



Cerebrovascular and microglial states are not altered by functional neuroinflammatory gene variant

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Abstract

The translocator protein, a microglial-expressed marker of neuroinflammation, has been implicated in Alzheimer's disease, which is characterized by alterations in vascular and inflammatory states. A *TSPO* variant, rs6971, determines binding affinity of exogenous radioligands *in vivo*; however, the effect of these altered binding characteristics on inflammatory and cerebrovascular biomarkers has not been assessed. In 2345 living subjects (Alzheimer's Disease Neuroimaging Initiative, n = 1330) and postmortem brain samples (Religious Orders Study and Memory and Aging Project, n = 1015), we analyzed effects of rs6971 on white matter hyperintensities, cerebral infarcts, circulating inflammatory biomarkers, amyloid angiopathy, and microglial activation. We found that rs6971 does not alter translocator protein in a way that impacts cerebrovascular and inflammatory states known to be affected in dementia.

Keywords

Microglia, Alzheimer's, MRI, genetics, risk factors, inflammation, white matter disease

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Introduction

Late-onset Alzheimer's disease (AD) is characterized by the accumulation of A β plaques and neurofibrillary tangles, chronic inflammation, and progressive neurodegeneration.^{1–4} The inflammation hypothesis of AD posits that chronic over-activation of microglia in response to the buildup of amyloid pathology results in a positive feedback loop whereby pro-inflammatory conditions sustain the elevated expression of factors that further exacerbate amyloidogenesis.^{1,5–7} In addition to the damaging effects of “traditional” neuroinflammation, defined by the activation of resident

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microglia and infiltration of peripheral monocytes, the immune response also serves to protect against neurodegeneration; studies show that increased expression of certain inflammatory cytokines may reduce A β deposition⁸ and promote cell survival and neurovascularization.^{4,9}

The convergence of inflammation and cerebrovascular pathology in AD is becoming increasingly clear, yet the mechanisms behind this convergence are poorly understood. It is known that cerebral ischemia is a strong activator of the immune response,^{10,11} and that, in parallel, vascular irregularities are themselves risk factors for AD.^{12,13} A β deposition within neurovasculature, known as cerebral amyloid angiopathy (CAA), may contribute to brain ischemia and lead to cognitive impairment.^{14,15} Cerebral infarcts are regions of ischemic damage associated with aging and present to a greater degree in individuals who develop AD than in those who do not.¹⁶ The presence of both macro- and micro cerebral infarcts, caused largely by cerebral small vessel disease (SVD),¹⁷ have been shown to influence risk for AD as well as global cognitive performance,^{18–20} and may be a consequence of accumulating cerebrovascular insults (and accompanying hypoperfusion²¹) that are implicated in neurodegenerative processes.²² At the neuroimaging level, white matter lesions associated with SVD can be imaged *in vivo* as regions of increased signal on T2-weighted MR images; these white matter hyperintensities (WMHs) have been associated with increased age, the presence of cerebral infarcts, multiple vascular disease risk factors, AD and mild cognitive impairment (MCI) diagnosis, and the progression from MCI to AD.^{23,24}

At the molecular genetic level, the translocator protein 18 kDa (TSPO) has recently become a focus of interest in the investigation of psychiatric and neurological illnesses with potential immune mechanisms of illness. TSPO is expressed in activated microglia and used as a biomarker for neuroinflammation^{25,26}; it is found in the outer mitochondrial membrane where it is thought to regulate cholesterol transport, steroidogenesis, and apoptosis.²⁷ *In vivo* PET imaging studies using TSPO radioligands show increased binding in patients with acute brain injury,²⁸ multiple sclerosis,^{29–33} MCI,³⁴ and AD.^{25,35} A single genetic polymorphism located in exon 4 of the *TSPO* gene, rs6971 (Ala147Thr), reliably determines the binding affinity of second generation TSPO radioligands in the brain,^{36,37} where A/A, A/G, and G/G genotypes correspond to high, medium, and low-affinity binding phenotypes (herein referred to as HABs, MABs, and LABs, respectively). It is hypothesized that the Alanine to Threonine substitution at position 147 results in a conformational change in TSPO structure that influences its interaction with ligands.^{36,38,39}

Differences in binding affinity may have important implications for the etiopathology of AD; TSPO ligands have been shown to ameliorate neuroinflammation *in vitro*,⁴⁰ reverse neuropathology and behavioral decline in AD mouse models,⁴¹ reduce A β ₄₂-induced neurodegeneration in drosophila,⁴² as well as confer neuroprotective and regenerative effects *in vivo* and *in vitro*.^{43–46}

Despite this potential involvement of TSPO in the etiopathology of neurodegenerative disorders, and the well-known links between inflammation, cerebrovascular disease, and AD, no studies to date have investigated the effect of rs6971 on MRI-based phenotypes or inflammatory biomarkers. We therefore sought to test whether *TSPO* rs6971 genotype was associated with *in vivo* structural imaging measures of cerebrovascular disease and neuroinflammation in two large clinical samples (the Alzheimer's Disease Neuroimaging Initiative (ADNI) and the Religious Orders Study/Memory and Aging Project (ROS/MAP)). To identify potential mechanisms of action, we also analyzed the effect of genotype on levels of plasma inflammatory biomarkers in living subjects, as well as on cerebral infarcts, CAA, and densities of active microglia from postmortem brain tissue. Due to the anti-inflammatory action of TSPO ligands,⁴⁷ we hypothesize that low-affinity binding groups would have exacerbated pathology and increased levels of pro-inflammatory biomarkers vs. medium- and high-affinity binding groups, as determined by genotype.

Materials and methods

Subject characteristics and genetics

ADNI. ADNI is a large multicenter collaboration established in 2003, in which elderly subjects at various stages of cognitive impairment are assessed longitudinally for multimodal imaging, neuropsychiatric test performance, and fluid biomarkers. Details on subject recruitment and inclusion/exclusion criteria are reported elsewhere.⁴⁸ A total of 699 individuals from ADNI 1 and 631 individuals from ADNI GO and 2, all self-reported Caucasian, were included in analyses ($n_{\text{total}} = 1330$; see Table 1 for details). Data were extracted from the ADNI website (<http://adni.loni.usc.edu>). ADNI 1 subjects were genotyped for rs6971 using the Quad 610 BeadChip (Illumina Inc., San Diego, CA, USA), and ADNI GO and 2 subjects were genotyped using the HumanOmniExpress BeadChip (Illumina Inc., San Diego, CA, USA). *APOE* ϵ 4 status was obtained separately by genotyping rs429358 and rs7412, according to previously published methods.⁴⁹ ADNI ethics approval was obtained for each institution involved including the Research

Table 1. Summary statistics for ADNI samples by diagnosis.

ADNI 1 (n = 699)	CN (n = 195)	SMC	EMCI	LMCI (n = 338)	AD (n = 166)	Diff P
WMHv ^a (SD)	0.8 (2)	–	–	0.9 (3)	1.3 (3)	0.07
CI (+/–) ^b	18+,177–	–	–	30+,308–	13+,153–	0.90
Age, Y (SD)	75 (7)	–	–	75 (7)	76 (7)	0.22
Sex (F/M)	88 F, 107 M	–	–	117 F, 221 M	75 F, 91 M	0.018
BP (systolic)	133 (15)	–	–	133 (17)	135 (18)	0.35
BP (diastolic)	73 (10)	–	–	74 (10)	73 (9)	0.61
MMSE (SD)	29 (1)	–	–	27 (2)	23 (2)	<0.001
Education, Y (SD)	16 (3)	–	–	16 (3)	15 (3)	<0.001
APOE ε4 status (+/–)	53+,142–	–	–	185+,153–	112+,54–	<0.001
rs6971 genotype (LAB/MAB/HAB)	20/88/87	–	–	25/135/178	18/66/82	0.33
ADNI GO and 2 (n = 631)	(n = 129)	(n = 40)	(n = 242)	(n = 122)	(n = 98)	Diff P
WMHv ^c (SD)	6.8 (13)	8.5 (11)	7.3 (9)	7.7 (10)	8.7 (10)	0.70
CI (+/–) ^b	6+,113–,10 NA	40 NA	20+, 184–, 38 NA	6+, 75–, 41 NA	1+, 35–, 62 NA	0.35
Age, Y (SD)	74 (6)	72 (5)	71 (7)	72 (8)	75 (8)	<0.001
Sex (F/M)	62 F, 67 M	27 F, 13 M	102 F, 140 M	52 F, 70 M	38 F, 60 M	0.025
BP (systolic)	134 (16)	131 (17)	132 (17)	132 (18)	131 (17)	0.76
BP (diastolic)	74 (10)	72 (9)	73 (9)	73 (10)	74 (10)	0.87
MMSE (SD)	29 (1)	29 (1)	28 (2)	28 (2)	23 (2)	<0.001
Education, Y (SD)	16 (3)	17 (3)	16 (3)	16 (3)	16 (3)	0.16
APOE ε4 status (+/–)	34+,95–	12+,28–	105+,137–	69+,53–	66+,32–	<0.001
rs6971 genotype (LAB/MAB/HAB)	19/51/59	5/20/15	26/108/108	9/56/57	10/42/46	0.75

^aVolumes are in mm³, normalized to a standard space. ^bSample sizes for MRI cerebral infarcts (CI) are different than those indicated in column headers, some non-overlapping subjects were evaluated for CI: ADNI1 (n_{CN} = 196, n_{LMCI} = 339, n_{AD} = 167), ADNI GO and 2 (n_{CN} = 123, n_{EMCI} = 207, n_{LMCI} = 83, n_{AD} = 36). ^cVolumes are in mm³. Continuous variables (age, education, MMSE, and BP) were analyzed for diagnosis group differences using ANOVA (two-tailed). Factor variables (sex, CI, APOE ε4 status, and rs6971 genotype) were analyzed using Fisher's exact test (two-tailed). LAB: low affinity binders (rs6971 A/A); MAB: medium affinity binders (rs6971 A/G); HAB: high affinity binders (rs6971 G/G); MAF: minor allele frequency; WMHv: white matter hyperintensity volume; CI: cerebral infarcts detected by MRI; MMSE: mini mental status examination; APOE: Apolipoprotein E; BP: blood pressure (mmHg); SD: standard deviation; M: male; F: female; Y: years; CN: cognitively normal; SMC: some memory concern; EMCI: early mild cognitive impairment; LMCI: late mild cognitive impairment; AD: Alzheimer's disease.

Ethics Board at the Centre for Addiction and Mental Health (Toronto, Canada). All aspects of the study were performed in accordance with the Declaration of Helsinki, US 21CFR Part 50 – Protection of Human Subjects, and Part 56 – Institutional Review Boards, and federal HIPAA regulations. All subjects provided written informed consent.

ROS/MAP. ROS and MAP are community-based cohort studies of aging and dementia. Participants in ROS are older nuns, priests, and brothers from across the USA,⁵⁰ and those in MAP are older residents of north-eastern Illinois.⁵¹ Both studies were approved by the Institutional Review Board of Rush University Medical Center in compliance with the Health Insurance Portability and Accountability Act and enroll older persons without dementia who agree to annual evaluation and autopsy. All participants provided written informed consent. Follow-up rates exceed 90% and autopsy rates exceed 85%. A

neuroimaging substudy was initiated in 2009.⁵² A total of 1015 postmortem brains were available for analysis at time of study. Full details on sample characterization and assessments have been previously published⁵³ (supplementary methods). All subjects were genotyped for rs6971 using the Affymetrix (Santa Clara, CA, USA) Genechip 6.0 platform. APOE (rs7412 and rs429358) genotypes were imputed from MACH (version 1.0.16a) and HapMap release 22 CEU (build 36), as previously described.⁵⁴

In vivo neuroimaging

ADNI. Structural MR images (including T1, T2, PD) were acquired for all subjects using a standard protocol on 1.5T scanners across multiple sites. Correction for gradient non-linearity was performed using gradwarp and standardization across sites and platforms was performed as previously published.⁵⁵ The presence of cerebral infarcts was evaluated for 1151 subjects in

ADNI 1, GO, and 2 using the same method, outlined by De Carli et al. on the ADNI website⁵⁶ (supplementary methods). For ADNI 1, WMHv estimates were derived from T1, T2, and PD images using a method described elsewhere^{57,58} (supplementary methods); for ADNI GO and 2, volumes of CSF, gray matter, white matter, and WMH were calculated from fluid attenuated inversion recovery (FLAIR) and T1-weighted images using a four-tissue Bayesian segmentation method,^{59,60} outlined by De Carli et al. on the ADNI website⁶¹ (supplementary methods).

ROS/MAP. A subset of subjects with genetic data ($n = 291$) underwent a multimodal neuroimaging protocol that included high-resolution T1-weighted magnetization-prepared rapid acquisition gradient-echo (MPRAGE) and T2-weighted FLAIR scans. Detailed scan acquisition parameters have been published elsewhere.⁶² WMHv estimates were extracted from scans using an automated pipeline, as described^{62,63} (supplementary methods).

In vivo plasma biomarkers

ADNI 1. Data were available from a subset of individuals from ADNI 1 ($n = 520$) who contributed plasma aliquots for proteomic analysis using the 190-analyte Luminex xMAP platform by Rules-Based Medicine (Myriad RBM, Austin, TX, USA) (supplementary methods). Concentrations of five inflammatory biomarkers were available that were also assayed in the ROS/MAP sample: tumor necrosis factor alpha (TNF α), interleukin-6 receptor (IL6R), C-reactive protein (CRP), vascular cell adhesion protein 1 (VCAM1, CD106), and matrix metalloproteinase 9 (MMP-9).

ROS/MAP. A subset of subjects ($n = 394$) had blood drawn and plasma inflammatory biomarkers quantified as previously described.⁶² Highly sensitive multiplexed sandwich ELISA arrays (Endogen Searchlight technologies, Billerica, MA) were used to detect plasma concentrations of five inflammatory proteins, listed above.

Postmortem neuropathology, ROS/MAP

Neuropathological evaluation. Brain autopsies were conducted at predetermined sites across the United States and neuropathological examinations were performed at Rush University Medical Centre. Full details of autopsy and pathological evaluation procedures have been previously published.^{18,64} Micro and Macro cerebral infarcts were quantified from fixed brain slabs using hematoxylin/eosin staining (supplementary methods) and coded as either present (one or more infarcts) or absent, as previously reported.¹⁹ Degree of cerebral

amyloid angiopathy (CAA; none, mild, moderate, and severe) was assessed using immunohistochemical labeling as previously described⁶⁵ (supplementary methods). A subset of brains were also analyzed for quantification of microglia at three levels of activation, across four brain regions (inferior temporal, midfrontal, posterior putamen, and ventral medial caudate), as described⁶⁶ (supplementary methods). Average density (across all stages of activation), as well as the square root-transformed ratio of most active (stage 3) to least active microglia (stage 1), was calculated for each region.

Statistical analysis

All analyses were performed using R statistical software (version 3.0.2). Dichotomous outcomes (presence or absence of cerebral infarcts both *in vivo* and postmortem) were tested using logistic regression, with *TSPO* genotype (additive model), sex, age, diastolic blood pressure (hypertension y/n in ROS/MAP), education (total years), and *APOE* $\epsilon 4$ status as predictors. Continuous outcomes (WMHv, plasma biomarkers, microglial density) were modeled similarly using linear regression. Due to important differences in WMHv estimation methods between ADNI 1, ADNI GO and 2, and ROS/MAP, each dataset was analyzed separately. Outcomes were tested for normality using the Shapiro-Wilk test⁶⁷ and corrected, if necessary (Shapiro-Wilk $P < 0.01$), using Box-Cox power transformations.⁶⁸ Since the ADNI 2 FLAIR-derived WMHv estimates were not normalized to a standard space, models of this data also co-varied for total intracranial volume. Ordinal regression was used to evaluate the degree of amyloid angiopathy using the same modeling approach and set of covariates as above. Due to their biologically related functions in cholesterol transport⁶⁹ and *TSPO*'s ability to up-regulate *APOE*,⁷⁰ interactions between *APOE* $\epsilon 4$ status and *TSPO* genotype were also tested in each model. For all analyses, P -values are two-tailed and reported as Bonferroni-corrected (P_{cor}) or uncorrected (P_{raw}).

Data handling

All analyses in this study were performed using de-identified data from centrally curated databases. ADNI data are stored and maintained in the Laboratory of Neuro Imaging (LONI) Image Data Archive (IDA) at University of Southern California (Los Angeles, CA, USA). Clinical, imaging, and genetic data, ROS/MAP data are stored and maintained at the Rush Alzheimer's Disease Center (RADC) at the Rush University Medical Center (Chicago, IL, USA). For clinical evaluation in ROS/MAP, a PhD level neuropsychologist with expertise in late-life cognitive function, AD and

dementia, blinded to age, gender, race and clinical data other than education, occupation, information about sensory or motor deficits and effort, reviews the results, and summarizes impairment in each of five clinical domains (orientation, attention, memory, language and perception) as probable, possible or not present and renders an opinion regarding the presence of dementia and AD. For neuropathology, JAS is one of three board-certified neuropathologists involved in reviewing and confirming postmortem assessments. Microglia counts were obtained by trained research assistants and postdoctoral fellows – inter-rater reliability is excellent. For all protocols, neuropathological examiners were blind to all clinical data including but not limited to age, gender, education, and diagnosis at time of death. In ADNI, RBM was blinded to the clinical status of subjects who contributed plasma samples. For ROS/MAP, all available data on each phenotype of interest were extracted by a trained database manager and transferred securely to CAMH for statistical analyses. For ADNI, data were downloaded directly from the LONI IDA with institutional permission.

Results

In vivo cerebral infarcts and white matter hyperintensities (ADNI and ROS/MAP)

Table 1 summarizes demographic characteristics of the ADNI 1 and ADNI GO and 2 cohorts analyzed, according to *TSPO* rs6971 genotype.

In the ADNI 1, GO, and 2 combined Caucasian sample ($n = 1151$), rs6971 genotype was not associated with the presence of cerebral infarcts (Wald $\chi^2_1 = 1.98$, $P_{\text{raw}} = 0.16$) (Figure 1). However, there was a nominal association with infarcts in the LMCI group (Wald

$\chi^2_1 = 3.98$, $P_{\text{raw}} = 0.047$), whereby likelihood of having infarcts increased stepwise with decreasing binding affinity (O.R._{LAB:HAB} = 2.97, C.I._{95%} = (1.01, 8.70)). No associations were observed in the CN (Wald $\chi^2_1 = 2.67$, $P_{\text{raw}} = 0.10$), EMCI (Wald $\chi^2_1 = 0.01$, $P_{\text{raw}} = 0.94$), or AD group (Wald $\chi^2_1 = 1.13$, $P_{\text{raw}} = 0.29$), though there were no AD subjects who had both infarcts and the rs6971 LAB genotype, making additive model inference in this subgroup impossible.

In three independent samples (ADNI 1, ADNI GO and 2, and ROS/MAP), there were no significant associations of rs6971 genotype with WMHv (Figure 2), though in ADNI 1 ($n = 699$), rs6971 genotype showed a trend-level association with WMHv (additive $F_{1,692} = 2.87$, $P_{\text{raw}} = 0.091$). The direction of this trend in ADNI 1 was consistent with that observed in the combined sample for infarcts, with HAB subjects showing both lower risk for presence of infarcts and greater WMHv (though neither result was significant at $P < 0.05$). Also, in ADNI, GO, and 2 ($n = 631$), there was a weak association of rs6971 with WMHv in the EMCI group ($n = 242$, additive $F_{1,234} = 3.55$, $P_{\text{raw}} = 0.061$); however, the allelic pattern of this trend was in the opposite direction as in ADNI 1, whereby LAB subjects had lower WMHv. No interactions of rs6971 genotype and *APOE* $\epsilon 4$ status were found (all $P_{\text{raw}} > 0.1$).

Plasma inflammatory biomarkers (ADNI and ROS/MAP)

In the ADNI 1 subsample with plasma inflammatory biomarker data ($n = 520$), no main effect of rs6971 genotype was found for any biomarker (all $P_{\text{raw}} > 0.1$) (supplementary Figure 1). Post-hoc testing revealed effects of genotype on TNF α levels in the AD subjects ($n = 104$, additive $F_{1,99} = 10.92$, $P_{\text{raw}} = 0.0013$);

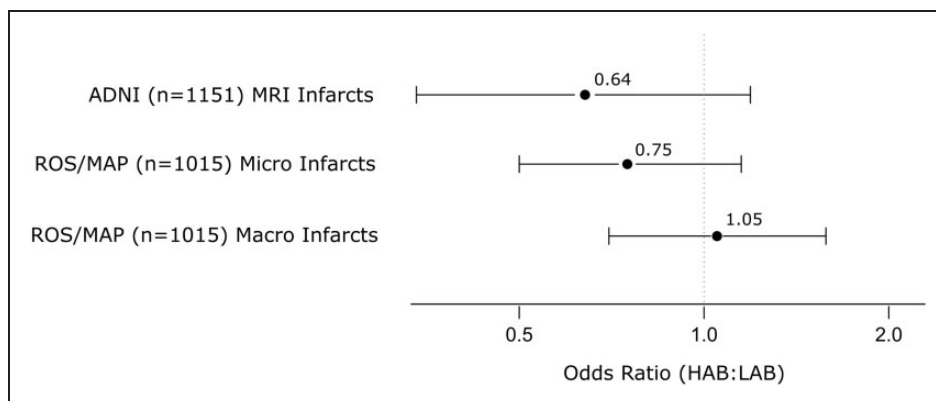


Figure 1. Effects of rs6971 on cerebral infarcts *in vivo* and postmortem.

Odds ratios (O.R.s) for rs6971 effect on presence of cerebral infarcts in the ADNI 1, GO, and 2 combined sample ($n = 1151$) and ROS/MAP ($n = 1015$) samples. O.R.s are shown for the additive model homozygote contrast (i.e. HABs vs. LABs).

ROS/MAP: Religious Orders Study/Memory and Aging Project; ADNI: Alzheimer's Disease Neuroimaging Initiative; HAB: high-affinity binding genotype; MAB: medium-affinity binding genotype; LAB: low-affinity binding genotype.

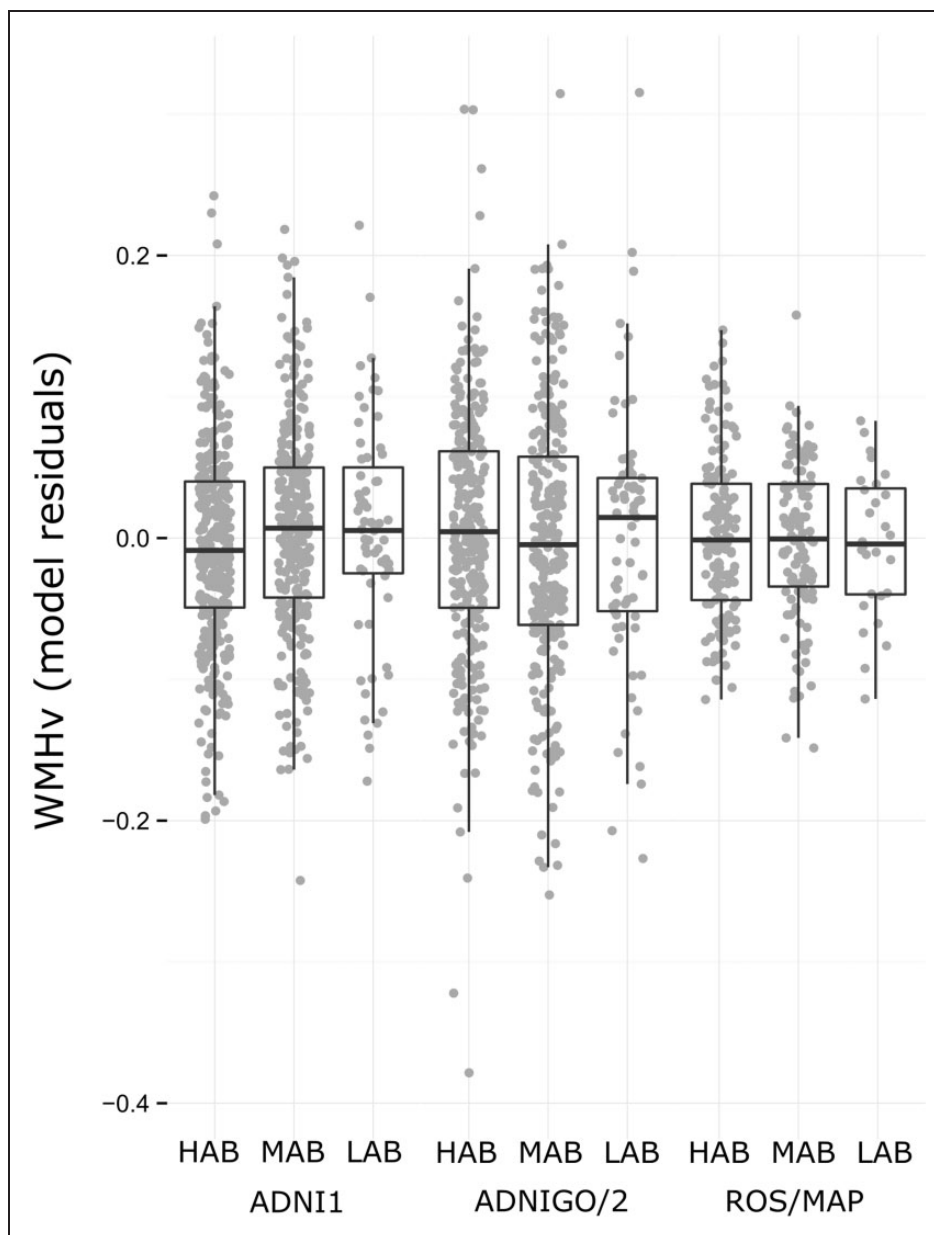


Figure 2. Effects of rs6971 on *in vivo* white matter hyperintensities. White matter hyperintensity volume (WMHv) plotted by rs6971 genotype group in ADNI 1 ($n = 699$), ADNI GO and 2 ($n = 631$), and ROS/MAP ($n = 291$) samples. WMHv is plotted as the residuals of linear models that include the following co-variables: age, sex, diastolic blood pressure (hypertension y/n for ROS/MAP), *APOE* $\epsilon 4$ status, education, and total intracranial volume (ADNI 2 only). No results show evidence to reject null association (all additive $P > 0.05$).

ROS/MAP: Religious Orders Study/Memory and Aging Project; ADNI = Alzheimer's Disease Neuroimaging Initiative; HAB: high-affinity binding genotype; MAB: medium-affinity binding genotype; LAB: low-affinity binding genotype.

however, this effect was not replicated in the ROS/MAP AD subsample ($n = 85$, additive $F_{1,80} = 0.34$, $P_{\text{raw}} = 0.24$). Further, there were no main effects of rs6971 genotype on any biomarker in the ROS/MAP sample subset or within any diagnostic group separately, and no significant interactions of rs6971 and *APOE* $\epsilon 4$ status were found in either subsample (all $P_{\text{cor}} > 0.05$) (supplementary Figure 1).

Postmortem neuropathology and microglial activation (ROS/MAP)

In the whole sample ($n = 1015$), rs6971 was not associated with macro or micro cerebral infarcts (all $P_{\text{raw}} > 0.1$, see Figure 1). No significant main effects of rs6971 genotype were found for degree of amyloid angiopathy; however, there was an uncorrected trend

toward significance (additive Wald $\chi^2_1=3.43$, $P_{\text{raw}}=0.064$), whereby HAB subjects were less likely to have greater CAA severity (O.R._{HAB:LAB}=0.72, C.I._{95%}=(0.51,1.02)) (supplementary Figure 2). This trend was seen only in the CN group (additive Wald $\chi^2_1=3.34$, $P_{\text{raw}}=0.068$); raw association P -values in the MCI and AD groups were 0.51 and 0.28, respectively. Finally, we found no associations of rs6971 with either the density of active microglia at any stage of activation nor the ratio of highly active microglia to relatively inactive microglia, in any region tested (all $P_{\text{raw}} > 0.05$) (supplementary Figure 3). Interaction analyses revealed no interactions of rs6971 and *APOE* $\epsilon 4$ status on any post-mortem pathological measure (all $P_{\text{raw}} > 0.1$).

Discussion

Our study is the first to analyze variation in *TSPO* (rs6971) with respect to structural neuroimaging and plasma biomarkers related to neuroinflammation. To our knowledge, we are also the first to analyze the effect of rs6971 on microglial activation and post-mortem neuropathology. We found no significant effects of *TSPO* genotype with respect to postmortem or *in vivo* cerebral infarcts (Figure 1). While we found a marginally protective effect ($0.05 < P < 0.1$) of genotype on *in vivo* WMH burden (ADNI 1), this finding did not replicate in either of two independent samples (ADNI GO and 2, ROS/MAP) (Figure 2). Further, we found diagnosis-dependent effects of *TSPO* genotype on plasma levels of TNF α (ADNI 1) that did not replicate in an independent sample (ROS/MAP). After correction for multiple testing, we found no significant association of *TSPO* genotype with either degree of cerebral amyloid angiopathy or microglial activation in post-mortem tissue. Exploratory analyses found no interactions of *TSPO* and *APOE* genotypes.

We hypothesized that genotype-driven alterations in *TSPO* structure may influence its downstream neuroprotective action,^{43–46} thereby conferring early and enduring risk across the lifespan for atherosclerotic damage, SVD, and potentially inflammatory dysregulation. The observed trend effects of *TSPO* genotype on vascular phenotypes (as well as amyloid angiopathy) were most prominent in the CN subgroups of both samples; this suggests that the effects of *TSPO* variation may be dependent on the time course of illness, with the greatest genotype differences observed in pre-symptomatic or early stage AD subjects. Interestingly, the HAB subjects appeared to be both marginally protected from micro infarcts as well as from increased CAA severity in the ROS/MAP sample, though neither result achieved statistical significance at $P < 0.05$. While association between micro infarcts and severe CAA has been previously shown,⁷¹

it is possible that the level of neuropathology across MCI and AD groups in the ROS/MAP sample is not high enough to observe a similarly significant pattern of *TSPO* effects on these measures; ROS subjects are priests, nuns, and brothers with well-documented healthy lifestyles and greater than average lifespan.⁷²

In our ADNI 1 analysis of inflammatory biomarkers, we found a diagnosis-dependent effect of *TSPO* genotype on TNF α , whereby HABs had higher levels if they were diagnosed with AD. TNF α is generally regarded as a “traditional” pro-inflammatory cytokine, but recent evidence suggests that TNF α may have regulatory roles in a diverse set of processes within the central nervous system including cell viability, synaptic plasticity, and learning and memory.^{73,74} This finding, however, was not replicated in ROS/MAP, suggesting that the association is likely null. This null association is further supported by our negative observations for the other four biomarkers tested. Despite evidence for the cross-talk of peripheral and central immune systems,⁷⁵ it is possible that blood-based inflammatory biomarkers were unable to capture brain-specific molecular effects of *TSPO* variation.

In line with our primarily null findings, the evolving literature on *TSPO* function is somewhat equivocal, and genome-wide association studies of AD and AD-related phenotypes have not demonstrated significant associations with *TSPO* variants. It is possible that the relationship between *TSPO* genotype and AD is modulated by additional factors that have not yet been identified. Our main effect results align with a very recent investigation of *TSPO* genotype effects on *in vivo* amyloid and cognition in the ADNI cohort,⁷⁶ which found no differences between HAB, MAB, and LAB subjects across or within diagnostic subgroups. At the molecular level, the role of *TSPO* in steroidogenesis has recently been called into question⁷⁷ following two studies: one showing that testicular production of testosterone was unaltered in a conditional *TSPO* knockout mouse,⁷⁸ and the other showing that the global knockout mouse was viable⁷⁹ (contrary to previous reports⁸⁰). These studies, however, do not preclude the possibility that, when present, different forms of *TSPO* may function in potentially divergent ways. To test this directly in the context of immune system activation, we examined the effect of genotype on microglial activation in human postmortem brain tissue, finding no genotypic group differences. Importantly, these results suggest that the mechanism via which *TSPO* genotype may influence downstream pathology and disease risk does not involve the modulation of microglial activation.

There are several limitations to this study. First, while efforts were made to ensure genotype groups

were matched for socio-demographic factors, potential confounding effects of unaccounted-for subclinical pathology or other environmental factors cannot be ruled out. Second, population stratification due to ethnic diversity is a concern in any genetic analysis and must be considered as a potential confounder. The observed minor allele frequencies (MAFs) for *TSPO* rs6971 in the ROS/MAP and ADNI samples were 0.32 and 0.31, respectively, consistent with observations in other Caucasian populations (HapMap-CEU MAF = 0.29 (http://hapmap.ncbi.nlm.nih.gov/cgi-perl/snp_details_phase3?name=rs6971&source=hapmap28_B36&tmpl=snp_details_phase3)). Third, we recognize that the literature shows discordance between patterns of inflammatory biomarkers measured in serum vs. CSF,⁸¹ and thus our plasma biomarker results may not apply to brain-specific inflammation. Fourth, M1- and M2-like microglial phenotypes^{82,83} have been shown to have distinct and often opposing roles in disease.^{84,85} Given that we did not quantify the relative densities of M1- and M2-like microglia, it is possible that a genotype effect on either specific cellular population may have been missed. Fifth, AD is being increasingly recognized as a pathologically heterogeneous disorder,⁸⁶ and great variability exists even at the level of our well-defined endophenotypes (i.e. location and type of infarcts and hyperintensities); therefore, heterogeneity between subjects and within diagnostic groups likely contributed to our negative findings. Finally, study design differences between ADNI and ROS/MAP should be acknowledged when considering our findings. While inter-study variability in image processing pipelines (for WMHv) and plasma protein quantification methods could potentially drive differences between sample results, the fact that we found no significant effects of genotype in either sample (other than for plasma TNF α in ADNI AD subjects only) should alleviate concerns regarding false negatives.

In conclusion, our findings do not support genetic variation in *TSPO* at rs6971 as a cerebrovascular and inflammatory risk factor related to neurodegeneration. In particular, the observed null effects of rs6971 on levels of microglial density across brain regions suggest that *TSPO* structural changes due to genotype likely do not alter interactions with endogenous ligands in a manner that influences microglial activation. While we provide the first insight into the potential effects of rs6971 variation on structural imaging and postmortem brain pathology in humans, continued *in vitro* and animal model experiments of differential *TSPO* binding would be required to verify the pathway-level impact of this variation with respect to AD etiology.

Authors' note

Data used in preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.usc.edu). As such, the investigators within the ADNI contributed to the design and implementation of ADNI and/or provided data but did not participate in analysis or writing of this report. A complete listing of ADNI investigators can be found online (http://adni.loni.usc.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf).

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Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. ANV had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Authors' contribution

Dr. Felsky reports no relevant disclosures and was responsible for analytical study design, data compilation and organization, final data analyses, and manuscript writing and editing.

Dr. De Jager reports no relevant disclosures and was responsible for study design of the genetic component of ROS/MAP and manuscript editing.

Dr. Schneider reports no relevant disclosures and was responsible for neuropathological data collection and quality control for the ROS/MAP dataset, data processing, and manuscript editing.

Dr. Arfanakis reports no relevant disclosures and was responsible for data collection of the ROS/MAP imaging sub-study, data processing, and manuscript editing.

Dr. Fleischman reports no relevant disclosures and was responsible for ROS/MAP study design and data collection, including imaging and inflammatory biomarker sub-studies, and manuscript editing.

Dr. Arvanitakis reports no relevant disclosures and was responsible for study design of ROS/MAP inflammatory biomarker sub-study and manuscript editing.

Dr. Honer reports no relevant disclosures and was responsible for study design in the MAP sample and manuscript editing.

Ms. Pouget reports no relevant disclosures and was responsible for collaborative input into overall study design and manuscript editing.

Dr. Mizrahi reports no relevant disclosures and was responsible for collaborative input into overall study design and manuscript editing.

Dr. Pollock reports no relevant disclosures and was responsible for collaborative input into overall study design, facilitating data transfer, and manuscript editing.

Dr. Kennedy reports no relevant disclosures and was responsible for collaborative input into overall study design and manuscript editing.

Dr. Bennett reports no relevant disclosures and was responsible for overall ROS/MAP study design, clinical data collection, facilitation of data transfer, and manuscript editing.

Dr. Voineskos reports no relevant disclosures and was responsible for assisting in the development of the overall study design, facilitating collaboration, assisting in data analysis and interpretation, and manuscript editing.

Supplementary material

Supplementary material for this paper can be found at <http://jcbfm.sagepub.com/content/by/supplemental-data>

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