

# Estrogen Receptor Genes, Cognitive Decline, and Alzheimer Disease

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## Abstract

### Background and Objectives

Lifetime risk of Alzheimer disease (AD) dementia is twofold higher in women compared with men, and low estrogen levels in postmenopause have been suggested as a possible contributor. We examined 3 *ER* (*GPER1*, *ER2*, and *ER1*) variants in association with AD traits as an indirect method to test the association between estrogen and AD in women. Although the study focus was on women, in a comparison, we separately examined *ER* molecular variants in men.

### Methods

Participants were followed for an average of 10 years in one of the 2 longitudinal clinical pathologic studies of aging. Global cognition was assessed using a composite score derived from 19 neuropsychological tests' scores. Postmortem pathologic assessment included examination of 3 AD (amyloid- $\beta$  and tau tangles determined by immunohistochemistry, and a global AD pathology score derived from diffuse and neurotic plaques and neurofibrillary tangle count) and 8 non-AD pathology indices. *ER* molecular genomic variants included genotyping and examining *ER* DNA methylation and RNA expression in brain regions including the dorsolateral prefrontal cortex (DLPFC) that are major players in cognition and often have AD pathology.

### Results

The mean age of women ( $N = 1711$ ) at baseline was 78.0 ( $SD = 7.7$ ) years. In women, *GPER1* molecular variants had the most consistent associations with AD traits. *GPER1* DNA methylation was associated with cognitive decline, tau tangle density, and global AD pathology score. *GPER1* RNA expression in DLPFC was related to cognitive decline and tau tangle density. Other associations included associations of *ER2* and *ER1* sequence variants and DNA methylation with cognition. RNA expressions in DLPFC of genes involved in signaling mechanisms of activated ERs were also associated with cognitive decline and tau tangle density in women. In men ( $N = 651$ , average age at baseline: 77.4 [ $SD = 7.3$ ]), there were less robust associations between *ER* molecular genomic variants and AD cognitive and pathologic traits. No consistent association was seen between *ER* molecular genomic variations and non-AD pathologies in either of the sexes.

### Discussion

*ER* DNA methylation and RNA expression, and to some extent *ER* polymorphisms, were associated with AD cognitive and pathologic traits in women, and to a lesser extent in men.

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## Glossary

**ACC** = anterior cingulate cortex; **AD** = Alzheimer disease; **A $\beta$**  = amyloid- $\beta$ ; **DLPFC** = dorsolateral prefrontal cortex; **ER** = estrogen receptor; **FDR** = false discovery rate; **GPER1** = G protein-coupled estrogen receptor; **IRB** = Institutional Review Board; **JNK** = c-Jun N-terminal kinase; **MAP** = Memory and Aging Project; **MCI** = mild cognitive impairment; **PCC** = posterior cingulate cortex; **ROS** = Religious Orders Study.

Women comprise more than 60% of Americans with Alzheimer dementia,<sup>1</sup> and lifetime risk of Alzheimer dementia is 2-fold higher in women compared with men.<sup>2</sup> As older women lose their main source of estrogen after menopause, low estrogen levels<sup>3</sup> have been proposed a possible contributor for vulnerability of older women for Alzheimer dementia.<sup>4</sup> Previous studies suggested that older age at menopause and longer reproductive age were associated with higher levels of cognition in older women.<sup>5,6</sup> Low bioavailable levels of estradiol in older women were associated with a higher risk of cognitive impairment.<sup>7</sup> However, results of randomized clinical trials that examined postmenopausal hormone therapy for prevention of cognitive decline have found no benefits.<sup>8,9</sup> These inconsistent findings could stem from different reasons including locally generated estrogen that affects brain function<sup>10</sup> above and beyond circulating estrogen. Therefore, other avenues are needed to explore to clarify whether estrogen is related to cognition in older women.

Estrogen has 1 transmembrane receptor, G protein-coupled estrogen receptor (GPER1), and 2 steroid receptors, estrogen receptor  $\alpha$  (ER1) and  $\beta$  (ER2), that are the main receptors mediating estrogen activity. Because of the possible association between estrogen and cognition,<sup>5,7</sup> several cross-sectional<sup>11,12</sup> and few longitudinal studies<sup>13-15</sup> have examined ER1 and ER2 polymorphisms with cognitive impairment or cognitive decline. In these studies, only a few selected polymorphisms were examined. In this study, the overarching research question was to examine associations between ER molecular genomic variants with cognition in older women, and we extended previous studies in 4 areas. First, we examined common single-nucleotide sequence variants in and flanking ER1 and ER2. Second, we also examined GPER1 whose sequence variants were associated with hormone-sensitive diseases<sup>16,17</sup> but were not examined in relation to cognition. Third, we examined ER sequence variants with Alzheimer disease (AD) and related dementia pathologic indices. Fourth, we examined DNA methylation and RNA expression of the ER genes with cognitive decline, and with the pathologic indices. Although the study focus was on women, in a comparison, we separately examined ER molecular variants in men.

## Methods

Participants were enrolled in one of the 2 longitudinal studies of community-dwelling older adults: The Religious Orders Study (ROS) and the Rush Memory and Aging Project (MAP). One of the authors, David A Bennett, is the principle

investigator of both studies. ROS began enrollment in 1994 and enrolls nuns, priests, and brothers across the United States. MAP began enrollment in 1997 and enrolls older adults living in personal accommodations or retirement centers across Northeastern Illinois. Eligible participants were older adults without known dementia at enrollment who consented to annual cognitive and clinical assessment and for brain donation at the time of death. Implementing the same protocols and performing the cognitive and clinical assessments by the same trained personnel facilitated joint analysis of ROS and MAP whose details are described elsewhere.<sup>18</sup>

We included 1,711 women without dementia at baseline, with available genotype data, and with at least 2 cognitive assessments. Of the 1,711 women, 917 died and had available postmortem pathologic examinations. Postmortem ER methylation data were available in 420 and ER expression levels in 738. The corresponding numbers of men were 651 with genotype data, 222 with methylation data, and 323 with RNA expression data. Flowcharts of the analytic samples are illustrated in eFigures 1–2, [links.lww.com/WNL/C615](https://links.lww.com/WNL/C615). Venn diagrams illustrate sample sizes across different data sets (eFigures 3–4).

### Genotype Processing

DNA was extracted from peripheral blood or frozen brain tissue. Genotyping was performed on the Affymetrix Genome-Wide Human SNP Array6.0 or the Illumina Omni Quad Express platform. After passing quality control steps,<sup>19</sup> sequence variant dosages were imputed on the Haplotype Reference Consortium panel. In this study, we examined sequence variants located in the 3 ER genic regions and their 10-kilobase flanking areas. Sequence variants were excluded if they had minor allele frequency <1%, imputation score <0.3, or missing rate >5%. Because the study's primary objective was at the gene level, rather than the sequence variant level, we did not prune sequence variants based on their linkage disequilibrium. This yielded 141 sequence variants in GPER1, 697 in ER2, and 1,547 in ER1 regions to be examined in this study.

### DNA Methylation Processing

Frozen samples of the dorsolateral prefrontal cortex (DLPFC) were used to obtain DNA methylation data. DLPFC was selected as the brain region for molecular assessments because it is crucially involved in cognitive activities and frequently has AD pathology in older adults.

DNA was extracted from DLPFC gray mater using the Qiagen QIAamp DNA mini protocol. DNA methylation was generated using a bead assay (Infinium HumanMethylation450;

Illumina). Raw methylation data were processed further for quality control,<sup>20</sup> which resulted in distinct DNA methylation values for 420,132 cytosine-phosphate-guanine (CpG) sites. In this study, we examined CpG sites located in the 3 *ER* genic regions and their 10-kilobase flanking areas. This yielded 84 CpG sites in *GPER1*, 50 in *ER2*, and 76 in *ER1* regions to be examined in this study.

### RNA Expression Processing

DLPFC was the first brain region at which RNA extraction and processing was performed.<sup>21</sup> RNAs were extracted from frozen samples of DLPFC using Qiagen's miRNeasy mini kit and the RNase free Dnase Set. RNA samples that passed quality control criteria were sequenced on an Illumina HiSeq platform after RNA-seq library preparation using the strand-specific deoxyuridine triphosphate method with poly(A) selection. RNA-seq data were processed further keeping only highly expressed genes, which resulted in 13,484 genes.

Recently, we generated TruSeq stranded Total RNA Library libraries (Illumina, 20020599) from DLPFC, posterior cingulate cortex (PCC), and anterior cingulate cortex (ACC) using a Zephyr G3 next generation sequencing workstation (Perkin Elmer) according to the manufacturer's instructions with minor modifications.<sup>22</sup> Libraries were sequenced on an Illumina NovaSeq 6000 platform at 40–50 M reads, 2 × 150 bp paired end. Although expression levels of *GPER1* and *ER2* were available, the *ER1* expression level did not meet quality control criteria.

### Cognition Assessment

Annually, 21 neuropsychological tests (eMethods, links.lww.com/WNL/C615) were administered by research assistants who worked in both cohorts and were trained by a single trainer. Two tests were used only for diagnostic purposes, and scores of the remaining 19 tests, which were the same tests across ROS and MAP participants and across visits, were standardized using means and SDs of all participants' scores at baseline. The global cognition score is the mean of the 19 standardized neuropsychological scores. We have used these composite measures of cognition in our previous studies<sup>23,24</sup> because composite variables derived from multiple indicators are more appropriate for use in longitudinal analysis<sup>25</sup> where we need variables with less random errors including floor and ceiling effects.

At each annual visit, a neurologist and a neuropsychologist reviewed cognitive and clinical data and adjudicated the presence of mild cognitive impairment (MCI) or dementia based on established criteria.<sup>26</sup>

### Postmortem Pathologic Assessment

The median (Q1–Q3, range) of time interval between death and autopsy was 6.8 (5.1–10.2, 1–76.3) hours. Details of the procedures are described elsewhere.<sup>18,27</sup> One hemisphere was frozen for multiomics and biochemical analyses, and the next hemisphere was fixed in 4% formaldehyde solution. The fixed hemisphere was cut into 1 cm slabs, and tissue sections were

prepared from predetermined brain regions that were examined for AD and non-AD pathologies by experts blinded to clinical data.

### AD

Sections from 8 brain regions were examined by immunohistochemistry for the assessment of amyloid- $\beta$  (A $\beta$ ) and tau-labeled tangles using antibodies against A $\beta$  and phosphorylated tau.<sup>28</sup> Using image analysis, compartments labeled by A $\beta$  antibodies were quantified in each brain region as the percent area occupied by the labeled compartment, which was averaged across brain regions to yield the overall A $\beta$  load. Similarly, the overall tau tangle density of brain was calculated by averaging regional tau-labeled tangles, which were counted using stereologic mapping techniques.

A modified Bielschowsky silver stain was used to visualize neuritic plaques, diffuse plaques, and neurofibrillary tangles in 5 cortical brain regions. A global AD pathologic score was developed by making an average of the summary measures of the 3 indices. Moreover, a board-certified neuropathologist blinded to clinical data determined pathologic AD diagnosis using established criteria.<sup>29</sup>

### Non-AD Pathologies

We also examined indices of 3 neurodegenerative (Lewy bodies, transactive response DNA binding protein 43 [TDP-43], and hippocampal sclerosis) and 5 cerebrovascular disease (macroinfarcts, microinfarcts, atherosclerosis, arteriolosclerosis, and cerebral amyloid angiopathy) pathologies, which are described in the eMethods, links.lww.com/WNL/C615 and in previous studies.<sup>27</sup>

### Covariates

Sex, race, and years of education were obtained using self-report at baseline. Age at baseline and age at death were calculated using dates of birth (obtained at baseline), baseline interview, and death.

### Statistical Analysis

Characteristics of participants in different analytic data sets were compared using analysis of variance,  $\chi^2$ , and Kruskal-Wallis tests. Linear mixed-effects models were used to examine cognitive decline over years of follow-up. The core model had terms for intercept (level of cognition), time (rate of cognitive decline), age, education, and interactions of age and education with time. Time was treated differently in models examining sequence variants compared with models examining DNA methylation or RNA expression. In models with sequence variants, the time term of the core mixed-effects model was analyzed prospectively. Time was zero at study baseline and was positive thereafter over years of follow-up. By contrast, levels of DNA methylation and RNA expression were determined at death and time was zero at death and negative in years before death.

Then, in separate mixed-effects models, we examined the association of each sequence variant's dosage with the level

**Table 1** Characteristics of Study Participants

Variable	Sequence variants analyses		DLPFC RNA expression analysis		DLPFC DNA methylation analyses		p Value
	Women	Men	Women	Men	Women	Men	
Sample size	1711	651	738	323	420	222	
Age at baseline, y, mean (SD)	78.0 (7.7)	77.4 (7.3)	80.9 (6.7)	79.1 (6.9)	81.5 (6.6)	78.9 (6.9)	F = 32.5, <i>p</i> < 0.001
Education, y, mean (SD)	15.8 (3.4)	17.2 (4.0)	15.8 (3.2)	17.1 (3.9)	16.1 (3.4)	17.6 (3.6)	F = 28.5, <i>p</i> < 0.001
White non-Hispanic, n (%)	1,559 (91)	615 (94)	724 (98)	318 (98)	407 (97)	216 (97)	$\chi^2 = 68.1$ , <i>p</i> < 0.001
Mini Mental State Examination score at baseline, mean (SD)	28.3 (1.8)	28.0 (2.1)	28.1 (1.8)	27.8 (2.2)	27.9 (1.9)	27.7 (2.3)	KW $\chi^2 = 46.4$ , <i>p</i> < 0.001
Global Cognition at baseline, mean (SD)	0.08 (0.53)	0.04 (0.54)	-0.02 (0.49)	0.00 (0.51)	-0.13 (0.48)	-0.07 (0.54)	F = 13.8, <i>p</i> < 0.001
Cognition status, n (%)							$\chi^2 = 32.0$ , <i>p</i> < 0.001
No cognitive impairment	1,294 (76)	467 (72)	538 (73)	225 (70)	271 (65)	141 (64)	
Mild cognitive impairment	417 (24)	184 (28)	200 (27)	98 (30)	149 (35)	81 (36)	
Dementia	0	0	0	0	0	0	
Years of follow-up, mean (SD)	9.7 (5.3)	9.7 (5.6)	9.0 (4.6)	9.0 (4.8)	7.2 (3.5)	7.2 (3.6)	F = 25.8, <i>p</i> < 0.001
Sample size of postmortem examinations	917	422	738	323	420	222	
Age at death, y, mean (SD)	90.2 (6.5)	88.0 (6.5)	90.5 (6.5)	88.2 (6.5)	89.0 (6.6)	86.2 (6.2)	F = 25.3, <i>p</i> < 0.001
Years between last clinical evaluation and death, Mean (SD)	1.03 (1.59)	0.78 (0.95)	0.92 (1.30)	0.67 (0.75)	0.69 (0.66)	0.69 (0.80)	KW $\chi^2 = 17.6$ , <i>p</i> = 0.004
Cognition status-last visit, n (%)							$\chi^2 = 14.9$ , <i>p</i> = 0.135
No cognitive impairment	302 (33)	151 (36)	252 (34)	117 (36)	138 (33)	78 (35)	
Mild cognitive impairment	216 (24)	120 (28)	179 (24)	92 (28)	105 (25)	64 (29)	
Dementia	399 (43)	151 (36)	307 (42)	114 (35)	177 (42)	80 (36)	
Pathologic diagnosis of Alzheimer disease, n (%)	609 (66)	238 (56)	491 (67)	176 (54)	263 (63)	119 (54)	$\chi^2 = 34.1$ , <i>p</i> < 0.001
Square root of global Alzheimer disease pathology score, mean (SD)	0.79 (0.38)	0.67 (0.38)	0.79 (0.38)	0.66 (0.38)	0.77 (0.38)	0.65 (0.40)	F = 14.9, <i>p</i> < 0.001
Square root of amyloid- $\beta$ load, mean (SD)	1.62 (1.13)	1.40 (1.13)	1.67 (1.14)	1.42 (1.17)	1.48 (1.07)	1.25 (1.12)	F = 8.1, <i>p</i> < 0.001
Square root of tau tangle density, mean (SD)	1.68 (1.30)	1.26 (1.07)	1.68 (1.28)	1.19 (0.97)	1.52 (1.15)	1.16 (1.02)	F = 18.8, <i>p</i> < 0.001
Lewy bodies, n (%)	223 (24)	116 (27)	179 (24)	93 (29)	98 (23)	53 (24)	$\chi^2 = 5.0$ , <i>p</i> = 0.411
TDP-43 in hippocampus or neocortex, n (%)	308 (34)	98 (23)	240 (33)	75 (23)	122 (29)	46 (21)	$\chi^2 = 28.1$ , <i>p</i> < 0.001
Hippocampal sclerosis, n (%)	91 (10)	30 (7)	68 (9)	26 (8)	32 (8)	14 (6)	$\chi^2 = 5.8$ , <i>p</i> = 0.323
One or more macroinfarcts, n (%)	319 (35)	155 (37)	254 (34)	118 (37)	137 (33)	83 (37)	$\chi^2 = 2.6$ , <i>p</i> = 0.759
One or more microinfarcts, n (%)	271 (30)	134 (32)	208 (28)	98 (30)	104 (25)	61 (27)	$\chi^2 = 6.1$ , <i>p</i> = 0.298
Moderate to severe atherosclerosis, n (%)	291 (32)	133 (32)	233 (32)	102 (32)	186 (44)	98 (44)	$\chi^2 = 36.9$ , <i>p</i> < 0.001
Moderate to severe arteriolosclerosis, n (%)	303 (33)	107 (25)	246 (33)	85 (26)	166 (40)	74 (33)	$\chi^2 = 25.9$ , <i>p</i> < 0.001
Moderate to severe cerebral amyloid angiopathy, n (%)	325 (35)	145 (34)	263 (36)	104 (32)	143 (34)	70 (32)	$\chi^2 = 2.6$ , <i>p</i> = 0.761

Abbreviations: DLPFC = dorsolateral prefrontal cortex. *p*-values were derived from analysis of variance,  $\chi^2$ , and Kruskal-Wallis tests comparing the 6 different subsets in continuous, categorical, and rank data, respectively. Degree of freedom in all the analyses was 5.

**Table 2** Summary of Associations of the 3 Estrogen Receptor (*ER*) Genes With Longitudinal Changes of Cognition and Underlying Pathologies in Older Women

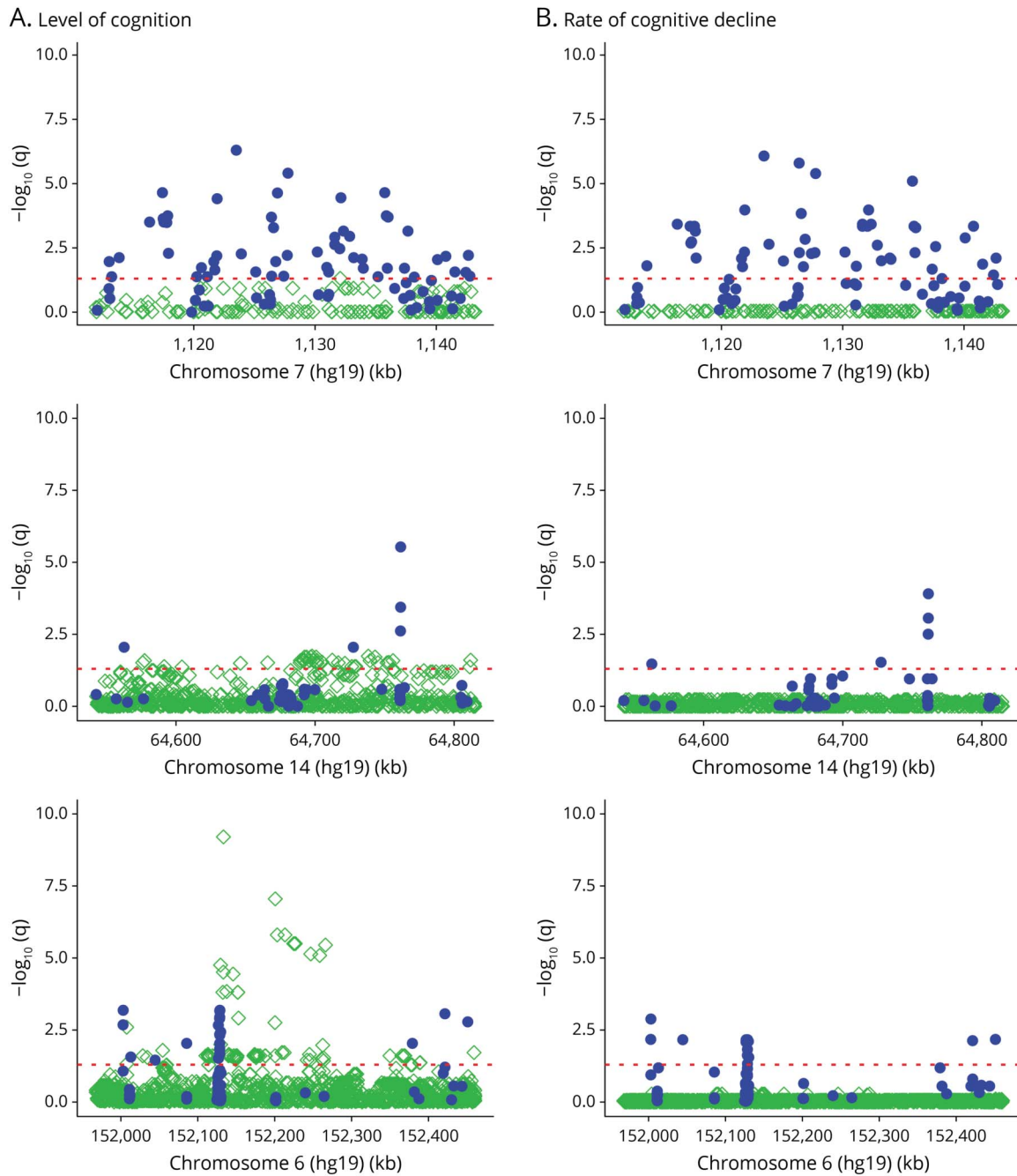
<i>ER</i>	Variant	Cognition		AD pathologic indices			Non-AD pathologies									
		Level	Rate of decline	A $\beta$	Tau	Global AD score	Lewy body	TDP-43	HS	Macroinfarct	Microinfarct	Atherosclerosis	Arteriolosclerosis	CAA		
<b><i>GPER1</i></b>	Sequence variants	0.028 <sup>a</sup>	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.038	NS	NS	NS	
	DNA methylation	<0.001 <sup>a</sup>	<0.001 <sup>a</sup>	NS	0.013 <sup>a</sup>	0.013 <sup>a</sup>	NS	NS	NS	NS	NS	NS	NS	NS	NS	
	RNA	DLP	-0.181 (0.045)	-0.014 (0.004)	0.065 (0.046)	0.140 (0.051)	0.028 (0.015)	0.139 (0.015)	NS	NS	NS	NS	NS	NS	NS	NS
		PCC	-0.201 (0.076)	-0.015 (0.007)	0.118 (0.083)	0.134 (0.096)	0.047 (0.029)	0.152 (0.029)	NS	NS	NS	NS	NS	NS	NS	NS
		ACC	-0.124 (0.102)	-0.012 (0.009)	0.069 (0.110)	-0.045 (0.112)	0.014 (0.036)	0.696 (0.036)	NS	NS	NS	NS	NS	NS	NS	NS
<b><i>ER2</i></b>	Sequence variants	0.009 <sup>a</sup>	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
	DNA methylation	<0.001 <sup>a</sup>	<0.001 <sup>a</sup>	NS	0.013 <sup>a</sup>	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
	RNA	DLP	-0.047 (0.057)	-0.005 (0.005)	0.125 (0.057)	0.041 (0.064)	0.048 (0.019)	0.035 <sup>a</sup>	NS	NS	NS	NS	NS	NS	NS	NS
		PCC	-0.166 (0.078)	-0.015 (0.007)	0.184 (0.085)	0.174 (0.099)	0.074 (0.029)	0.035 <sup>a</sup>	NS	NS	NS	NS	NS	NS	NS	NS
		ACC	0.018 (0.097)	0.005 (0.009)	0.028 (0.103)	0.024 (0.105)	0.036 (0.034)	0.358 (0.034)	NS	NS	NS	NS	NS	NS	NS	NS
<b><i>ER1</i></b>	Sequence variants	<0.001 <sup>a</sup>	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
	DNA methylation	<0.001 <sup>a</sup>	<0.001 <sup>a</sup>	NS	0.016 <sup>a</sup>	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	

Abbreviations: ACC = anterior cingulate cortex; CAA = cerebral amyloid angiopathy; DLP = dorsolateral prefrontal cortex; FDR = false discovery rate; HS = hippocampal sclerosis; NS = not significant; PCC = posterior cingulate cortex.

Cells with single values indicate q-values (FDR-corrected *p*-values) derived from omnibus tests that combine *p*-values of the associations of sequence variants or CpG sites with the outcomes. Cells with 3 values indicate estimate (SE), q-value (FDR corrected *p*-value) derived from mixed-effects models (cognition as the outcome) or linear regression models (AD pathology indices as the outcomes).

<sup>a</sup>Significant associations.

**Figure 1** Association of Polymorphisms and DNA Methylation at Estrogen Receptor Genes *ER1*, *ER2*, and *GPER1*, With the Level of Global Cognition and Rate of Cognitive Decline

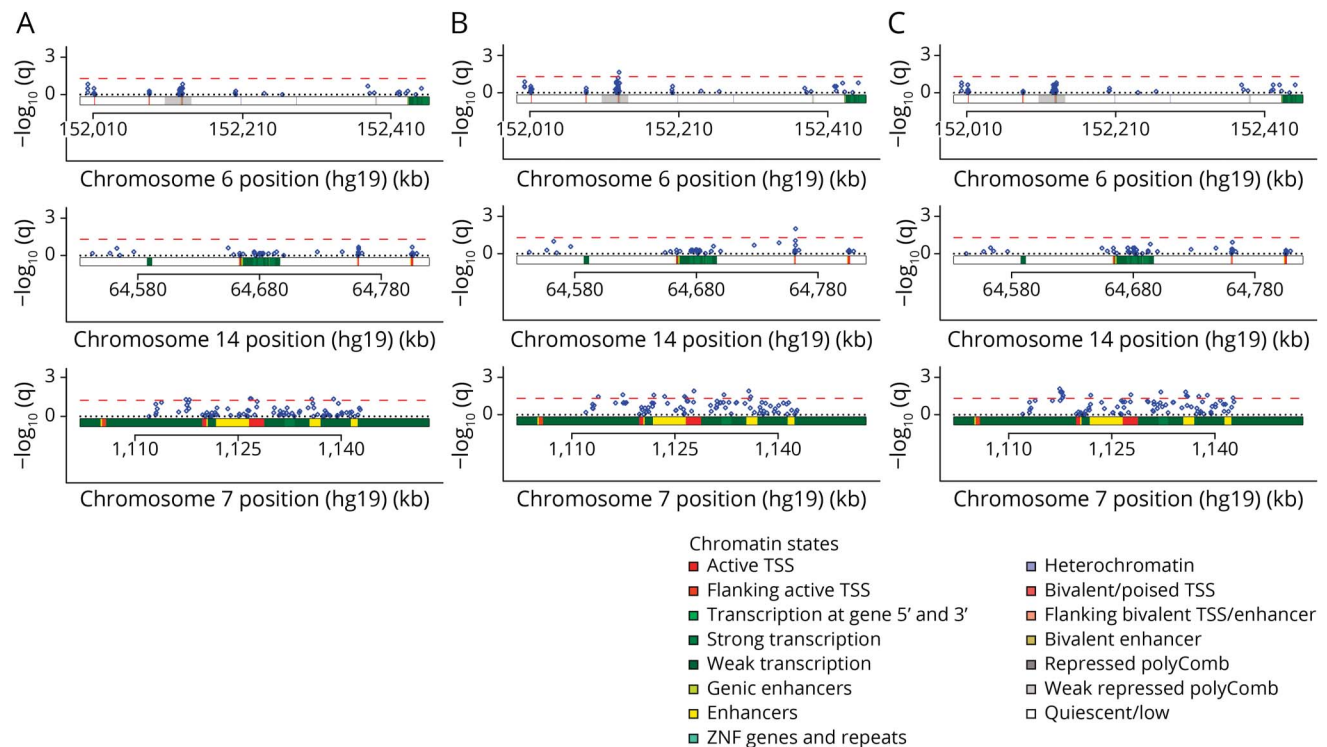


In each plot, the association of all sequence variants (green circles) and CpG sites (blue circles) in the examined genetic region with the level of global cognition (A) or rate of cognitive decline (B) is illustrated. X-axis indicates the chromosomal position of the sequence variants/CpG sites, and the Y-axis indicates the FDR-corrected  $p$ -value of the association between the SNP/CpG site and the outcome. The horizontal red dashed lines show the level above which the associations are statistically significant. *GPER1* = G protein-coupled estrogen receptor; FDR = false discovery rate.

and rate of cognitive decline by addition of terms for the sequence variant and its interaction with time, respectively.  $p$  Values of the associations of sequence variants in each *ER* genic region were combined separately for the level and rate of cognitive decline using the Fisher product method,<sup>30</sup> as described previously.<sup>31</sup> This omnibus method tests a null hypothesis that none of the examined sequence variants at an *ER* gene were associated

with the outcome (level or rate of cognitive decline). The omnibus test can handle arbitrary dependency structures and is powerful and computationally efficient for boosting power in sequencing studies.<sup>32</sup> When an omnibus test indicated the association of gene's polymorphisms with an outcome, we subsequently examined each sequence variant to identify specific sequence variants associated with the outcome. In further analyses, we replaced sequence

**Figure 2** Association of DNA Methylation at Estrogen Receptor Genes With Alzheimer Disease Pathology Indices



In each plot, the association of individual CpG sites in the examined genetic region with the level of amyloid- $\beta$  load (A), tau tangle density (B), or global AD pathology score (C) is illustrated, derived from linear regression. The Y-axis indicates the FDR-corrected  $p$ -value of the association, and the X-axis illustrates the location of the CpG site in the genome. The color bands in the X-axis represent the chromatin state of the genetic region. (A) Amyloid- $\beta$  load, (B) tau tangle density, and (C) global AD pathology score. FDR = false discovery rate.

variants with the DNA methylation level at each CpG site or with mRNA expression levels of *ER* genes.

Then, we examined associations of *ER* polymorphisms, methylation, and expression levels with AD and non-AD pathologic indices. We replaced mixed-effects models with linear regressions, when examining AD pathology indices, or logistic regressions, when examining non-AD pathologies.

Model assumptions were assessed both graphically and numerically. We applied Benjamini and Hochberg false discovery rate (FDR)<sup>33</sup> to reduce inflation of type I error due to multiple testing. For parsimony, only FDR-corrected  $p$ -values (q-values) are reported, and q-values less than 0.05 were indicators for rejection of null hypotheses.

### Standard Protocol Approvals, Registrations, and Patient Consents

All participants signed an informed consent and an Anatomical Gift Act. Rush University Medical Center Institutional Review Board (IRB) approved each study. The IRB approval numbers are L91020181 (ROS) and L86121802 (MAP).

### Data Availability

The data can be obtained through the Rush Alzheimer's Disease Center Research Resource Sharing Hub at radc.

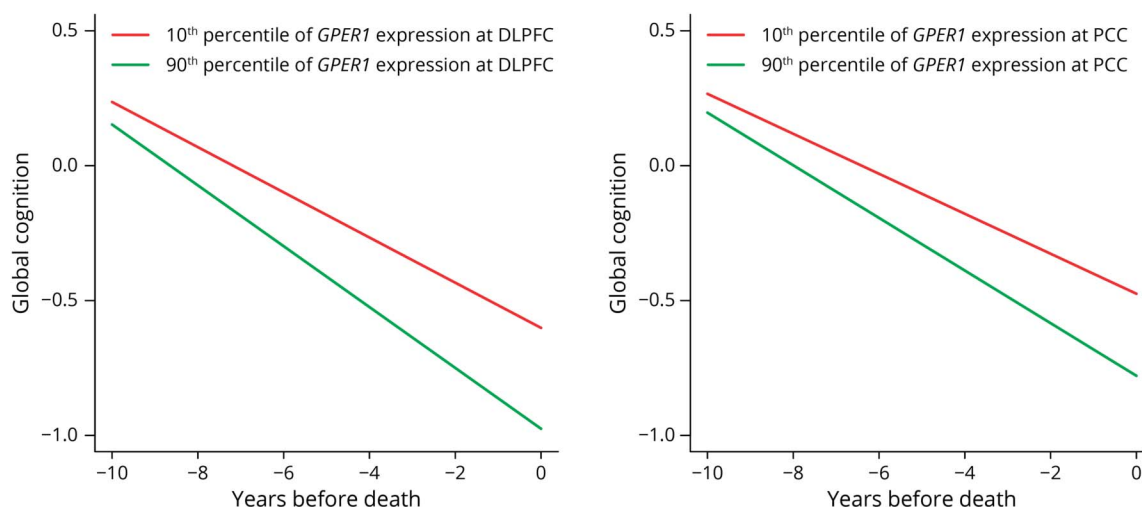
rush.edu. To access data, an application must be filled including study premise and a brief description of the research plan.

## Results

Demographic and other characteristics of the 3 data sets of women used for analyzing sequence variants, DNA methylation, and RNA expression are summarized in Table 1. At baseline, the average age of women in the sequence variants data set was 78 years old, which was 3 years younger than the other 2 data sets that were composed of deceased participants (eTable 1, links.lww.com/WNL/C615). Although the level of education was not different across the 3 data sets, the level of cognition at baseline was the highest in the sequence variants data set consistent with being youngest at baseline (eTable 1).

Women in the sequence variants data set had the longest follow-up, which was 10 years on average. At baseline, 24% met MCI diagnosis and none had dementia, whereas at the last visit, performed on average 1 year before death, 24% had MCI and 43% had dementia. Dementia was more frequent in participants with AD pathologic diagnosis than without (55% vs 21%,  $\chi^2 = 97.5$ ,  $df = 1$ ,  $p < 0.001$ ).

**Figure 3** Association of *GPER1* Expression Level With Cognitive Decline Before Death



In each plot, the association of the expression level of *GPER1* at DLPFC (left panel) or at PCC (right panel) with cognitive decline in the past 10 years before death is illustrated. Each plot illustrates cognitive trajectory in an average 90-year-old woman with 16 years of education with either low (10th percentile) or high (90th percentile) expression level of the *ER* gene. DLPFC = dorsolateral prefrontal cortex; *GPER1* = G protein-coupled estrogen receptor; PCC = posterior cingulate cortex.

Associations of *ER* polymorphisms, DNA methylation, and RNA expression in women are summarized in Table 2, and are explicated below.

### ***GPER1*-Women**

#### **Polymorphisms-Cognition**

Using mixed-effects models and subsequent omnibus tests indicated that *GPER1* polymorphisms were associated with the level of global cognition at baseline ( $q = 0.028$ ) but not with the rate of cognitive decline. Examining individual sequence variants indicated that 1 (1%) sequence variant was associated with the level of global cognition (Figure 1; eTable 2, [links.lww.com/WNL/C615](https://links.lww.com/WNL/C615)).

#### **Polymorphisms-Pathologies**

The omnibus tests indicated that *GPER1* polymorphisms were not associated with AD pathologic indices. In examining associations with non-AD pathologies, *GPER1* polymorphisms were associated with only microinfarcts ( $q = 0.038$ ). Examining individual sequence variants separately, 11 sequence variants were associated with microinfarcts after FDR correction (eTable 3, [links.lww.com/WNL/C615](https://links.lww.com/WNL/C615)).

#### **DNA Methylation-Cognition**

The rate of cognitive decline ( $q < 0.001$ ) and cognition level at death ( $q < 0.001$ ) were both related to *GPER1* DNA methylation measured postmortem. Examining individual CpG sites indicated that most of the significant CpG sites were related to both rate of cognitive decline and cognition level (Figure 1; eTables 4–6, [links.lww.com/WNL/C615](https://links.lww.com/WNL/C615)).

#### **DNA Methylation-Pathologies**

DNA methylation at *GPER1* was associated with the tau tangle density ( $q = 0.013$ ) and global AD pathology score ( $q = 0.013$ ).

Examining individual CpG sites indicated that DNA methylation values of 10 and 14 CpG sites at *GPER1* were associated with the tau tangle density and global AD pathology score, respectively, after FDR correction (Figure 2, eTable 7, [links.lww.com/WNL/C615](https://links.lww.com/WNL/C615)). DNA methylation at *GPER1* was not associated with any of the non-AD pathologies.

#### **RNA Expression-Cognition**

Both rate of cognitive decline (Estimate =  $-0.014$ , standard error [SE] =  $0.004$ ,  $q = 0.005$ ) and final level of cognition at death (Estimate =  $-0.181$ , SE =  $0.045$ ,  $q < 0.001$ ) were related to *GPER1* expression levels in postmortem DLPFC (Figure 3). To contextualize the effect size, we used model-derived estimates (eTable 8, [links.lww.com/WNL/C615](https://links.lww.com/WNL/C615)) to compare the rate of cognitive decline in 2 representative women with different levels of DLPFC *GPER1* RNA expression. Cognitive decline in a woman with a high (90<sup>th</sup> percentile = 14.9) level of *GPER1* RNA expression was 35% faster than a woman with a low (10<sup>th</sup> percentile = 12.8) level.

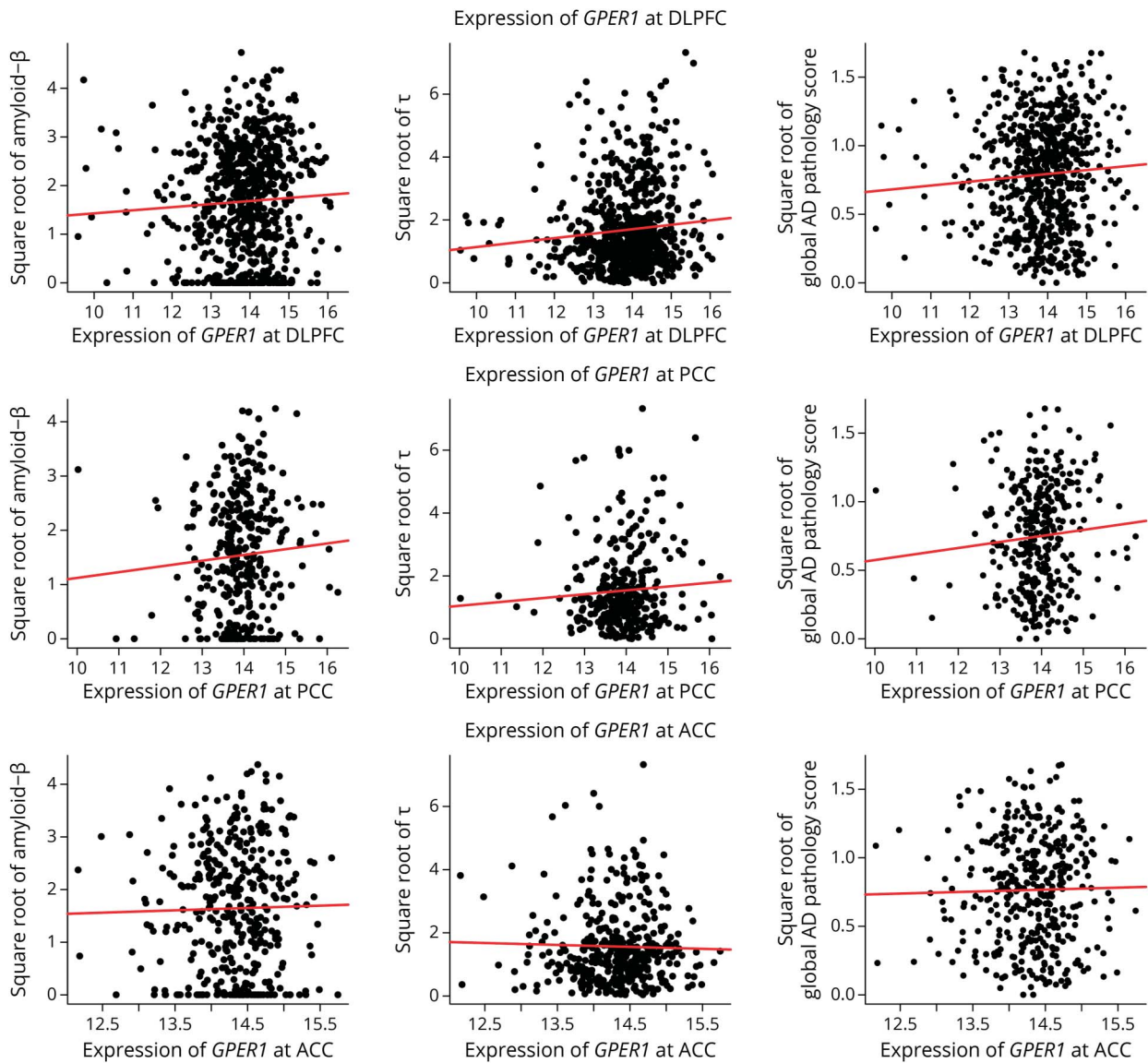
Only the level of cognition (Estimate =  $-0.201$ , SE =  $0.076$ ,  $q = 0.026$ ), not the rate of decline (Estimate =  $-0.015$ , SE =  $0.007$ ,  $q = 0.083$ ), was related to expression levels of *GPER1* at PCC (Figure 3). *GPER1* expression levels at ACC were not associated with cognitive decline.

#### **RNA Expression-Pathologies**

Examining associations of *GPER1* expression levels with AD pathologic indices indicated that the level of expression in DLPFC was associated with the tau tangle density (Estimate =  $0.140$ , SE =  $0.051$ ,  $q = 0.039$ ) but not with the A $\beta$  load or global AD pathology score (Figure 4). To contextualize the



**Figure 4** Association of *GPER1* Expression Levels at the Dorsolateral Prefrontal Cortex (DLPFC), Posterior Cingulate Cortex (PCC), and Anterior Cingulate Cortex (ACC) With AD Pathology Indices



*GPER1* = G protein-coupled estrogen receptor.

effect size, we used model-derived estimates (eTable 9, links.lww.com/WNL/C615) to compare tau tangle density in 2 representative women with different levels of DLPFC *GPER1* RNA expression. Tau tangle density was 19% higher in a woman with a high (90th percentile) level of *GPER1* RNA expression compared with a low (10th percentile) level.

No association was seen between expression levels of *GPER1* at PCC or ACC and AD pathologic indices. In examining non-AD pathologies, *GPER1* expression was not associated with any of them.

#### DNA Methylation-RNA Expression

Because both DNA methylation and RNA expression of *GPER1* in DLPFC were related to cognitive decline and tau tangle density,

we examined their association by themselves. DNA methylation and RNA expression of *GPER1* in DLPFC were not related.

#### ER2-Women

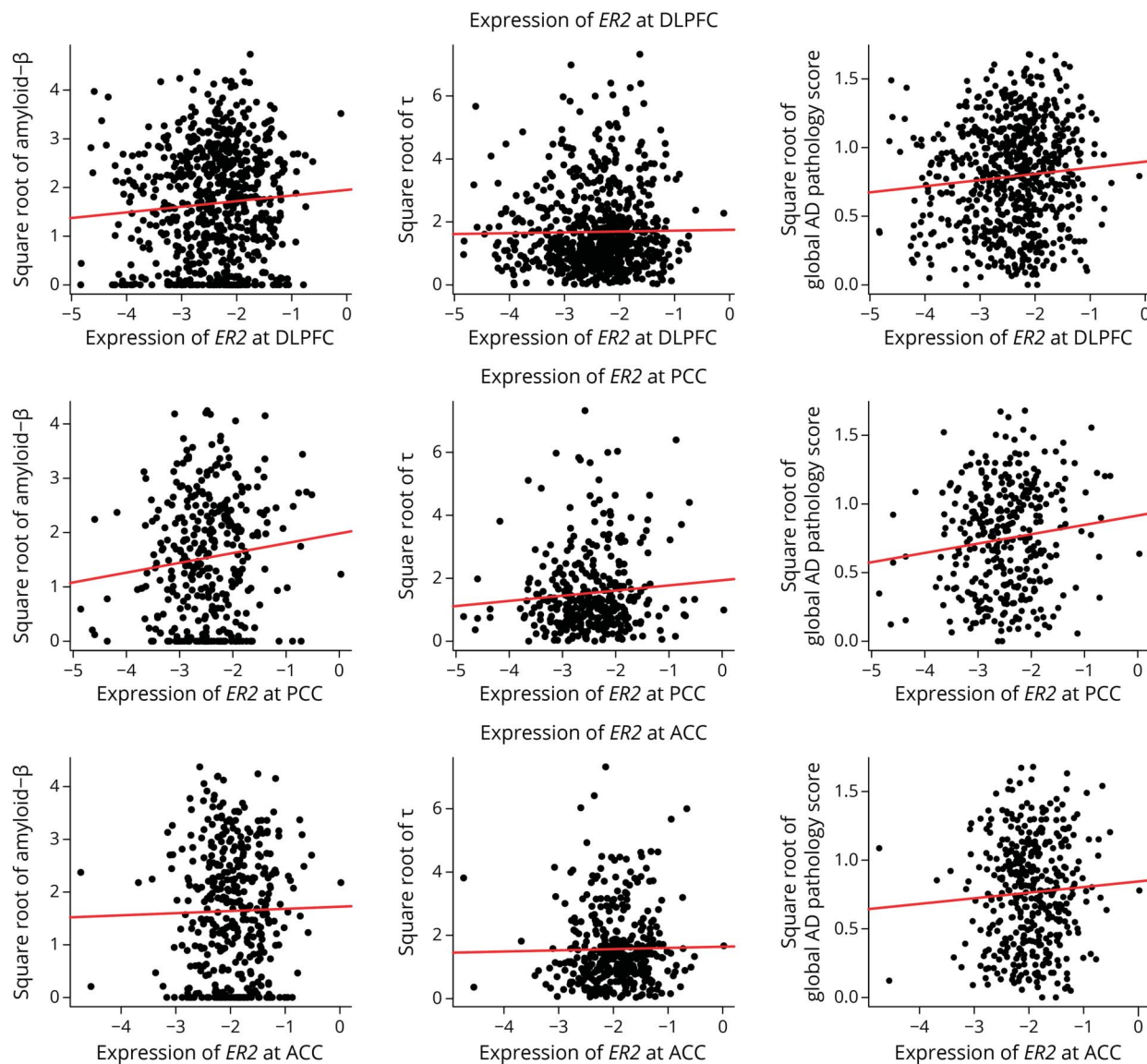
##### Polymorphisms-Cognition

*ER2* polymorphisms were associated with the level of global cognition at baseline ( $q = 0.009$ ) but not with the rate of cognitive decline. Examining individual sequence variants indicated that 42 (6%) of sequence variants were associated with the baseline level of global cognition (Figure 1; eTable 2, links.lww.com/WNL/C615).

##### Polymorphisms-Pathologies

*ER2* polymorphisms were associated neither with AD nor with non-AD pathologies.

**Figure 5** Association of *ER2* Expression Levels at the Dorsolateral Prefrontal Cortex (DLPFC), Posterior Cingulate Cortex (PCC), and Anterior Cingulate Cortex (ACC) With AD Pathology Indices



AD = Alzheimer disease; ER = estrogen receptor.

### DNA Methylation-Cognition

Like *GPER1* DNA methylation, the rate of cognitive decline ( $q < 0.001$ ) and cognition level at death ( $q < 0.001$ ) were related to levels of *ER2* DNA methylation measured postmortem. Examining individual CpG sites indicated that all 5 significant CpG sites were related to both rate of cognitive decline and cognition level (Figure 1; eTables 4–6, [links.lww.com/WNL/C615](https://links.lww.com/WNL/C615)).

### DNA Methylation-Pathologies

DNA methylation at *ER2* CpG sites was associated with the tau tangle density ( $q = 0.013$ ) but not with the  $A\beta$  load or global AD pathology score. By examining individual CpG sites, we found that the DNA methylation value of 1 CpG site at *ER2* was associated with tau tangle density (Figure 2, eTable 7, [links.lww.com/WNL/C615](https://links.lww.com/WNL/C615)).

DNA methylation at *ER2* was not associated with any of the non-AD pathologies.

### RNA Expression-Cognition

In mixed-effects models, *ER2* expression levels were not associated with cognitive decline.

### RNA Expression-Pathologies

Expression levels of *ER2* at DLPFC and PCC were associated with an only global AD pathology score (DLPFC: Estimate = 0.048, SE = 0.019,  $q = 0.035$ ; PCC: Estimate = 0.074, SE = 0.029,  $q = 0.035$ ) not  $A\beta$  or tau tangles (Figure 5). The expression level of *ER2* at ACC was not associated with any of the AD pathology indices.

Examining non-AD pathologies, the *ER2* expression level was not associated with any of them.

## ER1-Women

### Polymorphisms-Cognition

*ER1* polymorphisms were associated with the level of global cognition at baseline ( $q < 0.001$ ) but not with the rate of cognitive decline. Examining individual sequence variants indicated that 82 (5%) sequence variants were associated with the level of global cognition (Figure 1; eTable 2, [links.lww.com/WNL/C615](https://links.lww.com/WNL/C615)).

### Polymorphisms-Pathologies

*ER1* polymorphisms were associated neither with AD nor with non-AD pathologies.

### DNA Methylation-Cognition

Like *GPER1* and *ER2* DNA methylation, the rate of cognitive decline ( $q < 0.001$ ) and cognition level at death ( $q < 0.001$ ) were related to levels of *ER1* DNA methylation measured postmortem. Examining individual CpG sites indicated that more than half of significant CpG sites were related to both rate of cognitive decline and cognition level (Figure 1; eTables 4–6, [links.lww.com/WNL/C615](https://links.lww.com/WNL/C615)).

### DNA Methylation-Pathologies

Gene-based analysis indicated that DNA methylation at *ER1* was associated with tau tangle density ( $q = 0.016$ ) but not with the A $\beta$  load or global AD pathology score. Examining individual CpG sites indicated that the DNA methylation value of 1 CpG site at *ER1* was associated with tau tangle density (Figure 2, eTable 7, [links.lww.com/WNL/C615](https://links.lww.com/WNL/C615)).

DNA methylation at *ER1* was not associated with any of the examined non-AD pathologies.

## Sensitivity Analyses

Because *GPER1* RNA expression in DLPFC had the most consistent association with AD phenotypes in women, we did 4 sensitivity analyses to confirm this finding. First, we separately examined *GPER1* RNA expression in DLPFC with cognitive decline in women with and without AD pathologic diagnoses. The findings confirmed our hypothesis that the associations of *GPER1* RNA expression with cognitive decline were stronger in women with an AD pathologic diagnosis (eTable 10, [links.lww.com/WNL/C615](https://links.lww.com/WNL/C615)). Second, we hypothesized that if *GPER1* RNA expression was associated with AD traits should also be RNA expressions of genes of signaling pathways of activated ERs. We examined DLPFC RNA expressions of 3 signaling pathways of activated ERs: c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase, and Akt kinase.<sup>34</sup> We found that the most robust associations with cognitive decline and tau tangle density were derived from analyzing RNA expression of genes of the JNK pathway, which are more specifically involved in downstream signaling of activated *GPER1* rather than *ER1* and *ER2*<sup>34</sup> (eTables 11–12). Third, we removed the first 3 global cognition assessments to address a learning effect that occurs during the first annual visits,<sup>35</sup> as performed previously.<sup>36</sup> However, the removal of the first 3 cognition assessments did

not change our findings that the rate of cognitive decline (Estimate =  $-0.012$ , SE = 0.005,  $p = 0.030$ ) and final level of cognition at death (Estimate =  $-0.012$ , SE = 0.005,  $p = 0.030$ ) were related to *GPER1* expression levels in postmortem. Fourth, we randomly selected 100 genes whose RNA expression levels in DLPFC passed quality-control criteria. Then, in separate models, we examined associations of the 100 RNA expressions with cognitive decline and AD pathology indices (eTables 13–14). The analyses indicated that 4 genes had the same type of associations that *GPER1* had: their RNA expressions were associated with faster cognitive decline and more tau tangles. The 4 genes were *ZC3H11A*, *FURIN*, *SMARCD3*, and *PPP4R2*, and 2 of them were previously reported associated with gray matter volume (*ZC3H11A*)<sup>37</sup> and AD (*FURIN*).<sup>38</sup> These sensitivity analyses supported the association of *GPER1* RNA expression in DLPFC with AD phenotypes in women.

## ER-Men

As a comparison, we examined associations of the *ER* molecular variants with cognitive decline and the pathology indices in men (eTable 15, [links.lww.com/WNL/C615](https://links.lww.com/WNL/C615)) whose characteristics are summarized in Table 1. *ER1* sequence variants were the only *ER* sequence variants associated with cognition (eTable 16). Moreover, *ER1* DNA methylation sites were the only ones associated with cognition and AD pathology indices (eTables 17–18). RNA expressions of *GPER1* and *ER2* were not associated with cognition or AD pathology indices in men. None of molecular variants of *ER* genes were associated with non-AD pathologies. These findings suggest that in men, there were less robust associations between *ER* molecular genomic variants and AD traits.

## Discussion

In this study, *ER* polymorphisms were not associated with the rate of cognitive decline in women, which was supported by lack of association between *ER* polymorphisms and AD pathologic indices and is in line with meta-analyses indicating lack of association between *ER* polymorphisms and dementia in European ancestry.<sup>12,39</sup> Although few longitudinal studies reported association between some *ER1* and *ER2* polymorphisms and cognitive decline,<sup>14,15</sup> their samples were younger than ours and were followed for fewer years. To our knowledge, this is the first study that examined *ER* polymorphisms with AD and non-AD brain pathologies or examined *ER* DNA methylation and expression levels with cognitive decline and brain pathologies.

In contrast to polymorphisms, DNA methylation at all 3 *ER* genes in women was associated with the rate of cognitive decline and level of tau tangle density, which is the major AD pathologic index that drives cognitive decline.<sup>40</sup> Moreover, *GPER1* expression levels in women were associated with the rate of cognitive decline and with some indices of AD pathology. These results are consistent with previous studies in

women in which ER mediates effects of estrogen and DNA methylation is necessary for estrogen to enhance memory consolidation.<sup>34</sup> In fact, our finding that levels of DNA methylation and RNA expression of *GPER1* in DLPFC were not related, while both were associated with cognitive decline and tau tangle density, indicates that the associations of ER DNA methylation with AD traits were not through alteration of ER expression but were rather through other mechanisms. Several mechanisms have been attributed to estrogen's possible effect on cognition, which may also underlie the association of cognition with *GPER1* RNA expression as a mediator of estrogen effects, including modulation of neurotransmitters (acetylcholine<sup>41</sup> and dopamine<sup>42</sup>), enhancement of memory consolidation pathways (long-term potentiation and depression and dendritic spine density<sup>43</sup>), and neuroprotective mechanisms.<sup>44</sup> However, few studies have examined whether estrogens or ER is associated with the production or clearance of A $\beta$  load or tau tangle density.<sup>45,46</sup> Although randomized trials examining estrogen for the prevention of cognitive decline in women<sup>8,9</sup> were negative, several factors may underlie these negative findings including type of menopause of studied women, women's age at enrollment, type of treatment, its dosage, or its mode of delivery. More studies are required to uncover whether the current findings are reproducible and valid.

Compared with *ER1* and *ER2*, *GPER1* DNA methylation and RNA expression had the most consistent associations with AD pathologic indices, cognition level, and cognitive decline rate in older women. Although more consistent associations of *GPER1* may be due to its specific downstream signaling mechanisms including the JNK pathway,<sup>34</sup> it may also be due to the brain regions examined in this study. Previous studies indicated that *GPER1* had the highest level in the prefrontal cortex compared with *ER1* and *ER2*.<sup>42</sup> *ER1* was located primarily in amygdala and hypothalamus,<sup>47</sup> and higher levels of *ER2* were present in hippocampus compared with *ER1*.<sup>42,47</sup> Therefore, brain regions with the highest levels of *ER1* and *ER2* were not examined in this study, which might have favored preferential associations of *GPER1* with AD traits in women.

Although the study focus was more on women, we also separately examined ER in men. In men, there were less robust associations between ER molecular genomic variants and AD traits. Moreover, they were *ER1*, rather than *GPER1* and *ER2*, sequence variants and DNA methylation that had associations with cognitive decline and AD pathology indices. This finding is corroborated by a meta-analysis suggesting the association of an *ER1* sequence variants with cognition in men not women.<sup>11</sup> Future studies are needed to clarify whether this heterogeneous association of ER molecular variants with AD cognitive and pathologic phenotypes across sexes is related to higher levels of estrogen in older men compared with postmenopausal women.

We did not find a consistent association between the 3 ER genes' polymorphisms, DNA methylation, or RNA expression and non-AD brain pathologies including vascular pathologies in women or men. To our knowledge, no previous studies

examined these associations. Previous studies examining ER genes in relation to peripheral vessels' atherosclerosis or clinical vascular events did not report consistent associations either.<sup>48,49</sup> Therefore, further studies are required to untangle whether no association exists between ER genes and non-AD brain pathologies, or some associations will be found by using more granular metrics for non-AD pathologic assessments.

Clinicians confront conflicting studies about potential benefits of estrogen on cognition. This study suggests that there is substantial heterogeneity among ER molecular genomics in relation to AD cognitive and pathologic traits in older women, which may underlie the conflicting relationship between estrogen and cognition. We examined ER molecular genomics in DLPFC that strongly contributes to cognition. Higher expressions of *GPER1*, not *ER2*, were related to faster cognitive decline. Moreover, higher levels of all 3 ER DNA methylation were related to cognitive decline through unidentified mechanisms. Eventually, translating this kind of work to living humans will be required to make our findings actionable for clinicians.

Some study strengths underlie our findings. Data came from well-characterized longitudinal studies of older adults with high follow-up and autopsy rates. Staff involved in the assessment of brain pathologies or in the measurement of genetic variations were blinded to clinical data. However, some limitations require further studies to confirm our findings. The participants were selected, not representative of the US population. They were mostly non-Hispanic White volunteers with a high education level, which requires findings to be replicated in more diverse populations, including more participants of minorities and more participants with low socioeconomic status, for generalizability. The cohorts did not include questions to differentiate cisgender from transgender women or men. The postmortem interval was not short, which might have affected ER DNA methylation and RNA expression levels. Not everyone with genetic information had available DNA methylation or transcriptomic data. Due to the postmortem assessment of DNA methylation or RNA expression, a temporal order of their occurrence in relation to the examined outcomes, cognitive decline or AD pathologies, cannot be inferred. Our DNA methylation data were produced using Illumina 450K array that targets regions with a high frequency of CpG sites, which is a small fraction of the whole epigenome. Although we developed a composite measure of global cognition from 19 neuropsychological tests, the measure might still have suffered from distributional properties, including floor and ceiling effects, of the included tests.

In conclusion, we found that ER molecular variants were associated with AD phenotypes in women. ER polymorphisms were associated with the baseline level of cognition. ER gene methylation and expression levels, specifically *GPER1* in DLPFC, were associated with rate of cognitive decline, level of cognition at death, and AD pathology indices. In men, there were less robust associations between ER molecular genomic variants and AD traits. Nonetheless, no consistent association was seen

between ER and non-AD pathologies in either of the sexes. Further studies are required to confirm the above findings.

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## Disclosure

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Name	Location	Contribution
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<b>Jingyun Yang, PhD;</b>	Rush University Medical Center	Analysis and interpretation of data; revising the manuscript for intellectual content
<b>Lei Yu, PhD</b>	Rush University Medical Center	Analysis and interpretation of data; revising the manuscript for intellectual content
<b>Dominika Burba, MS</b>	Rush University Medical Center	Analysis of data
<b>Woojeong Bang, MS</b>	Rush University Medical Center	Analysis of data
<b>Shinya Tasaki, PhD</b>	Rush University Medical Center	Analysis and interpretation of data; revising the manuscript for intellectual content
<b>Fran Grodstein, PhD</b>	Rush University Medical Center	Interpretation of data; revising the manuscript for intellectual content
<b>Yanling Wang, PhD</b>	Rush University Medical Center	Acquisition of data; revising the manuscript for intellectual content
<b>Jinying Zhao, PhD</b>	University of Florida	Revising the manuscript for intellectual content
<b>Philip L. De Jager, MD, PhD</b>	Columbia University Irving Medical Center	Acquisition of data; revising the manuscript for intellectual content
<b>Julie A. Schneider, MD</b>	Rush University Medical Center	Acquisition of data; revising the manuscript for intellectual content
<b>David A. Bennett, MD</b>	Rush University Medical Center	Acquisition of data; design and conceptualized study; interpretation of the data; revising the manuscript for intellectual content

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