Interaction between variants in *CLU* and *MS4A4E* modulates Alzheimer's disease risk

Mark T.W. Ebbert^a, Kevin L. Boehme^a, Mark E. Wadsworth^a, Lyndsay A. Staley^a, for the Alzheimer's Disease Neuroimaging Initiative*, Alzheimer's Disease Genetics Consortium, Shubhabrata Mukherjee^b, Paul K. Crane^b, Perry G. Ridge^a, John S.K. Kauwe^{a,*}

^aDepartment of Biology, Brigham Young University, Provo, Utah 84602, USA; ^bDepartment of Medicine, University of Washington, Seattle, Washington 98105, USA

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Corresponding Author:

John S. K. Kauwe
4102 LSB
Brigham Young University
Provo, UT 84602
Phone: 801-422-2993
email: kauwe@byu.edu

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Abstract

INTRODUCTION: Ebbert et al. reported gene-gene interactions between rs11136000-rs670139 (*CLU-MS4A4E*) and rs3865444-rs670139 (*CD33-MS4A4E*). We evaluate these interactions in the largest dataset for an epistasis study.

METHODS: We tested interactions using 3837 cases and 4145 controls from ADGC using meta- and permutation analyses. We repeated meta-analyses stratified by *APOEε4* status, estimated combined OR and population attributable fraction (cPAF), and explored causal variants.

RESULTS: Results support the *CLU-MS4A4E* interaction and a dominant effect. An association between *CLU-MS4A4E* and *APOEε4* negative status exists. The estimated synergy factor, OR, and cPAF for rs11136000-rs670139 are 2.23, 2.45 and 8.0, respectively. We identified potential causal variants.

DISCUSSION: We replicated the *CLU-MS4A4E* interaction in a large case-control series, with *APOEε4* and possible dominant effect. The *CLU-MS4A4E* OR is higher than any Alzheimer's disease locus except *APOEε4*, *APP*, and *TREM2*. We estimated an 8% decrease in Alzheimer's disease incidence without *CLU-MS4A4E* risk alleles and identified potential causal variants.

1. Introduction

Alzheimer's disease (AD) is a complex neurodegenerative disease, and is the third leading cause of death in the United States [1]. AD is characterized by the accumulation of amyloid plaques and neurofibrillary tangles in the brain. Many genetic loci exist that modify AD risk, but collectively, they explain only a fraction of AD's heritability [2] and are not diagnostically useful [3,4]. Rare variants with large effects and epistatic interactions may account for much of the unexplained AD heritability, but are largely unknown due to limitations in traditional GWAS studies. While rare variant and epistatic effects on AD are poorly understood, recent studies suggest that gene-gene interactions play a critical role in AD etiology and progression [3,5–7].

A previous study [3] reported evidence of two gene-gene interactions that increase AD risk. Specifically, Ebbert et al. reported interactions between rs11136000 C/C (*CLU*; minor allele = T, MAF = 0.38) and rs670139 G/G (*MS4A4E*; minor allele = T, MAF = 0.38) genotypes (synergy factor (SF) = 3.81; $p = .016$), and the rs3865444 C/C (*CD33*; minor allele = A, MAF = 0.21) and rs670139 G/G (*MS4A4E*) genotypes (SF = 5.31; $p = .003$). All three variants have been implicated in numerous AD GWAS studies [8–13] and are on the “AlzGene Top Results” list [14], which summarizes the most established genes associated with AD.

MS4A4E and *CLU* were recently replicated in a large meta-analysis of 74046 individuals, but *CD33* did not replicate [15]. Despite *CD33* failing to replicate, several studies demonstrated that *CD33* is involved in AD-related pathways and pathology, giving convincing evidence that *CD33* is somehow involved in AD. Three specific studies demonstrated that *CD33* alters monocyte function, amyloid uptake, and that *CD33* expression is associated with clinical dementia ratings [16–18]. rs3865444 is located in the 5'UTR of *CD33*.

The association between *CLU* and AD status has been strongly established by both genetic and biological data. Recent studies demonstrated that rs11136000—an intronic SNP within *CLU*—is associated with AD-related pathology in healthy individuals including neural inefficiency [19] and decreased white matter integrity [20].

MS4A4E is a member of the membrane-spanning 4-domains subfamily A, but little else is known about the gene. However, rs670139—located in the *MS4A4E* 3'UTR according to gene model XM_011545416.1—is consistently associated with AD [15,18,21].

In this study, we attempted to replicate these gene-gene interactions using the largest dataset used in an epistasis study, to date [22]. We performed an independent meta-analysis of datasets from the Alzheimer's Disease Genetics Consortium (ADGC) using 3837 cases and 4145 controls, followed by a combined meta-analysis that included the original Cache County results [3] with an additional 326 cases and 2093 controls. We also tested for dosage or dominant effects and an *APOEε4* effect. Finally, we explored possible causal variants using whole-genome sequence data from the Alzheimer's Disease Neuroimaging Initiative (ADNI).

2. Methods

2.1. Data description

We used SNP data from the ADGC, which consists of 32 studies collected over two phases and includes 16000 cases and 17000 controls. All subjects are self-reported as being of European American ancestry. More information about this dataset can be found in Naj et al. [8] and the ADGC data preparation description [23].

Genotype data from 2419 individuals from the Cache County Study on Memory Health and Aging were also used in this study. The full cohort of 5092 individuals represented approximately 90% of the Cache County population aged 65 and older when the study began in 1994 [24]. The Cache County data consists exclusively of individuals of European American ancestry. Exactly 2673 individuals were excluded from the original Cache County analysis because of incomplete genotype or clinical data [3]. Additional information on this dataset can be found in previous reports [3,24].

Whole-genome data from 747 (223 controls, 195 cases, 329 MCI) individuals were used in this article and were obtained from the ADNI database (adni.loni.usc.edu). ADNI is a large collaboration from several academic and private institutions, and subjects have been recruited from over 50 sites across the U.S. and Canada. Currently, over 1500 adults (ages 55 to

90) participate, consisting of cognitively normal older individuals, people with early or late MCI, and people with early stage AD. For up-to-date information, see www.adni-info.org.

2.2. SNP data preparation and statistical analysis

As gene-gene interactions are challenging to identify and replicate, we used the highest quality data possible. For each ADGC dataset, we filtered SNPs imputed with low information ($\text{info} < 0.5$) and converted the IMPUTE2/SNPTEST format files to PLINK format, using PLINK v1.90b2i [25,26]. We used the default PLINK uncertainty cutoff of 0.1, meaning any imputed call with uncertainty greater than 0.1 was treated as missing. We included SNPs with a missing genotype rate less than 0.05 and individuals with a missing rate less than 0.01. We then extracted the SNPs of interest: rs3865444 (*CD33*), rs670139 (*MS4A4E*), and rs11136000 (*CLU*) and tested Hardy-Weinberg equilibrium [27,28]. Using R v3.1.1 [29], we excluded samples without complete data for all covariates including age, gender, case-control status, *APOEε4* dose, and the two SNPs being tested in the corresponding interaction. Entire datasets missing the respective SNPs or covariates after data cleaning were excluded from further analysis. The requirement of complete data for both SNPs and all covariates is necessary for this analysis. Unfortunately, this requirement led to the exclusion of 23 and 24 entire datasets for the *CD33-MS4A4E* and *CLU-MS4A4E* interactions, respectively. We also excluded the ADC1 dataset because it contained only one AD case, likely making it biased.

Following data preparation, we tested the individual interactions in each dataset using logistic regression. We defined the R models as “case_control ~ rs3865444 + rs670139 + rs3865444:rs670139 + apoe4dose + age + sex” and “case_control ~ rs11136000 + rs670139 + rs11136000:rs670139 + apoe4dose + age + sex” for the *CD33-MS4A4E* and *CLU-MS4A4E* interactions, respectively. Case-control status, SNPs, and sex were coded as factors, age was numeric, and apoe4dose was an ordered factor from 0-2.

Using results from each study, we performed a meta-analysis to test replication across the ADGC datasets using METAL (version 2011-03-25) [30], and performed a second meta-analysis including the original Cache County results to provide synergy factor and odds ratio estimates from the largest number of samples possible. We tested the originally reported interactions and heterozygous interactions (rs11136000 C/C—rs670139 G/T and rs3865444 C/C—rs670139 G/T) to test for potential dosage or dominant effects based on suggestive evidence found in the original Cache County study

(Supplemental Table 1). We assessed whether there is a dosage or dominant effect based both on whether the heterozygous interaction is significant and a t-test comparing two means. Specifically, we tested for a significant difference between the homozygous and heterozygous effect sizes. A significant difference would suggest a dosage effect, whereas an insignificant difference would suggest the effect might be dominant.

Following the meta-analyses, we performed a permutation analysis with 10000 permutations for interactions that replicated independently. For each ADGC dataset, we randomly permuted case-control status across all individuals, tested the interaction, and reran the meta-analysis. We stored the p-values from each of the 10000 meta-analyses and calculated the empirical p-value by finding the original p-value's rank in the distribution of p-values divided by the number of permutations. We also calculated the combined population attributable fraction (cPAF) as previously described [3,8].

Results are represented using both odds ratios and synergy factors [6,31] and their associated 95% confidence intervals and p-values. Synergy factors represent the ratio between the *observed* and *expected* odds ratios for the two interacting SNPs (Equation 1). The *expected* odds ratio for the interaction assumes there is no synergy between the SNPs (i.e., the SNPs are independent) and equals the product of the individual odds ratios (the denominator of Equation 1) [6,31]. Essentially, the synergy factor measures how strongly the *observed* and *expected* odds ratio relationship deviates from linearity, as the synergy factor deviates from one. A synergy factor equal to one suggests no synergy; rather, there is no evidence of statistical epistasis.

Because synergy factors less than 1 can be challenging to interpret, we present interaction synergy factors in the direction greater than 1. Consequently, we performed all interaction analyses using each gene's homozygous minor allele as the reference group, which is opposite the direction standardly used in genome-wide association studies. This also has the added advantage that the interaction's odds ratio is presented in the risk direction for easy comparison to top AD risk loci. To calculate the interaction's odds ratio, we used each SNPs individual odds ratio as previously reported in a larger dataset [21], *but we had to invert the individual odds ratios to be the same direction as our analyses*. We then calculated the interaction's *observed* odds ratio (Equation 1) using the inverted odds ratios. We also estimated each synergy factor's 95% confidence interval using rmeta [32].

Based on results from the interaction replication, we performed a synergy factor analysis using the Cortina-Borja synergy factor Calculator [31] to test for an *APOEε4* effect for the *CLU-MS4A4E* interaction. Specifically, we stratified the combined ADGC and Cache County data by *APOEε4* status and tested for an association between the interaction and case-control status within each stratum. Alleles rs11136000 C and rs670139 G were used as the exposed groups.

2.3 Exploring causal variants

As a follow up analysis, we explored causal variants for replicated interactions using 747 (223 controls, 195 cases, 329 MCI) ADNI whole genomes that were sequenced, aligned to hg19, and variants identified by Illumina using their internal analysis procedure. We used linkage disequilibrium, Regulome DB (accessed November 2014) [33], and functional annotations from wAnnovar [34] to isolate SNPs of interest. We first extracted all SNPs within approximately 50 kilobases of each SNP of interest, calculated linkage disequilibrium using Haploview [35], and retained all SNPs with a $D' \geq 0.99$. Using Regulome DB and wAnnovar, we annotated each remaining SNP for: (1) known regulation and functional effects; (2) minor allele frequencies from the 1000 Genomes Project [36], 6500 Exomes Project [37], and the ADNI dataset; and (3) corresponding MutationTaster predictions [38]. We retained all nonsynonymous SNPs, SNPs located in untranslated regions (UTRs), and SNPs with a Regulome DB score less than 4. For each retained SNP, we tested individual associations with case-control status in the 223 controls and 195 cases using the VarStats tool in the Variant Tool Chest (VTC) [39] and subsequently tested their interaction with all SNPs in the other interacting gene using logistic regressions in R.

3. Results

3.1 Sample and dataset demographics

Sample demographics and minor allele frequencies for rs11136000, rs670139, and rs3865444 are presented for each dataset (Table 1). Eight of the 32 datasets with 3837 cases and 4145 controls passed quality controls for the *CD33-MS4A4E* interaction while seven datasets with 3140 cases and 2713 controls passed for *CLU-MS4A4E*. The remaining

datasets were either missing required SNP(s), missing a covariate, or consisted of only controls and could not be included in the analysis. All SNPs passed Hardy-Weinberg equilibrium in all remaining datasets for both cases and controls.

3.2 Homozygous and heterozygous interaction meta-analysis results

The heterozygous interaction between the rs11136000 C/C (*CLU*) and rs670139 G/T (*MS4A4E*) genotypes did not replicate in the independent analysis, though it is suggestive (SF = 1.58, $p = 0.07$, Figure 1a; Supplemental Table 1). Although the heterozygous interaction did not replicate independently in ADGC, the combined meta-analysis including Cache County is significant (SF = 1.90, $p = 0.01$, Figure 1a; Supplemental Table 1). The originally reported homozygous *CLU-MS4A4E* interaction between the rs11136000 C/C (*CLU*) and rs670139 G/G (*MS4A4E*) genotypes replicates in the independent meta-analysis (SF = 1.79, $p = 0.008$, Figure 1b; Supplemental Table 1). The combined meta-analysis is also significant (SF = 2.23, $p = 0.0004$, Figure 1b; Supplemental Table 1). The individual SNP odds ratios, as previously reported for rs11136000 and rs670139 [21], are 0.83 and 1.09, respectively. The inverted individual SNP odds ratios for rs11136000 and rs670139, are 1.20 and 0.92, respectively. The expected odds ratio for the interaction is $1.20 * 0.92 = 1.10$, thus the observed odds ratio is $2.23 * 1.10 = 2.45$. Empirical p-values obtained from permutations support the main interaction (ADGC: $p = 0.035$ with Cache: $p = 0.002$) and the cPAF for *CLU-MS4A4E* is 8.0. Comparing means to determine whether there is a dosage or dominant effect between the heterozygous and homozygous interactions was not significant ($p = 0.22$).

We found an association between the *CLU-MS4A4E* interaction and case-control status in *APOEε4* negative subjects in the combined ADGC and Cache County data (SF = 2.08, $p = 0.004$, Figure 2a; Supplemental Table 2) that did not exist with *APOEε4* positive subjects (SF = 1.19, $p = 0.26$, Figure 2b; Supplemental Table 2). The *CD33-MS4A4E* interaction failed to replicate in either the independent or combined meta-analyses (Figures 3a and 3b, Supplemental Table 1).

3.3 Exploring causal variants

We explored causal variants in the *CLU* and *MS4A4E* regions using the ADNI whole-genome data. There were 36 and 32 SNPs that fit the inclusion criteria previously described for SNPs near rs11136000 and rs670139, respectively (Supplemental Tables 3 and 4). Most of the SNPs are rare (MAF < 0.01) according to the 1000 Genomes, 6500 Exomes, and ADNI datasets. None of the SNPs were significantly associated with case-control status individually or in the

pairwise interactions. We identified two SNPs in *MS4A4E* (rs2081547 and rs11230180) that have a Regulome DB score of ‘1f’ and have been shown to modify *MS4A4A* expression [40], the gene upstream from *MS4A4E*. A score of ‘1f’ means they are known to modify expression and are known DNase and transcription factor binding sites.

4. Discussion

In this study we attempted to replicate two gene-gene interactions and their association with AD case-control status in the largest dataset used in an epistasis study, to date. The *CD33-MS4A4E* interaction failed to replicate and may have resulted from over-fitting in the Cache County data as previously described by Ebbert et al. [3] Over-fitting happens when a model identifies random data patterns as significant when they are not truly relevant to the question at hand. While there is substantial evidence that CD33 function is related to AD pathways and pathology [16–18], our data do not support an interaction with *MS4A4E* that impacts AD risk.

We replicated the *CLU-MS4A4E* interaction, demonstrated an association in *APOEε4* negative subjects, and reported evidence of a possible dominant effect for *MS4A4E*. The homozygous interaction between rs11136000 (*CLU*) and rs670139 (*MS4A4E*) replicates independently in the ADGC datasets, supporting its validity. To provide synergy factor and odds ratio estimates from the largest number of samples possible, we report the combined meta-analysis synergy factor and odds ratio including the Cache County data. Given the broad sampling and large sample size used for this analysis, our results are likely to be generalizable to other populations of European ancestry. Further investigating this interaction in other ethnic groups is warranted.

Comparing the *CLU-MS4A4E* odds ratio of 2.45 to top AD risk alleles according to AlzGene.org [14] along with *APP*, *PLD3*, and *TREM2* adds greater perspective. Momentarily ignoring *APOEε4*, *APP*, *PLD3*, and *TREM2*, the highest individual odds ratio is from *APOEε2* (OR = 1.61) [3,14] when inverting to its respective risk allele, followed by *ABCA7* (OR = 1.23) [3,14], both of which are dramatically lower than 2.45. Of known AD risk loci, only *APOEε4* (OR = 3.68), *APP* (OR = 5.29), and *TREM2* (OR = 5.05) have ORs greater than 2.45 [3,14,41,42]. The *CLU-MS4A4E* odds ratio is even greater than the *PLD3* Val232Met mutation (OR = 2.10) [43]. These results suggest the *CLU-MS4A4E* interaction may play an important role in AD etiology.

A distinction must be made regarding statistical and biological epistasis, however [22]. While there is evidence that *CLU*, like *CD33*, interacts indirectly with *MS4A2* [3], little is known about *MS4A4E* itself and we do not know whether it biologically interacts with *CLU*. *MS4A2* indirectly modifies *BCL2L1* activation or expression [3], which physically interacts with *CLU*. Research suggests *CLU* prevents amyloid fibrils and other protein aggregation events [44] while *MS4A4E* may facilitate aggregation as a membrane-spanning protein. Membrane-spanning proteins play diverse roles in cell activity including transport and signaling. Experiments will be required to determine whether there is biological epistasis between *CLU* and *MS4A4E*, and whether the interaction affects amyloid fibril formation. Our results indicate further investigative efforts in gene-gene interactions (and protein-protein interactions) may be important to resolve AD etiology.

Comparing means for the effect estimates to assess whether there is a dosage or dominant effect between the *CLU-MS4A4E* heterozygous and homozygous interactions was not significant, suggesting there may be a dominant effect for the rs670139 G allele. A dominant effect has important epidemiologic and heritability implications. Since the *CLU-MS4A4E* interaction increases risk, heterozygous individuals may be at equal risk compared to homozygous individuals. We found an association between the *CLU-MS4A4E* interaction and case-control status in *APOEε4* negative subjects in the combined ADGC and Cache County data that did not exist with *APOEε4* positive subjects. This potential three-way interaction may provide valuable insight into AD risk and protective factors. A recent paper by Jun et al. [45] found *CLU* has a stronger association in *APOEε4* positive individuals while the region surrounding *MS4A4E* has a stronger association in *APOEε4* negative individuals. Further statistical and biological studies will be necessary to clarify these potential associations. Since all analyses in this study used each gene's homozygous minor allele as the reference group, the interaction between *CLU-MS4A4E* major alleles is framed as a risk factor, meaning the interaction between the minor alleles is protective. Since the tested heterozygous interaction also increases risk, the protective association may only apply to the interaction between the homozygous minor alleles.

We report several rare potential causal variants linked to rs11136000 or rs670139 with a $D' \geq 0.99$ in the ADNI whole-genome data. No individual variants were significantly associated with AD risk in the ADNI data, but the analysis was likely underpowered with only 240 controls and 202 cases. Two particularly interesting variants, rs11230180 and rs2081547, are known to affect *MS4A4A* expression. We believe further analysis of these variants is necessary to better

understand their involvement in AD. Exploring the effects of rs670139, itself, may also be important. Little is known about *MS4A4E*, including the gene's chromosomal structure. According to gene model XM_011545416.1, rs670139 is in the *MS4A4E* 3'UTR, but other gene models differ. 3'UTR variants can affect transcription and translation.

The cPAF for *CLU-MS4A4E* is 8.0, suggesting there would be an approximate 8% decrease in AD incidence across the population if both major alleles were eliminated. In reality, this estimate is for the causal variants that rs670139 and rs11136000 may be tagging, but the overall effect is nontrivial. Identifying a targeted treatment in the associated pathways could have a significant impact.

A major gap in AD literature to date is the lack of known causal variants. Several SNPs have repeatedly turned up in genome-wide association studies, but the tagSNPs themselves are unlikely to play a direct role in AD etiology. What is more likely is that the tagSNPs are in close linkage disequilibrium with one or more causal variants. We hypothesize two possible explanations: (1) the SNPs are linked to multiple rare variants that drive AD development and progression; or (2) there is another common variant in the region with functional effects that remain unknown. In either case, given the biological complexity of AD and results presented in this study, we believe epistasis plays a critical role in AD etiology. As such, the community must continue to identify and vet these and other interactions that are supported in the literature.

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35 **Table 1. Sample demographics by dataset.** For each dataset the following information is provided: percent cases,
 36 females, age, *APOEε4* positive percentage, and minor allele frequencies for rs670139, rs3865444, and rs11136000.

37 **Figure 1. Forest plot showing heterozygous (Panel A) and homozygous (Panel B) *CLU-MS4A4E* interaction**
 38 **replication with potential dominant effect.** We tested the original homozygous interaction between the rs11136000 C/C
 39 (*CLU*; minor allele = T, MAF = 0.38) and rs670139 G/G (*MS4A4E*; minor allele = T, MAF = 0.38) genotypes, which
 40 replicated in ADGC independently (synergy factor = 1.79, $p = 0.008$, Panel B). We also report the combined meta-
 41 analysis including the original Cache County results to present a synergy factor estimate from the largest number of
 42 samples possible, which is also significant (synergy factor = 2.23, $p = 4e-04$, Panel B). We also tested for a dosage or
 43 dominant effect based on suggestive evidence in the original Cache County results (Panel A) by testing the heterozygous
 44 interaction between the rs11136000 C/C (*CLU*) and rs670139 G/T (*MS4A4E*) genotypes, which did not replicate
 45 independently, but is suggestive (synergy factor = 1.58, $p = 0.07$, Panel A). The combined analysis, including the original
 46 Cache County results, is significant (synergy factor = 1.90, $p = 0.01$, Panel A).

47 **Figure 2. Forest plot showing *APOEε4* negative association with Alzheimer's disease case-control status.** We tested
 48 for an *APOEε4* association with the *CLU-MS4A4E* interaction using the Cortina-Borja Synergy Factor Calculator [31].
 49 Specifically, we stratified the combined ADGC and Cache County data by *APOEε4* status and tested for an association
 50 between the interaction and case-control status within each stratum. Alleles rs11136000 C and rs670139 G were used as
 51 the exposed groups. We found an association in the *APOEε4* negative stratum (synergy factor = 2.08, $p = 0.004$, Panel A)
 52 that did not exist in the *APOEε4* positive stratum (synergy factor = 1.19, $p = 0.26$, Panel B), suggesting an *APOEε4* effect
 53 exists for this interaction.

54 **Figure 3. Forest plot showing failed replication for heterozygous (Panel A) and homozygous (Panel B) *CD33-***
 55 ***MS4A4E* interaction.** We tested the original homozygous interaction between the rs3865444 C/C (*CD33*; minor allele =
 56 A, MAF = 0.21) and rs670139 G/G (*MS4A4E*; minor allele = T, MAF = 0.38), which did not replicate in ADGC
 57 independently ($p = 0.81$, Panel B) and was not significant in the combined meta-analysis ($p = 0.28$, Panel B). We also
 58 tested the heterozygous interaction, which also was not significant (without Cache: $p = 0.64$, Panel A; with Cache: $p =$
 59 0.44, Panel A).

20 **Equation 1. The synergy factor describes the relationship between the expected odds ratio (denominator) and the**
 21 **observed odds ratio (numerator) for interacting variants.** The expected odds ratio (denominator) assumes that both
 22 variants are independent (i.e., there is no synergistic, or non-linear effect on the phenotype) while the observed odds ratio
 23 (numerator) is the actual effect. A synergy factor that deviates from 1 indicates a statistical interaction between the
 24 variants that affects the phenotype.

$$SF = \frac{OR_{12}}{OR_1 \times OR_2}$$

A)

B)

A)

B)

A) **B)**

Study	N	Cases (%)	Females (%)	Age	APOE 4 + (%)	rs670139 MAF (T)	rs3865444 MAF (A)	rs11136000 MAF (T)
ACT1	1858	487 (26.2)	1068 (57.5)	82.28	526 (28.3)	0.41	0.33	NA
ADC2	681	566 (83.1)	365 (53.6)	79.38	394 (57.9)	0.42	0.33	0.39
ADNI	371	230 (62.0)	157 (42.3)	77.82	201 (54.2)	0.45	0.31	0.37
LOAD	2965	1515 (51.1)	1882 (63.5)	78.22	1667 (56.2)	0.43	0.31	0.38
TARC1	388	244 (62.9)	244 (62.9)	78.96	189 (48.7)	0.43	0.32	0.41
UMVUMSSM_A	1058	450 (42.5)	676 (63.9)	75.48	451 (42.6)	0.43	0.31	0.38
UMVUMSSM_B	390	135 (34.6)	236 (60.5)	73.99	118 (30.3)	0.41	0.33	0.38
UMVUMSSM_C	271	210 (77.5)	160 (59.0)	74.77	167 (61.6)	0.42	0.29	NA