL has also been reported as a risk factor for ischaemic stroke [3]. Genes involved in lipid metabolism have been previously studied in the stroke population. Some studies suggest *APOE**ε4 and *APOE**ε2 alleles are associated with higher risk and lower risk of ischaemic stroke respectively, although this has not been confirmed in all studies [4,5].

Paraoxonase, a 45 kDa glycoprotein, is well known for its ability to detoxify organophosphates [6]. The process of atherosclerosis is thought to begin with inflammatory changes at the endothelial level, and oxidated LDL is believed to be pro-inflammatory. Oxidated lipids have also been associated with progression of carotid atherosclerosis [7,8]. As an HDL associated protein, paraoxonase protects LDL against oxidation [9], and thus may protect against atherosclerosis [10].

The paraoxonase gene maps to chromosome 7 (q21-23) and has three isoforms, *PON1*, *PON2* and *PON3* [11]. Some studies suggest *PON* polymorphisms might predispose to CHD or ischaemic stroke [12-14], while other studies are less conclusive [15]. *PON1* has two common polymorphisms at codon 192 [A/G: Gln (Q)/Arg (R)] and 55 [T/A: Leu(L)/Met (M)]. These have been shown to correlate with enzyme activity [16,17]. Increased enzyme activity has been associated with higher HDL levels in some populations [18,19], but not in all studies [20]. The *PON 192* and 55 polymorphisms have been associated with risk of ischaemic stroke in a small study of younger adults [13] and older patients respectively [21]. There are two common polymorphisms of the *PON2* gene, *PON2*-148 [C/G: Ala (A)/Gly (G)] and *PON2*-311 [C/G: Cys (C)/ Ser (S)], and the *PON2*-148 "G" (G variant) is associated with higher fasting blood glucose in NIDDM patients [22]. The *PON2*-311 "G" (S allele) has been associated with coronary artery diseases in different populations [23]. *PON3* is thought to have a similar role to *PON1*[24].

The purpose of this study was to assess the distribution of polymorphisms of the *PON1*, *PON2* and *PON3* genes in well-phenotyped patients with ischaemic stroke and matched control subjects, and to expand on previous studies by examining haplotype distribution in these samples. We also examined the association between genotype and full lipid profile for a subgroup of controls.

Table 1: TOAST (Trial of Org 10172 in Acute Stroke Treatment) Classification of stroke patients

Subtype	All Stroke Samples	
	Number	%
Large Vessel Disease (LVD)	93	23.4%
Small Vessel Disease (SVD)	87	21.9%
Cardioembolic	99	25%
Other/Unclassified	118	29.7%
TOTAL	397	100%

Methods

Subjects

After ethical committee approval and informed consent, 397 Caucasian patients with a diagnosis of ischemic stroke were recruited over a period of 3 years. Four hundred and five Caucasian individuals from the same region, without any clinical cerebrovascular or overt cardiovascular disease and matched for age and sex were used as controls. For a subgroup of controls (n = 181) detailed lipid parameters including total cholesterol, VLDL-Cholesterol, VLDL Triglyceride, IDL, LDL, HDL2, HDL3 were available for comparison with *PON* genotypes.

On the basis of clinical, laboratory and radiological parameters, all stroke cases were classified according to Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria [25] to determine stroke subtype (Table 1).

Markers and genotyping

DNA was extracted using Nucleon® DNA extraction kits. 5–10 ng DNA was amplified through Hot Start PCR protocol using Immolase Taq DNA polymerase®. We used different sets of primers for each SNP as follows:

1-*PON1*-192 (A/G):

PON1-192F: 5'-biotin-CTATTTTCTTGACCCCTACTTA-3'

PON1-192R: 5'-CGCTAAACCCAAATACATCTCC-3'

2-*PON1*-55 (T/A):

PON1-55F: 5'-biotin-CTGACAGAAACTGGCTCTGAAG-3'

PON1-55R: 5'-AAGCCAGTCCATTAGGTAGTAT-3'

3-*PON2*-311 (C/G):

PON2-311F: 5'-biotin-GGTTCTCCGCATCCAGAACATT-3'

PON2-311R: 5'-AACTGTAGTCACTGTAGGCTTCTC-3'

4-*PON2*-148 (C/G):

Table 2: Demographic parameters of cases and controls:

	Cases (n = 397)	Controls (n = 405)
Age (mean ± SD)	67.9 (± 11.5)*	67.2 (± 11.7)
Sex (M:F ratio)	1:1	1:1.3
Hypertension (%)	55.1	24.6
Total cholesterol (mmol/L) (mean ± SD)	5.6 (± 1.38)*	5.0 (± 2.0)
HDL (mmol/L) (mean ± SD)	1.21 (± 0.42)*	1.24 (± 0.6)
LDL (mmol/L) (mean ± SD)	3.65 (± 1.81)*	2.27 (± 1.49)
Triglycerides (mmol/L) (mean ± SD)	2.1 (± 1.6)*	1.9(± 1.1)
Atrial fibrillation	19.4%	-
Diabetes mellitus**	14.9%	3.2%
Previous IHD	33.5%	-
Carotid Stenosis (Out of 227 scanned, >50%, based on ACST criteria)	26%	-

* Non significant differences between cases and controls.
** History of diabetes or confirmed laboratory diagnosis.

PON2-148F: 5'-AGTGGAAATTTTTAAATTTGAAG-3'

PON2-148R: 5'-biotin-ACTGTTTTCAGATGCAACA-GAGA-3'

5-*PON3*-rs2074353 (*PON3*-1, A/G):

*PON3*b-2074353F: 5'-biotin-TCTTCCTTTGTAGTGTGT-TAGGAA-3'

PON3-2074353R: 5'-AATTTTTACATAACTCTTGAAACA-3'

6-*PON3*-rs2375003 (*PON3*-2, C/T):

*PON3*b-2375003F: 5'-biotin-CTGATCCCATGTAGAT-TAAATA-3'

PON3-2375003R: 5'-CGCTAGAAATCAGTGGTGGATT-3'

7-*PON3*-rs1053275 (*PON3*-3, A/G):

*PON3*b-1053275F: 5'-biotin-TGTCAAATCCACCACT-GATTTCTA-3'

PON3-1053275R: 5'-GAACAAAACCCAAGGGCACAAG-3'

Of the three *PON3* SNPs, only two SNPs [rs2074353 (*PON3*-1, A/G) and rs1053275 (*PON3*-3, A/G)] were confirmed in our population, with genotype frequencies in Hardy Weinberg equilibrium. All other SNPs were also in Hardy Weinberg equilibrium.

Genotyping assays for determining different alleles were based on the Dynamic Allele Specific Hybridisation (DASH) technique as described by the developers [26].

Data analysis
Association between markers and disease phenotype was tested with Pearson χ^2 test. Multiple Logistic Regression was used to adjust for differences between samples in age, sex, smoking and hypertension. ANOVA was used to test for associations between lipid profiles and allelic distribution. Haplotype frequencies for the six loci (*PON2*-148, *PON2*-311 *PON3*-3, *PON3*-1, *PON1*-55 and *PON1*-192) were estimated for cases and controls, using and comparing two different validated programs: EHPLUS and PHASE Ver.2 [27,28].

Results
Demographic parameters are shown in Table 2. Allele frequency for the six *PON* alleles investigated is shown in Table 3.

There was no significant difference between cases and controls by sex in genotype and allele frequencies. There was no significant difference in genotype and allele frequencies between cases and controls in terms of age stratification (≥65 and <65 years).

There was no significant difference in genotype or allele frequencies between cases and controls in the SVD group or LVD group, other than a small association between the GG genotype of *PON3* and females with LVD (p = 0.03, OR = 2). *PON2*-311 "CC" genotype (SS variant) was higher in male cardioembolic (CE) stroke patients than in male controls (p = 0.03, OR = 2.05).

There was no association between lipid profile (LDL, HDL, total cholesterol/HDL ratio or triglycerides) and *PON* genotype in the stroke population. We also studied the association between *PON* genotype and full lipid profile in 181 of the controls. No differences were found in

VLDL-Cholesterol, IDL, LDL, HDL2, HDL3, and total cholesterol concentrations in females or males between different genotypes of *PON1*-192, *PON1*-55, *PON2*-148 or *PON3*-1. VLDL-Triglyceride was higher in females with "GG" *PON3*-3 genotype (VLDL: AA genotype 69.8, "AG" genotype 77.8, "GG" genotype 116.0; p = 0.036).

Haplotype analysis was performed using PHASE version 2.02 and EHPLUS programs. The PHASE program [27], resulted in reconstruction of 16 different haplotypes in cases and controls. Overall haplotype frequencies in cases and controls were not significantly different (p = 0.19). However, some individual haplotypes were more frequent in one group than the other. Overall, the 112211 haplotype (1= frequent allele, 2= rare allele) was present at a higher frequency among cases than controls (16.62% vs 12.35%, p-value = 0.015, OR = 1.42 (1.07–1.87)). In contrast, 111121 was less frequent in cases than in controls (2.27% vs 4.81%, p = 0.006, OR = 0.46 (0.26–0.81)). There were also some differences in haplotype combination (genotype distribution) between cases and controls as follows: the "112211, 112221" haplotype (cases 10.6%, controls 5.2%: p = 0.005), the "111111, 112221" haplotype (cases 4%, controls 7.9%: p = 0.023).

We repeated analyses using EHPLUS [28] to compare haplotype arrangements in cases and controls. With this method, there was a significant difference (p = 0.005, df, 63) between groups. The most frequent haplotype in cases was 112211 and in controls 111111, while with PHASE2 the commonest haplotype for both groups was 112221. The EHPLUS program generated 64 different haplotypes both in cases and controls but only 32 had a frequency of more than 1%.

Discussion

PON1 and *PON2* are possible candidate genes for atherosclerotic diseases, especially coronary heart disease (CHD), but findings are inconsistent. In a recent meta-analysis of 43 studies [29], the 5 largest studies estimated the per-allele risk ratio at 1.05 for *PON1*-192 R (B) allele and combined analyses of studies of the *PON1*-55 M and *PON2*-311 C variants showed no significant overall associations with CHD. This differs from a recent study [30],

not included in the meta-analysis, which suggested that the picture remains complicated. While there have been fewer studies in ischaemic stroke, there is some evidence of a role for the *PON1*-192 RR (BB) genotype and also for the *PON1* (C-107T) allele in younger ischaemic stroke patients [13,14,31]. A recently published study of 81 stroke patients and 2053 controls from the CARE study also suggested a role for *PON1*-192 in predisposing to stroke, with no association with polymorphisms of the other *PON* genes. In contrast to this study, haplotype analysis or stroke subtyping was not performed [32]. There is evidence that paraoxonase (*PON1*) phenotype may predict vascular disease more accurately than genotype alone [33]. Due to logistical constraints in the current study it was not possible to measure *PON1* phenotype, but future vascular studies should consider this.

As there are ethical difficulties in gaining consent from incapacitated individuals, this is not a study based on incident stroke. It is acknowledged therefore that patients with more severe stroke may not be included. However, the TOAST classification has similar distribution to other studies. The distribution of other vascular risk factors shows that these are high risk individuals. They form a subgroup of a Scottish stroke population which has been demonstrated to have an association with 5-lipoxygenase activating protein, an important mediator of the activity of cellular 5-Lipoxygenase (5-LO) which is a key enzyme in the biosynthesis of leukotrienes, and may thus be involved in atherosclerosis by proinflammatory mechanisms [34]. While a large number of individuals were unclassifiable by TOAST criteria, they all had CT evidence of ischaemic stroke. Individuals with cardioembolic stroke were also included. While these individuals may have a different underlying mechanism for their event, the role of oxidative stress through lipids driving atherothrombosis is likely to underlie the cardiac disease in most of these individuals.

Other than weak associations in small subgroups (females with LVD stroke, males with CE stroke), our study found no significance in overall allele or genotype frequency, although haplotype analysis suggests that the combination of certain alleles may increase or decrease the risk of

Table 3: Allele Frequencies of 6 SNPs in *PON2*, *PON3* &*PON1* genes

	PON2-		PON3-		PON1-	
	148 A/G	311 C/G	3 A/G	1 A/G	55 T/A	192 A/G
Patients (n = 397)	0.76/0.24	0.77/0.23	0.48/0.52	0.48/0.52	0.64/0.36	0.71/0.29
Controls (n = 405)	0.73/0.27	0.74/0.26	0.52/0.48	0.51/0.49	0.63/0.37	0.71/0.29
P-value	*0.29*	*0.16*	*0.11*	*0.20*	*0.73*	*0.89*
OR * (95% CI)	1.16 (0.88–1.52)	1.18 (0.94–1.48)	0.85 (0.69–1.04)	0.87 (0.71–1.07)	1.04 (0.84–1.27)	0.98 (0.79–1.22)

* Adjusted for age, sex and hypertension.

ischaemic stroke in the whole population or in some specific subtypes. These results should be interpreted with caution due to the small numbers of individuals with specific haplotypes, but reinforces the need for very large scale studies to provide a definitive answer.

Although there are several programs based on different methods that can infer haplotypes, there is no consensus as to which method is the best [35]. Most programs, including EHPLUS, a modified version of EH developed by Ott, use the EM (Expectation-Maximisation) algorithm [28,35]. The program ignores all partial or missing genotype data so that the algorithm uses the full set of available data.

The PHASE program is based on coalescent models and uses Markov chain Monte Carlo methods [27,35]. These algorithms use a population genetics model to estimate different haplotype patterns, so a haplotype that is more similar to the commonly observed haplotype patterns is more likely to be inferred.

While the haplotype analysis is an important component of association studies in complex diseases [36], and the different computational methods have their individual strengths and weaknesses, significant haplotype differences in the absence of genotypic or allelic differences are difficult to interpret [35]. Our study highlights this further since one method (EHPLUS) suggests a statistically significant difference while PHSE fails to demonstrate an overall significant difference. It remains unclear which method is superior, but PHASE is believed to be more reliable for larger numbers of markers [35]. However, the level of significance is modest and could have arisen by chance.

In some genetic isolates certain *PON* genotypes can be associated with variation in plasma lipoproteins levels including LDL, HDL and total cholesterol [37-39]. In addition the physical relationship of *PON1* with HDL and the existence of cholesterol pathway regulatory elements at the *PON1* locus suggest a further relationship of *PON1* with lipoproteins, which may contribute to its role of vascular diseases [40]. However, apart from a minor correlation between VLDL-Triglyceride and *PON3* genotypes in female, we did not replicate these differences in our outbred Scottish population.

Conclusion
In conclusion, we found no significant genotypic or allelic frequency differences between stroke cases and controls for any of the structural polymorphisms of the *PON* genes tested. We identified specific haplotypes which may influence stroke risk in our population. Further work is

required in large cohorts to determine whether these differences are real or have arisen by chance.

Competing interests
The author(s) declare that they have no competing interests.

Authors' contributions
AP participated in study design, carried out the genotyping and the statistical analyses and drafted the manuscript. HRA and JC helped with sample preparation LW and DS contributed to the study design, sample collection and helped draft the manuscript. AC helped with study design and commented on the manuscript. MJM contributed to study design, coordinated recruitment and drafted the manuscript. Authors read and approved the final manuscript.

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