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A validation study of Vascular Cognitive Impairment genetics meta-analysis findings in an independent collaborative cohort

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Abstract

Vascular cognitive impairment (VCI), including its severe form vascular dementia (VaD), is the second most common form of dementia. The genetic aetiology of sporadic VCI remains largely unknown. We previously conducted a systematic review and meta-analysis (MA) of all published genetic association studies of sporadic VCI prior to 6 July 2012, which demonstrated that *APOE* (ε4, ε2) and *MTHFR* (rs1801133) variants were associated with susceptibility for VCI. *De novo* genotyping was conducted in a new independent relatively large collaborative European cohort of VaD (n_{max}= 549) and elderly non-demented samples (n_{max}= 552). Where available, genotype data derived from Illumina's 610-quad array for 1210 GERAD1 control samples were also included in analyses of genes examined. Associations were tested using the Cochran-Armitage trend test: *MTHFR* rs1801133 (OR= 1.36, 95% CI 1.16-1.58, p= <0.0001), *APOE* rs7412 (OR= 0.62, 95% CI 0.42-0.90, p= 0.01), and *APOE* rs429358 (OR= 1.59, 95% CI 1.17-2.16, p= 0.003). Association was also observed with *APOE* epsilon alleles; ε4 (OR= 1.85, 95% CI 1.35-2.52, p= <0.0001); and *APOE* ε2, (OR= 0.67, 95% CI 0.46-0.98, p= 0.03). Logistic Regression and Bonferroni correction in a subgroup of the cohort adjusted for gender, age and population maintained the association of *APOE* rs429358 and ε4 allele.

Introduction

Vascular Cognitive Impairment (VCI) represents a heterogeneous group of related conditions involving cognitive decline resulting from cerebrovascular disease or systemic disease that leads to inadequate cerebrovascular supply. Vascular dementia (VaD), an older and more commonly used term to describe more severe forms of VCI, is widely accepted to represent less than a fifth of all dementias and is arguably the second most common form of dementia after Alzheimer's disease (AD). However, there is ongoing debate regarding the validity and utility of distinguishing between AD and VaD given the very high presence of cerebrovascular disease in AD [1, 2]. Indeed, it has been proposed that VCI will become the foremost cause of dementia given the ageing demographics and escalating rates of stroke and ischaemic heart disease [3, 4].

Risk factors for common non-autosomal dominant inherited forms of VCI (i.e. sporadic VCI) strongly overlap with risk factors for those associated with AD. These include hypertension, diabetes mellitus, smoking, atrial fibrillation, positive family history, age and hypercholesterolaemia[5, 6]. However, as yet, little is still known about the extent to which genetic variation contributes to risk of VCI, which as is the case in AD, is likely to interact with various environmental influences.

Despite the extensive overlap between risk factors for AD, stroke and VCI, there has not been as much research into the molecular and genetic basis of VCI. This contrasts markedly with the volume of similar activity that has occurred, and proven beneficial in terms of AD research for over two decades[7]. The slower emergence of studies in VaD and VCI is most likely due to the lower prevalence and highly heterogeneous nature of VCI, both of which are factors that have also served to frustrate development of universally accepted means of VCI classification. This is highlighted by the numerous diagnostic and other research-based criteria and guidelines for VCI that have been developed to provide constructs for the classification of forms of VCI to facilitate research [4, 8-10] but in reality have been used to varying extents. Thus large-scale collaborative endeavours that are ordinarily needed to properly study diseases of lower prevalence such as VCI have not yet been regularly realised. Furthermore there are issues on the level of interpretation and inference that can be made across different smaller-scale studies. As a consequence of these factors, thus far, susceptibility genes of VCI, particularly those of small effect most likely remain undiscovered.

A useful, rapid and relatively low cost tool to explore and maximise the amount of information that can be extracted from what may be considered to be a number of inadequately powered individual studies of VCI is meta-analysis (MA). Recently we used MA to investigate a limited number of selected (i.e. most commonly studied) candidate genes, partly suggested by previously reported associations with AD, and having cardiovascular properties also relevant to VaD including;

Apoliopoprotein *(APOE),* methylenetetrahydrofolate reductase *(MTHFR)* and angiotensin converting enzyme *(ACE)* polymorphisms [11-14]. In our previous study [15], we used a combined systematic review and MA approach of all published candidate gene studies of sporadic VCI to identify potentially important candidate genes for VCI. This allowed us to try and address some of the shortcomings of previous studies in terms of statistical power, whilst acknowledging the heterogeneous nature of VCI. Associations with increased risk for VCI were found for *APOE* ε4 (OR= 1.818, 95% CI 1.611 to 2.053, p= <0.001; N= 3,554 cases, N= 12,277 controls) and *MTHFR* rs1801133 (OR= 1.323, 95% CI 1.061 to 1.650, p= 0.013; N= 659 cases, N= 981 controls). There was weak evidence of a protective effect for *APOE* ε2 (OR= 0.885, 95% CI 0.783 to 0.999, p= 0.048; N= 3,320 cases, N= 10,786 controls). MA of polymorphisms: rs4934 of Alpha-1-antichymotrypsin (*ACT, now formally referred to as SERPINA3)*; rs1799752 (intron 16 indel variant) of *ACE*; rs662 of Paraoxonase 1 (*PON-1)*; and the rs165932 variant of presenilin-1 *(PSEN-1)* showed no evidence for association [15].

In general, MA of multiple small studies may also suffer from between-study heterogeneity, or be of inferior methodological quality [16], including the use of different clinical diagnostic criteria. Many of these factors can give rise to disproportionally larger or small effect sizes for any gene found to be of interest. Considering these limitations, we attempted to validate our MA findings by genotyping the polymorphisms previously found to be interesting [15], in a uniformly diagnosed unprecedented cohort of archival DNA from VaD patients and healthy controls of European decent, thereby minimising variation in methodology and interpretation.

Materials and Methods

The collaborative cohort consists of archival samples from the United Kingdom (UK) (n=278) and Italy (n=823). Age and gender was available for a subsection of the cohort: mean age VaD 78.74 ± 0.32 (n= 509), 232 males/296 females; mean age controls 76.83 ± 0.43, (n= 513), 233 males/316 females. VaD cases were clinically diagnosed according to NINDS-AIREN criterion [8]. A sample of 549 VaD cases and 552 controls were genotyped using Sequenom iPLEX Gold (97.7% success rate) for a number of polymorphisms (see Table 1). Polymorphism rs4343 in *ACE* was genotyped as a proxy for the *ACE I/D* [17]. *SERPINA3 (ACT)* rs4934 was genotyped using Taqman (*TaqMan C_2188895_10)* and analysed using Sequence Detection Software version 2.4. All SNPs were concordant for HWE at P>0.001. SNPs rs429358 and rs7412 were directly genotyped and *APOE* epsilon alleles calculated – in brief the T allele at both *APOE* SNPs identified the ε2 allele, whereas the C allele at both positions constitutes the ε4 allele. The T allele at rs429358 and the C allele at rs7412 identify the ε3 allele, which is the most common allele in the general population[18]. Genotype data from an additional 1210 non-demented elderly individuals (451 males/759 females), from UK (n= 830), Northern Ireland (n= 110), Germany (n= 37) and United States (n= 233) that were collected as part of the GERAD1 consortium [19] that were used in AD Genome Wide Association Studies (GWAS) genotyped on the Illumina 610-quad chip (as described [19]), was available for inclusion in the analysis for *MTHFR* rs1801133 and *PON-1* rs662. Age was available for 1133 individuals; mean 76.34 ± 0.20. Genetic association of susceptibility to VaD were independently evaluated using the test for trend implemented in PLINK [20], with logistic regression (LR) analysis for age, gender, and population. A p-value <0.05 was considered nominal evidence for statistical significance and supportive of our previous findings [15]. SNP-SNP interactions were also analysed in 426 cases and 1730 controls using PLINK ALL x ALL epistasis mode with the odds ratio calculated for interaction and p values adjusted for the multiple tests performed.

Results

Associations were identified using the Cochran-Armitage trend test for *MTHFR* rs1801133 (OR= 1.36, 95% CI 1.16 to 1.58, p= 0.000095), *APOE* rs7412 (OR= 0.62, 95% CI 0.42 to 0.90, p= 0.01), and *APOE* rs429358 (OR= 1.59, 95% CI 1.17 to 2.16, p= 0.003) (Table 1). Association was also observed with

APOE epsilon alleles (ε4: OR= 1.85, 95% CI 1.35 to 2.52, p= 0.00005; ε2: OR= 0.67, 95% CI 0.46 to 0.98, p= 0.03); allelic distributions are provided in Table 2.

APOE and *MTHFR* associations were robust to Bonferroni correction, although logistic regression analysis in a subgroup of the cohort adjusted for gender, age and population reduced the strength of all associations (Table 1). However, associations for *APOE* rs429358 (OR= 1.66, 95% CI 1.19 to 2.32, p= 0.003) and ε4 allele (OR= 1.61, 95% CI 1.52 to 1.72, p= 0.0001) were robust to these adjustments.

There was no evidence of association between VCI risk and variants in a number of other genes that were included in our previous MA study and which here also showed lack of association: rs4934 of *ACT*; rs1799752 of *ACE*; rs662 of *PON-1*; and rs165932 of *PSEN-1* (Table 1).

Epistasis, defined typically as the interaction between different genes, has revealed many novel biological insights for complex disease genetics in recent years. The presence of an allele at one SNP loci may interact with alleles at other loci to exert a complementary or specific effect on gene expression and / or function. For example, genes involved may be part of multi-component proteins, the same biological pathway, or disease mechanism and exert modifier effects on phenotypes. As the genes investigated in this study have been suggested to contribute to VCI, we evaluated if SNPs demonstrated independent effects. Using the integrated epistasis approach implemented in PLINK (All x ALL command) we tested pairwise combinations of all SNPs across all genes for 426 case and 1730 control individuals; no SNP-SNP interaction was statistically significant following Bonferroni correction for multiple testing (data not shown).

Discussion

This study provides supportive evidence, in an independent European cohort of VaD patients and non-demented elderly, of association between variants in *APOE* and *MTHFR* and susceptibility for

VaD, that validate previous findings for these genes demonstrated by MA [15]. In agreement, we found no association for *PON-1* rs662, *SERPINA3 (ACT)* rs4934; *PSEN-1* rs165932 or *ACE* rs1799752 as investigated in our previous MA [15]. This supports the utility of MA as a method to maximise the amount of information that can be extracted from a series of published unrelated and small case-control association studies, which individually only allow for limited interpretation because of low statistical power. In a subsequently published MA, the same findings for these SNPs were reported, albeit with the inclusion of varying studies[14].

APOE

Apoliopoprotein (APOE) plays a key role in lipid metabolism of cholesterol and triglycerides used to support synaptogenesis and the maintenance of synaptic connections [21]. *APOE* has also been associated with increased risk of cerebral amyloid angiopathy [22, 23] as well as cardiovascular and cerebrovascular atherosclerosis, coronary heart disease, and high total serum cholesterol. Collectively the role of APOE in a number of vascular conditions, each of which could contribute to VCI, clearly makes *APOE* a strong candidate gene for VCI risk. There have also been numerous studies exploring possible association of *APOE* ε4 with ischaemic stroke, as recently reviewed by Stankovic and Majkic-Singh, however, results on this thus far are conflicting [24].

Our published MA of 63 cohorts totalling 3,554 cases and 12,277 controls showed that *APOE* ε4 was associated with increased risk of VCI[15]. Separate MA of people who were classified against specific definitions of VaD (OR= 1.913, 95% CI 1.683 to 2.173, p= <0.001; N= 2,422cases, N= 9,722 controls) also showed evidence of association. Stratification of *APOE* ε4 data by ethnicity also showed evidence of association with Asian, and European groups (OR= 1.939, 95% CI 1.576 to 2.386, p= <0.001; N= 1,268 cases, N= 4,078 controls). The association we previously identified by MA with susceptibility for VaD in Europeans was further supported in this study that comprised a large

European cohort; of particular note, the odds ratio in this study (OR= 1.85) is strikingly similar to that previously reported (OR= 1.82) for VCI.

Our previously published MA of *APOE* ε2 revealed a protective effect against VaD and in the analysis of all cohorts under a broader VCI term. The protective effect of *APOE* ε2 against VaD (OR= 0.812 95% CI 0.698 to 0.945, p= 0.007; N= 2,247 cases, N= 8,967 controls) was further supported in the current case-control study although statistical evidence was only weak (OR= 0.67, 95% CI 0.46 to 0.98, p= 0.03). *APOE* ε2 may have a protective effect in coronary artery disease, a disease mediated by altered lipoprotein levels, inflammatory and immune activities [25].

***MTHFR* rs1801133**

Methylenetetrahydrofolate reductase (MTHFR) is a key rate-limiting enzyme in the metabolism of homocysteine (Hcy). The rs1801133 (also known as C677T) polymorphism of *MTHFR* has been associated with reduced enzyme activity and increase serum Hcy levels [26]. It has also been linked to several vascular diseases including risk of coronary heart disease and hypertension [27], as well as with cognitive impairment [28]. One of the largest MA of 32 published articles (6110 cases/8760 controls) that investigated this polymorphism in relation to risk of ischaemic stroke/TIA, found that the T allele was associated with increased risk of stroke in a graded, dose-dependent manner (T allele pooled OR= 1.17; 95% CI 0.09–1.26; TT genotype pooled OR= 1.37; 95% CI 1.15–1.64)[29].

There have been conflicting findings regarding the association between this *MTHFR* polymorphism with stroke, however seven out of 11 MA of stroke that have been previously undertaken have shown an association for C677T [24]. The discrepancies may relate to the fact that the C677T polymorphism varies in different ethnic populations, ranging from less than 1% to 21% [30]. Furthermore, an association of *MTHFR* rs1801133 with AD was only found when the co-occurrence of *APOE* ε4 was also included in the analysis [31] suggesting an epistatic interaction. Indeed a similar observation was also found for *IL-6* rs1800795 in both AD and VaD [32]. Of particular interest is that analysis of the *MTHFR* rs1801133 by HaploReg version 2 [33] reveals multiple epigenetic effects for this SNP in a European population. Characterising the downstream effects of genetic mutations associated with disease is challenging. One approach is to evaluate associations between disease-associated SNPs and the expression levels of local genes (cis effects) and downstream consequences that are more distant from the target SNP (trans effects). Westra and colleagues [34] performed a systematic evaluation of expression quantitative trait loci (eQTLs) using next generation sequencing in >8,000 individuals; their results were published in *Nature Genetics* and made publicly available, thus providing a rich resource for researchers. We interrogated this valuable dataset for putative functionality of top-ranked SNPs in this study and presented relevant results in Table 3 to support biological insights for MTHFR. Rs1801133 has an impact on both epigenetics and the expression of several genes, with this association exceeding traditional genome-wide significance values (Table 3). The *cis*-eQTl links for blood are strongest for Mitofusin 2 gene (*MFN2*) central to mitochondrial fusion and important in the regulation of vascular smooth muscle cell proliferation[35], with defects associated with disorders of PNS [36], early events in ischaemic stroke and neurodegeneration [37-39].

The results of our MA for *MTHFR* rs1801133 in VCI also showed an association of the T allele and increased risk of VCI and in the smaller MA of VaD cases we also found association (OR= 1.309, 95% CI 1.121 to 1.528, p= 0.001; N= 616 cases, N= 981 controls). However, the majority of studies that were included in this MA were Asian and only 2 studies were Caucasian, with stratified analysis showing that Asian but not Caucasian cohorts (OR= 1.644, 95% CI 0.597 to 4.528), p= 0.336; N= 136 cases, N= 125 controls) were associated. The current European case-control study, which is larger than the combined sample size in the original MA, supported the presence of association with VaD and now provides evidence of association in Caucasians (OR= 1.36, 95% CI 1.16 to 1.58, p= 0.0001). The odds ratio in this study (OR= 1.36) is similar to the VCI association (OR= 1.32) previously reported, suggesting a similar effect size for both phenotypes, however it should be noted that the LR model accounting for age, gender and population in a subsection of the cohort did not show evidence of association in the current collection.

In this study of European subjects, there was no evidence of an epistatic interaction between *APOE* and *MTHFR* polymorphisms, supporting the hypothesis that *MTHFR* might serve as an independent genetic risk factor for VCI. In future studies of VCI, it is worth considering the testing of epistasis in a routine manner, where statistical power may allow, to avoid missing clues to risk variants that might otherwise be overlooked.

While there was no evidence of association for *SERPINA3 (ACT)* rs4934 and *PSEN-1* rs165932, the lack of association with either may relate to the fact most of the biological evidence of the potential involvement of both resultant proteins is more related to their roles in aspects of AD and in particular that of Aβ peptide pathology [40] that is not part of the main neuropathology of VaD. Yet, the function of *SERPINA3* might have been more plausible as an acute phase protein that is released in response to inflammatory stimuli that have been suggested in early stages of dementia [41].

The *PON-1* rs662 and *ACE* rs1799752 were arguably stronger candidate genes for VCI since Paraoxonase 1 (PON-1) has a vascular function as a component of high-density lipoprotein (HDL) but is also important for Hcy metabolism while angiotensin converting enzyme (ACE) plays an important part in the regulation of systemic blood pressure and fluid electrolyte balance with hypertension one of the biggest risk factors for VCI [42]. With respect to *PON-1,* despite links to Hcy metabolism, association with ischemic stroke [43] and synergistic interactions between genes of related processes shown in coronary artery disease patients [44], in this study we found no evidence of epistatic interactions. In contrast, the D allele of *ACE* has been suggested, albeit with many conflicting studies, to be associated with risk factors for VCI including; myocardial infarction, stroke, cardiovascular disease, essential hypertension, diabetes mellitus and leukoaraiosis in patients with ischemic stroke [45]. Yet we found no evidence here, nor in the previous MA study to support any association, in agreement with another recent study on VaD [11].

Study Limitations

Associations for *APOE* and *MTHFR* were robust to Bonferroni correction, although logistic regression analysis in a subgroup of the cohort adjusted for gender, age and population reduced the strength of all associations (Table 1). However, only a reduced number of cases could be included in this more detailed level of analysis, due to the lack of covariate data available for a substantial proportion of these archival samples, thus reducing the power to identify such associations. Furthermore, the identification of candidate genes by MA is limited and wholly determined by what studies have been previously conducted and are suitable for inclusion in the analysis. MA has limitations and biases in the same way as any hypothesis-driven approaches towards the discovery of risk genes. More recently the use of GWAS has been developed to address this, whilst itself still being limited by the amount of gene variants that are included.

Emerging evidence of the genetic aetiology from Genome Wide Associations Studies (GWAS)

Rs12007229, a single nucleotide variant of no known function located on the X chromosome, which is near the androgen receptor gene (OR= 3.7, 95% CI 2.3–5.8, per copy of the minor allele; *P*= 1.3×10^{-8})[46] was identified in 2012 in the first GWAS of VaD. Although a first for this disease it was somewhat limited as it involved data from a total of just over 300 incident and prevalent cases of VaD that were compared to a Dutch population-based discovery cohort of 5700 subjects. Another GWAS reported a novel association with an intronic variant of rs290227 within the spleen tyrosine kinase (SYK) gene [47] from a cohort of 87 Korean VaD patients and 200 controls. A comparison of these two GWAS could not identify common nucleotide variants[48], yet the potential power of GWAS to identify new variants, in relatively small cohorts is highlighted. However, in neither of these

GWAS (which are both considerably smaller in sample size to either the original MA or this replication study) was there any evidence found to support the involvement of *APOE* or *MTHFR* as a risk factor.

Conclusions

We report that variations in both *APOE* and *MTHFR* are associated with increased risk of VaD. While the potential involvement of *MTHFR* in VCI risk is interesting, it still needs further independent replication. However, the robust association seen here, and in previous MA for the *APOE* ε4 variant as a risk factor strongly support the validity of using *APOE* variants as a necessary co-variant in analysis of other genetic susceptibility factors of VCI, similar to how it has been widely applied in genetic studies of AD. GWAS is likely to serve as the most likely method by which to pursue the identification of new candidate genes for VCI in the future. However, future successes are also likely to be dependent upon the availability of large numbers of samples that will only come via collaboration such as has been seen to generate considerable success in recent years in the AD field where approximately 32 genes have now been identified [49].

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Cochran-Armitage trend test with Bonferroni correction						Logistic regression analysis	
Polymorphism	**Cases**	**Controls**	**OR (95% CI)**	**p - value**	**BONF**	**OR (95% CI)**	**p - value**
APOE rs429358 allele C	104/724	82/910	1.59 (1.17-2.16)	0.0026	0.018	1.66 (1.19-2.32)	0.003
APOE rs7412 allele T	43/779	81/905	0.62 (0.42-0.90)	0.012	0.084	0.70 (0.46-1.04)	0.078
MTHFR rs1801133 allele T	363/467	1252/2186	1.36 (1.16-1.58)	0.000095	0.0007	1.12 (0.93-1.34)	0.233
PON-1 rs662 allele G	252/586	1002/2446	1.05 (0.89-1.24)	0.563	1	1.12 (0.92-1.35)	0.255
ACE rs4343 allele A	339/489	396/600	1.05 (0.87-1.27)	0.618	1	1.07 (0.87-1.30)	0.524
PSEN-1 rs165932 allele C	333/489	385/577	1.02 (0.84-0.12)	0.833	1	1.04 (0.85-1.27)	0.706
ACT rs4934 allele G	378/366	469/471	1.04 (0.86 – 1.26)	0.710	1	1.01 (0.83-1.24)	0.917

Table 1: Summary analysis of *APOE*, *MTHFR, PON-1, ACE, PSEN-1 and ACT* variants in the European cohort. Cochran-Armitage trend test: no. of minor alleles in VaD cases; no. minor alleles in controls; Bonferroni corrected p-value (BONF). Logistic regression with age, gender and population origin as covariates odds ratio (95% confidence intervals) and resulting p-value for each respective analysis as detailed in the first column.

Alleles	Number of cases	Number of controls
Carries any ε2 allele	58	83
No ε2 allele	471	454
Carries any ε4 allele	138	86
No ε4 allele	391	451

Table 2: APOE epsilon allelic distributions within case and control groups; epsilon allele calls were derived from directly typed genotypes for rs429358 and rs7412 SNPs; APOE ε2, (OR= 0.67, 95% CI 0.46-0.98, p= 0.03) and APOE ε4 (OR= 1.85, 95% CI 1.35-2.52, p= <0.0001).

***Trans*-eQTLs**					
p-value	**SNP**	**Chromosome**	**Base position**	**Minor allele**	**Gene name**
2.062E-6	rs1801133	1	11778965	A	PTPRN2
5.145E-6	rs1801133	1	11778965	A	EXOSC6
1.393E-5	rs1801133	1	11778965	A	HOXD11
***Cis*-eQTLs**					
6.085E-100	rs1801133	1	11778965	A	MFN2
6.216E-80	rs1801133	1	11778965	A	PLOD1
7.818E-32	rs1801133	1	11778965	A	MTHFR, C1orf167
9.470E-13	rs1801133	1	11778965	A	KIAA2013, CLCN6
7.157E-9	rs1801133	1	11778965	A	KIAA2013, NPPA
0.002	rs1801133	1	11778965	A	C1orf187

Table 3: Blood eQTL browser (http://genenetwork.nl/bloodeqtlbrowser/) reports multiple *trans* and *cis* expression quantitative trait loci for *MTHFR* rs1801133: protein tyrosine phosphatase, receptor type, N polypeptide 2 (*PTPRN2*); exosome component 6 (*EXOSC6*); homeobox D11 (*HOXD11*); Mitofusin 2 (*MFN2*); procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 (*PLOD1*); methylenetetrahydrofolate reductase (*MTHFR*), chromosome 1 open reading frame 167; KIAA2013; chloride channel, voltage-sensitive 6 (*CLCN6*); natriuretic peptide A (*NPPA*); dorsal inhibitory axon guidance protein (*DRAXIN*).