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CSF protein changes associated with hippocampal sclerosis risk gene variants highlight impact of *GRN*/PGRN

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ABSTRACT

Objective: Hippocampal sclerosis of aging (HS-Aging) is a common cause of dementia in older adults. We tested the variability in cerebrospinal fluid (CSF) proteins associated with previously identified HS-Aging risk single nucleotide polymorphisms (SNPs).
Methods: Alzheimer's Disease Neuroimaging Initiative cohort (ADNI; n = 237) data, combining both multiplexed proteomics CSF and genotype data, were used to assess the association between CSF analytes and risk SNPs in four genes (SNPs): *GRN* (rs5848), *TMEM106B* (rs1990622), *ABCC9* (rs704180), and *KCNMB2* (rs9637454). For controls, non-HS-Aging SNPs in *APOE* (rs429358/rs7412) and *MAPT* (rs8070723) were also analyzed against Aβ1-42 and total tau CSF analytes.
Results: The *GRN* risk SNP (rs5848) status correlated with variation in CSF proteins, with the risk allele (T) associated with increased levels of AXL Receptor Tyrosine Kinase (AXL), TNF-Related Apoptosis-Inducing Ligand Receptor 3 (TRAIL-R3), Vascular Cell Adhesion Molecule-1 (VCAM-1) and clusterin (CLU) (all $p < 0.05$ after Bonferroni correction). The TRAIL-R3 correlation was significant in meta-analysis with an additional dataset ($p = 5.05 \times 10^{-5}$). Further, the rs5848 SNP status was associated with increased CSF tau protein – a marker of neurodegeneration ($p = 0.015$). These data are remarkable since this *GRN* SNP has been found to be a risk factor for multiple types of dementia-related brain pathologies.

1. Introduction

Studies of CSF analytes may provide biomarkers for dementia subtyping and also may provide clues about brain disease pathogenesis. These biomarker studies are all the more important as there are clearly many diseases in addition to Alzheimer's disease (AD) that underlie the clinical syndrome of dementia. Presently, individual AD "mimics" are challenging in any given patient to rule in or out. Clinical studies using neuroimaging and CSF analyses have identified a subset of individuals with evidence of neurodegeneration but lacking features of AD-type amyloidogenesis according to neuroimaging or biofluid studies. These cases have been termed "SNAP" (suspected non-amyloid pathology) and this biomarker profile has been observed in approximately 1/4th of cognitively impaired individuals (Jack et al., 2016)

Hippocampal sclerosis of aging (HS-Aging) is among the most common of the AD mimics (Nelson et al., 2013; Zarow et al., 2012), and prior studies emphasize the public health impact of this high-morbidity SNAP-type brain condition. HS-Aging is diagnosed at autopsy when neuron loss and astrocytosis are observed in the hippocampal formation, out of proportion to AD-type plaques and tangles (Amador-Ortiz et al., 2007a; Montine et al., 2012; Nelson et al., 2013). Unlike other diseases that share the diagnostic label of "hippocampal sclerosis", HS-Aging is distinguished clinically by the advanced age of the individuals afflicted, and by the usual lack of either seizure disorder or frontotemporal dementia symptoms clinically (Amador-Ortiz et al., 2007b; Lee et al., 2008; Nelson et al., 2011; Wilson et al., 2013). Further, HS-Aging has a pathological biomarker: TDP-43 pathology

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[1] 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 at: http://adni.loni.usc.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf.

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(Amador-Ortiz et al., 2007b; Neumann et al., 2006). HS-Aging affects up to 25% of the "oldest-old" (Leverenz et al., 2002; Nelson et al., 2011; Nelson et al., 2013; Zarow et al., 2012) and is associated with substantial disease-specific cognitive impairment (Brenowitz et al., 2014, Nelson et al., 2010). Even at state-of-the-art research institutions, HS-Aging tends to be misdiagnosed as AD clinically because of overlapping symptoms (Brenowitz et al., 2014, Nelson et al., 2011, Pao et al., 2011).

Genetic risk factors for HS-Aging have recently been characterized, comprising four specific gene variants that are the focus of the present study. The genes that harbor these risk-associated variants are: *GRN*, *TMEM106B*, *ABCC9*, and *KCNMB2*. The goal of the present study was to test the hypothesis that the specific gene variants associated with HS-Aging pathology also are associated with variation in the biochemical composition of CSF.

In terms of the specific risk alleles, gene-focused studies found that SNPs were associated with HS-Aging that previously had been linked to frontotemporal lobar degeneration with TDP-43 inclusions (FTLD-TDP), namely rs5848 (*GRN*) and rs1990622 (near *TMEM106B*) (Dickson et al., 2010, Murray et al., 2014, Rutherford et al., 2012, Van Langenhove et al., 2012). The *GRN* SNP was subsequently linked to other dementia-inducing disorders (Chang et al., 2013; Galimberti et al., 2012; Kamalainen et al., 2013; Pickering-Brown et al., 2008; Rademakers et al., 2008). Genome-wide association studies (GWAS) using large datasets have implicated two genes that encode potassium channel regulators — *ABCC9* (rs704180) and *KCNMB2* (rs9637454) – in HS-Aging pathology (Beecham et al., 2014; Nelson et al., 2014). Collectively these prior studies indicate that non-AD genes may have a strong impact on elderly individuals' brain structure and function, but much remains to be learned about these genes' roles in health and disease states. In contrast to AD, *APOE* gene variants are not associated with altered risk for HS-Aging (Brenowitz et al., 2014, Leverenz et al., 2002, Nelson et al., 2011, Pao et al., 2011, Troncoso et al., 1996), indicating that HS-Aging is a separate disease entity from AD.

The goal of the present study was to test the hypothesis that variability in CSF analytes is associated with HS-Aging risk alleles in a population of older adults, many of whom are cognitively impaired. This study analyzed data from the Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort and the Knight Alzheimer's Disease Research Center (ADRC) at Washington University Cohort. The CSF came from lumbar punctures in patients spanning the clinical spectrum from normal to demented subjects (see Ayton et al., 2015, Kang et al., 2015), and the average age of the research subjects when the samples were obtained was approximately 75 years. Our data provides support for the hypothesis that the *GRN* gene variant rs5848 is associated with neuroinflammatory brain changes in older adults.

2. Materials and methods

2.1. Subjects

Data used in the preparation of this article were obtained from the ADNI database (adni.loni.ucla.edu). The ADNI was launched in 2003 as a public-private partnership (Principal Investigator Michael W. Weiner). The original ADNI study aimed to recruit 800 adults, ages 55 to 90, to participate in the research to test whether serial magnetic resonance imaging (MRI), positron emission tomography (PET), other biological markers, and clinical and neuropsychological assessment can be combined to measure the progression of mild cognitive impairment (MCI) and early AD. Participants at each site provided informed written consent, and protocols were approved by the respective Institutional Review Boards (Petersen et al., 2010). We retrieved data from the ADNI database in September 2015. CSF data for the present study resulted from the ADNI Biomarkers Consortium Project "Use of Targeted Multiplex Proteomic Strategies to Identify Novel CSF Biomarkers in AD" which includes quantitative data on 83 separate CSF analytes from 310 individuals after quality control (QC) as detailed in the ADNI CSF protocol (the pre-QC data included 159 analytes from a MyriadRBM multiplex assay as discussed later). We also downloaded biomarker, genetic, demographic and diagnosis data and required that each participant have at most one genotype missing, resulting in a total of 237 participants' data being analyzed in the present study. This is detailed in the Genotypes and Imputation section and Supplemental Fig. 1.

2.2. CSF measurements

Collection and processing of ADNI CSF samples was described in detail elsewhere (Jagust et al., 2009; Shaw et al., 2011) (adni.loni.ucla.edu). The Myriad RBM (Mattsson et al., 2014) panel of 159 CSF analytes was processed on 337 samples, including 1 from a participant without diagnosis and 16 replicated samples used for test/retest QC. Analytes were filtered for sufficient dose, precision and reproducibility and were then log-transformed to better approximate a normal distribution. This resulted in a total of 83 post-QC CSF analytes from the MyriadRBM assay. Total tau and Aβ1-42 were assayed separately (Jagust et al., 2009). In cases where repeated lumbar punctures were performed, only the first measurement was used in the present study. Descriptive measures of these analytes across the 237 individuals with sufficient genotype data are provided in Supplementary Table 1.

2.3. Genotyping and imputation

The most recently generated GWAS genotyping data was acquired from the ADNI database September 2015. These data underwent extensive QC as part of the AD Big Data DREAM Challenge (Allen et al., 2016) (https://www.synapse.org/#! Synapse:syn2290704/wiki/60828). Briefly, samples from Illumina Human610-Quad BeadChip and Illumina HumanOmniExpress BeadChip arrays were mapped to hg19, converted to the positive strand and filtered for minor allele frequency (removed MAF < 0.05), SNP call rate (<0.98), sample call rate (<0.98), Hardy-Weinberg equilibrium ($p < 0.001$) and relatedness. SNPs and samples passing QC were then pre-phased using SHAPEITv2(O'Connell et al., 2014) and imputed to 1000 Genomes Phase 1 reference panel using default QC parameters (IMPUTE2 (Howie et al., 2012)).

To screen for potential ethnic outliers and protect against any potential subsequent spurious association, we LD pruned the GWAS data and examined principal component (PC) plots with 1000 Genomes data from the 5 "super populations" (African-AFR, Admixed American-AMR, East Asian-EAS, European-EUR and South Asian-SAS; Supplemental Fig. 4). No outliers were discovered, and PCs were retained for adjustment in regression models.

2.4. Statistical analysis

In order to discern correlations between HS-Aging genetic variants and the quantitative levels of CSF measurements, we performed linear regression analysis of each of the 83 post-QC log-transformed CSF analytes separately on each of the four HS-Aging SNPs. This was

Table 1
Sample demographics by diagnosis. Categorical outcomes were tested using a chi-square test. Continuous outcomes were tested with one-way ANOVA. An asterisk (*) denotes significance at $\alpha = 0.05$. Double asterisks (**) denote significance at $\alpha = 5 \times 10^{-8}$. NL = normal controls; MCI = mild cognitive impairment; AD = Alzheimer's disease; LP = lumbar puncture; Aβ1-42 is the 42-residue peptide of Aβ (Naslund et al., 1994).

	NL	MCI	AD
Sample size	63	119	55
Female (%)*	46%	29%	45%
Age at LP	75.8 (4.8)	74.9 (7.1)	74.2 (7.6)
Years of education	15.9 (2.7)	16.0 (3.0)	15.0 (3.1)
APOE-ε4 carrier (%)**	22%	55%	75%
Aβ1-42 (pg/mL)**	206.09 (56.89)	156.68 (48.94)	140.98 (34.40)
Total tau (pg/mL)**	69.5 (24.79)	104.37 (47.63)	125.25 (62.47)

Table 2
Genotype/diplotype counts by SNP. *APOE* = apolipoprotein E; *ABCC9* = ATP binding cassette subfamily C member 9; *TMEM106B* = *transmembrane protein 106B*; *GRN* = granulin; *KCNMB2* = potassium calcium-activated channel subfamily M regulatory beta subunit 2; *MAPT* = microtubule associated protein Tau. *APOE* genotypes are comprised of ε2, ε3, and ε4 alleles; for example, the first row (22) gives the count (0) of individuals with two ε2 alleles (ε2/ε2). *MAPT* diplotypes are defined by extended haplotypes, H1 and H2, using the SNP rs8070723. * = *APOE*.

SNP	Chromosome	Position (bp)	Gene	Genotype/diplotype	Count	Minor allele frequency
rs7412/rs429358	19	44,908,822/44,908,684	*APOE*			0.044
				22	0	
				23	18	
				24	3	
				33	98	
				34	89	
				44	29	
rs704180	12	21,994,111	*ABCC9*			0.485
				AA	57	
				AG	114	
				GG	64	
rs1990622	7	12,283,787	*TMEM106B*			0.416
				CC	83	
				CG	111	
				GG	43	
rs5848	17	42,430,244	*GRN*			0.327
				CC	99	
				CT	98	
				TT	23	
rs9637454	3	178,257,562	*KCNMB2*			0.278
				GG	118	
				AG	96	
				AA	16	
rs8070723	17	44,081,064	*MAPT*			0.192
				H1/H1	150	
				H1/H2	79	
				H2/H2	8	

conducted both without adjustment (i.e., marginally) and also correcting for gender, diagnosis, age at lumbar puncture, years of education and the first three PCs. The analysis was done with and without covariate adjustment as sensitivity analysis for potential confounding. In additional to these primary analyses, the positive controls of *APOE*-ε4 with Aβ1-42 and *MAPT* haplotypes with total tau were also analyzed similarly. Although a log transformation had already been made in the primary CSF data, any remaining violations of the normal distributional assumption (per a Shapiro-Wilks test for non-normality) were adjusted with a Box-Cox transformation.

Since 83 individual CSF analytes were tested for association with each of the four HS-Aging risk SNPs, we used a Bonferroni correction and considered any result significant when $p < 1.5 \times 10^{-4}$ ($= 0.05 / (83 * 4)$). We underscore that this is a conservative approach, especially

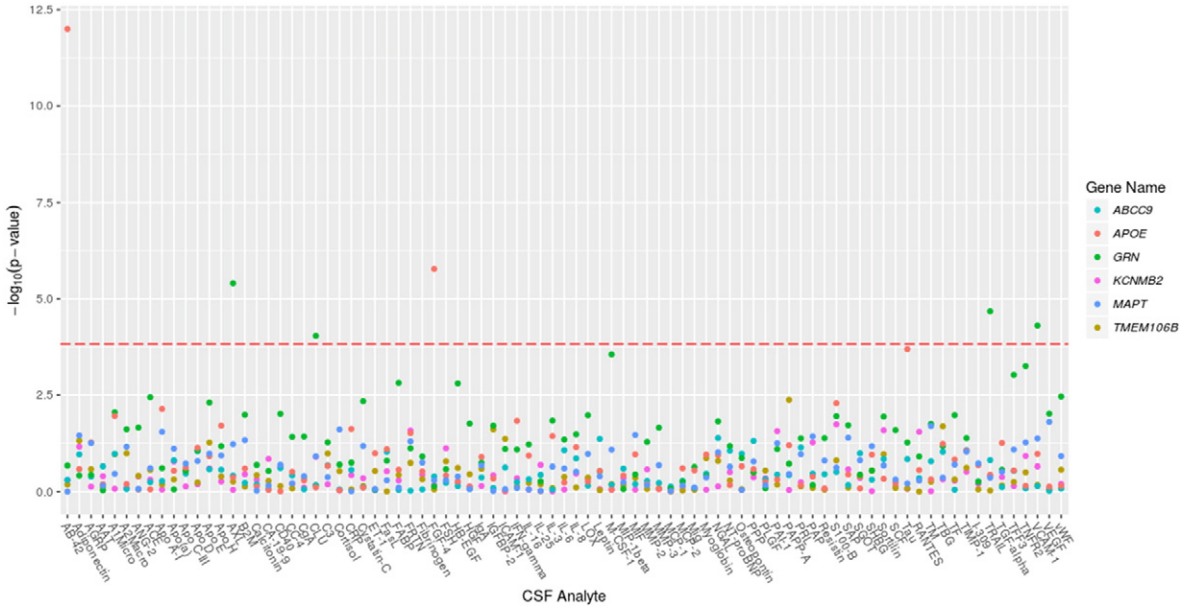

Fig. 1. Manhattan plot of all CSF analytes for each SNP. Linear regression results from each pair of log-transformed CSF analyte and SNP adjusted for gender, diagnosis, years of education, age at lumbar puncture and the first 3 principal components. Positive control variables are included with those from the primary analysis. These genes (SNPs) were tested: *ABCC9* (rs704180), *APOE* (rs429358/rs7412), *GRN* (rs5848), *KCNMB2* (rs9637454), *MAPT* (rs8070723), and *TMEM106B* (rs1990622).

Table 3
Significant CSF/SNP correlations. All significant results after Bonferroni adjustment were with the *GRN* SNP (rs5848_T). The parameter estimates (beta and std. err.) reflect the log-transformations of CSF analytes and can be exponentiated for a fold-change interpretation after adjustment for gender, diagnosis, years of education, age at lumbar puncture and the first 3 principal components. AXL = AXL receptor tyrosine kinase; TRAIL-R3 = TNF-related apoptosis-inducing ligand receptor 3; VCAM-1 = vascular cell adhesion molecule-1; CLU = clusterin.

CSF analyte	Beta est.	Std. err.	Nominal *p*-value	Bonferroni-corrected *p*-value
AXL	0.704	0.149	3.95E−06	0.001
TRAIL-R3	0.064	0.015	2.11E−05	0.007
VCAM-1	0.052	0.013	4.96E−05	0.016
CLU	0.062	0.016	9.19E−05	0.031

since the Bonferroni correction explicitly assumes uncorrelated tests, although many of the analytes are fairly highly correlated (Supplemental Fig. 2).

In addition to the primary analyses, additional analyses were performed after noting that the *GRN* SNP was significantly associated with several CSF analytes from the Myriad RBM panel: we investigated the relationship of this SNP with Aβ1-42 and total tau in the same regression framework.

2.5. Replication analysis

A post-hoc analysis was performed that included analogous CSF and genotype data from 286 subjects in the Knight ADRC at Washington University. All study-wide significant CSF/SNP relationships were meta-analyzed with Knight-ADRC data using the same statistical model and a inverse-variance weighted estimator to combine results.

3. Results

Subject demographics by diagnosis for the final data set are presented in Table 1. Age of individuals at the time of CSF draw was approximately the same, 75 years, whether the patients were cognitive normal, MCI, or demented ($p = 0.44$). Genotype SNP counts are presented in Table 2.

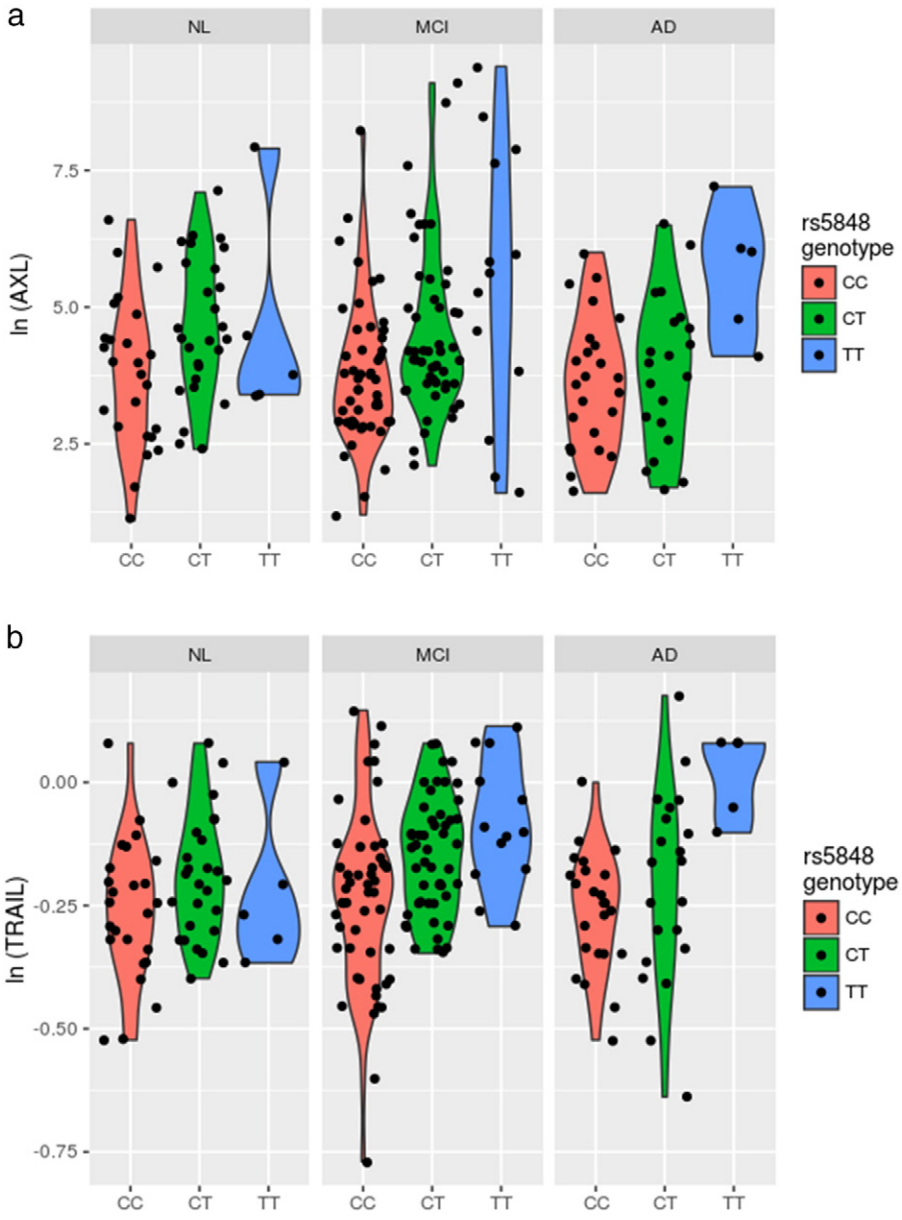

Fig. 2. a. Violin plot of top hit CSF/SNP combination stratified by diagnosis as determined by statistical significance. b. Violin plot of second hit by significance.

3.1. Significant associations between CSF analytes and HS-aging SNPs

The Manhattan-type plot (Fig. 1) displays log-transformed *p*-values for each CSF/SNP combination from regressing CSF on the SNPs' status with adjustment for other covariates (gender, diagnosis, age at lumbar puncture, years of education, and the first 3 principal components). Marginal results, i.e., without covariate adjustment, were similar and are not shown. The most significant association observed was for the positive control *APOE*-ε4 with Aβ1-42 ($p = 3.5 \times 10^{-12}$). The only HS-Aging gene to reach statistical significance with any CSF analyte after multiple testing adjustment was *GRN* (rs5848_T), which revealed four study-wide significant correlations. Table 3 shows these results along with the corresponding parameter estimates from the adjusted model. For example, an additional copy of the rs5848 T allele confers an estimated >2-fold increase ($2.02 = e^{0.704}$) of AXL after adjustment for gender, diagnosis, age at lumbar puncture and the first three principal components. The distributions of AXL measures by rs5848 genotype and diagnosis are shown in Fig. 2A, displaying the pattern of increased protein for each T allele. This is also shown for TRAIL-R3 (Fig. 2B), which is the only analyte that was study-wide significant after meta-analysis with the Knight ADRC data ($p = 5.05 \times 10^{-5}$). No HS-Aging gene other than *GRN* reached study-wide statistical significance with the conservative Bonferroni adjustment (incorporating the study design of 83 analytes × 4 SNPs assessed = 332 hypothesis tests). Supplementary Table 2 displays regression results for the top 4 associations for each of the other HS-Aging genes using nominal (uncorrected) *p* values.

Since the *GRN* rs5848_T allele was associated with CSF analytes from the RBM panel, we examined it in relation to tau and Aβ1-42, two canonical AD biomarkers, specifically to determine if the allele was associated with tau for a given level of Aβ1-42. In a model regressing tau level on the other covariates including Aβ1-42, the *GRN* risk allele showed

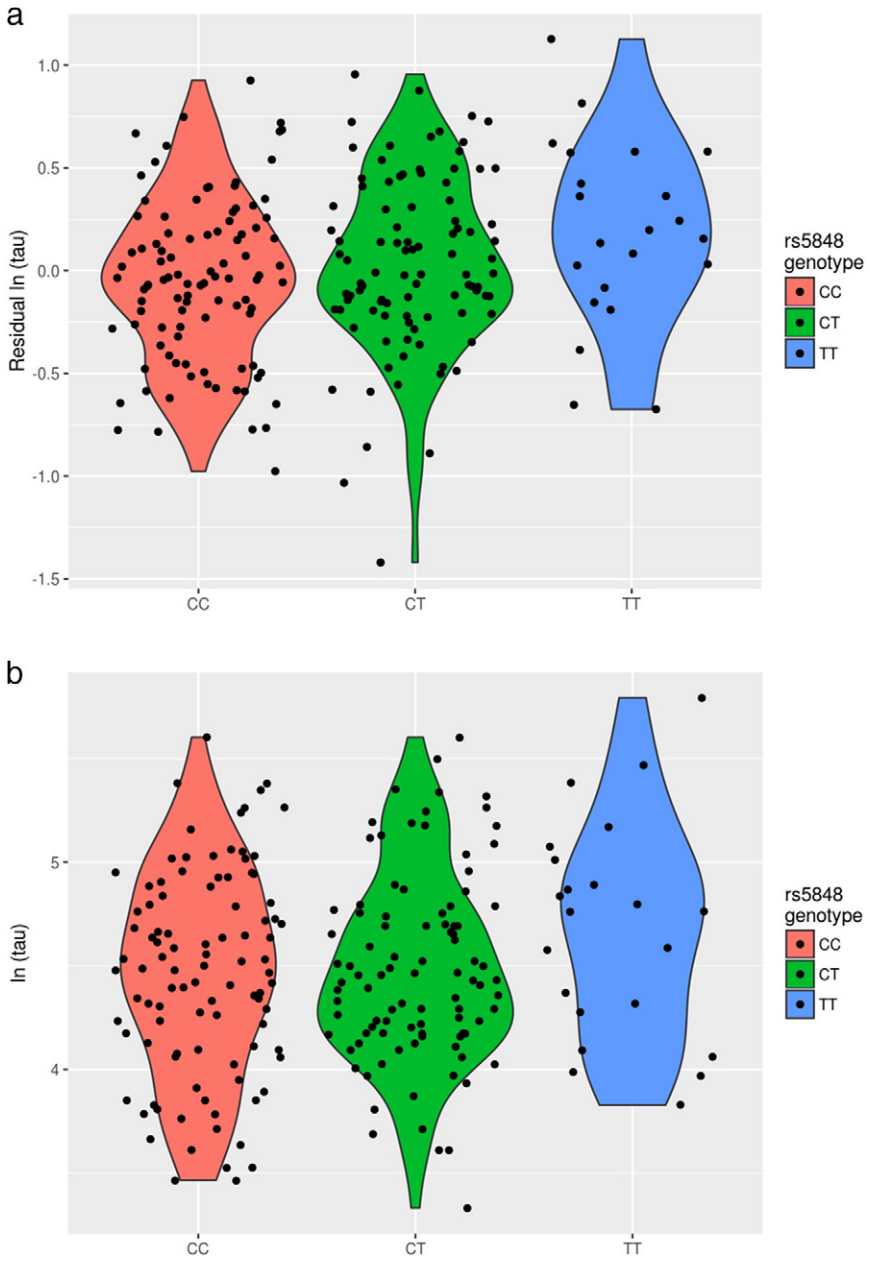

Fig. 3. a. Residuals of log-transformed tau vs. *GRN* genotype. Residuals from a model regressing log-transformed tau on Aβ1-42, gender, diagnosis, years of education, age at lumbar puncture and the first 3 principal components. b. Unadjusted log-transformed tau vs. *GRN* genotype.

association with tau levels ($p = 0.015$). Both biomarkers were log-transformed in the model due to skewness. Interestingly, without the adjustment for Aβ1-42, the SNP is no longer nominally significantly associated with total tau levels ($p = 0.054$). We show the residuals from a regression of log(tau) on Aβ1-42 and the adjustment covariates (Fig. 3A) and the unadjusted log(tau) values (Fig. 3B) against *GRN* genotype.

4. Discussion

We here report that the *GRN* risk SNP (rs5848) was associated with variation in detected levels of CSF proteins previously implicated in CNS inflammation in the ADNI data set (Aktas et al., 2007). The same *GRN* risk allele was also associated with increased CSF tau which may indicate directly related neurodegenerative changes. We found no direct evidence that other putative HS-Aging risk variants are associated with variation in CSF proteins in these samples.

An important caveat in interpreting our results is the limited sample size (n = 237 patients included) given the number of variables (83 analytes) being assessed. This sample size confers 80% power to detect an additive CSF analyte effect of 1.21 standard deviations with a nominal 5% level of significance, when adjusting for the $4 * 83 = 332$ hypothesis tests. Among the additional sources of variability is the fact that the research subjects span a broad spectrum of clinical states from "normal" to "dementia", which in itself probably introduces substantial variability related to patient activity, medication, diet, and other factors. There may be additional sources of variation due to possible preanalytical variables since the samples were collected from dozens of different clinical locations (see Materials And methods). Moreover, the non-Aβ (i.e., non-AD) pathogenetic elements in large autopsy series include α-synucleinopathy and primary age-related tauopathy (PART) which add to the phenotypic complexity (see for example Neltner et al. (2016)). These factors argue for caution in interpreting our data and heighten the likelihood of false-negative results; the study design is best tailored for high effect-size phenomena. Further, the CSF samples were obtained from relatively young individuals considering the age range of vulnerability to HS-Aging(Nelson et al., 2013). Another caveat relates to the basic characteristic of the ADNI cohort which is enriched for persons with AD risk per se. Multiple studies have found that HS-Aging tends to misdiagnosed as AD in the clinical setting (Brenowitz et al., 2014, Pao et al., 2011). However, we also note that the ADNI data set has been used productively by many other researchers to test hypotheses related to potential dementia biomarkers.

As far as we know this is the first study of CSF analytes in relation to HS-Aging genetic risk factors. The first gene variant to be linked to HS-Aging pathology, rs5848 (Rademakers et al., 2008) is physically located in the 3′ untranslated region of the *GRN* mRNA. The risk allele is associated with decreased plasma expression of *GRN*/PGRN (Dickson et al., 2010, Fenoglio et al., 2009).

Our results can be interpreted from different perspectives, reflecting how much is currently unknown. Whereas multiple *GRN* mutations cause FTLD-TDP (184–188), rs5848 is apparently a disease-modifying allele that alters the manifestation of multiple different diseases rather than affecting FTLD or HS-Aging specifically. For example, rs5848 has been linked to AD, Parkinson's disease, C9ORF72 neurodegeneration, and bipolar disorder (Chang et al., 2013, Galimberti et al., 2012, Kamalainen et al., 2013, Pickering-Brown et al., 2008, Rademakers et al., 2008, van Blitterswijk et al., 2014). Moreover, the SNAP profile – biomarker indication of a neurodegenerative process despite lack of Aβ-type amyloidogenesis – is linked to multiple different brain conditions. Hypothetically, one could have "neurodegeneration" in the CNS without tau protein in the CSF, especially in a disease like HS-Aging where the cell loss may occur without substantial tauopathy. The present study for the first time ties rs5848 related brain changes with increased CSF analytes in addition to CSF tau, indicating that neurodegeneration linked to TDP-43 pathology (the most specific pathological marker of HS-Aging) leads to increased CSF tau that can be detected in a complex background. A prior study reported that rs5848 status and PGRN levels in CSF were linked to CSF tau variance (Morenas-Rodriguez et al., 2015), whereas another study found that *GRN* mutant FTLD cases lacked increased tau in CSF (Carecchio et al., 2011).

Also interesting is the subset of CSF analytes that were found to be altered in association with rs5848 genotypes; these include *AXL*, *TRAIL*-R3, *VCAM*-1 and *CLU*. Each of these gene products has been the subject of extensive research, and collectively the prior studies appear to point towards a common theme that also is relevant to *GRN* itself. *GRN* has been implicated in microglial function and neuroinflammation (Cenik et al., 2012; Jian et al., 2013), and each of the above mentioned protein products also can be linked to neuroinflammatory pathways. For example, *CLU* (clusterin, also known as apolipoprotein J) has been described to play a role in microglial activation and Aβ uptake (Mulder et al., 2014; Xie et al., 2005). Since *GRN* is a risk factor for FTLD-TDP, with extensive TDP-43 pathology, as well as HS-Aging, which seems to be a different disease, it is possible that the GRN/PGRN protein and neuroinflammation play a contributory role in TDP-43 pathology per se. However, more work is required to test this hypothesis. Whether the analytes themselves are pathogenetic agents is another important question that remains to be answered.

We conclude that among subjects with CSF analyte and genotype data available in the ADNI cohort, the HS-Aging risk gene variants are mostly not found to be associated with CSF protein changes. However, the *GRN* risk SNP rs5848 shows some analyte variation that indicate high effect sizes, perhaps linked to neuroinflammatory phenotype. We now know that dementia has many causes and multiple pathologic comorbidities often are simultaneously expressed in elderly individuals. Future studies may elucidate other links between non-AD risk alleles and biomarkers to enable better diagnoses and to thus strengthen our ability to develop and test future therapeutic strategies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.exger.2017.01.025.

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