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The effects of the *TOMM40* poly-T alleles on Alzheimer's disease phenotypes

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Abstract

The *TOMM40* poly-T is a polymorphism in intron 6 of the *TOMM40* gene, which is adjacent to and in LD with *APOE*. Roses and colleagues identified the association between the length of *TOMM40* poly-T with the risk and age of onset of late-onset Alzheimer's disease (LOAD). Following the original discovery, additional studies found associations between the *TOMM40* poly-T and LOAD-related phenotypes independent of *APOE* genotypes, while others did not replicate these associations. Furthermore, the identity of the *TOMM40* poly-T risk allele has been controversial between different LOAD-related phenotypes. Here we propose a framework to address the conflicting findings with respect to the *TOMM40* poly-T alleles associations with LOAD-phenotypes and its functional effects. The framework is used to interpret previous studies as means to gain insights regarding the nature of the risk allele, VL vs. S. We suggest that the identity of the *TOMM40* poly-T risk allele depends on the phenotype being evaluated, the ages of the study subjects at the time of assessment, and the context of the *APOE* genotypes. In concluding remarks we outline future studies that will inform the mechanistic interpretation of the genetic data.

Keywords

TOMM40 poly-T; *APOE*; late onset Alzheimer's disease; cognitive performance; brain imaging

The association of genetic variation within the *TOMM40-APOE-C1-2-4* genomic region of Chr:19q13.32 with the risk and age of onset of late-onset Alzheimer's disease (LOAD) was established over 20 years ago by linkage analysis of pedigrees[1]. The *APOE* ϵ 4 allele of apolipoprotein E (*APOE*)[1] is the strongest genetic risk factor for LOAD and is associated with lower age of onset, and over the ensuing years these findings have been highly replicated[1–4]. LOAD genome-wide association studies (GWAS) conducted over the last decade have confirmed strong associations with this genomic region[5–10], and no other

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LOAD-association has remotely approached the same level of significance[5, 6, 11, 12]. Although some LOAD GWAS studies exclude all variants in this region because of their high linkage disequilibrium (LD) with the coding SNPs that define the *APOE* genotype, other LOAD genetic studies have focused on the association between variants and haplotypes based on the promoter and enhancer regions of genes in this region with LOAD phenotypes[13–20]. However, whether the association signal is attributable to additional variants and haplotypes within this LD region jointly with *APOE*ε4, is an enduring enigma in the field of LOAD genetics.

The *TOMM40* poly-T is a polymorphism in intron 6 of the *TOMM40* gene, which encodes Tom40 protein (Translocase of the Outer Mitochondrial Membrane, 40kD) and is adjacent to and in LD with *APOE*. In 2010, Roses *et al.* reported the association between the length of this highly polymorphic poly-T variant (rs10524523 aka '523) with the risk and age of onset of LOAD[21]. The high LD between DNA-nucleotide variants in the *APOE* genomic region impedes the assessment of independence by statistical conditional analysis [22]. Therefore, the association analysis in the original report was limited to individuals with the *APOE* ε3/4 genotype, to reduce genetic heterogeneity and eliminate confounding with the *APOE* SNPs. Subsequently, several studies have supported the hypothesis of an *APOE*-independent association of the *TOMM40* poly-T variant with several phenotypes related to Alzheimer's disease (AD), including hippocampal thinning[23], cognition and gray matter volume[24], cerebrospinal fluid (CSF) biomarkers for AD[25, 26], increased neuritic tangles and a higher likelihood of pathologically diagnosed AD[22], the age of onset of mild cognitive impairment (MCI) due to AD[27], and cognitive performance and rate of decline in non-demented elderly[28–32]. Recently, we tested the association of the *TOMM40* poly-T with cognitive decline, based on longitudinal measures of cognitive function, *e.g.* Montreal Cognitive Assessment (MoCA) obtained from an on-going study at Duke, the Bryan ADRC Prevention Screening Study and Database/Repository (PREPARE). This study population is comprised of a non-demented socioculturally diverse volunteer cohort from Durham, NC. In unpublished data, we found that the *TOMM40* poly-T was associated with changes in MoCA scores adjusting for different follow-up intervals, sex, age, and for *APOE* genotype.

Overall, the studies summarized in Table 1, determined the *APOE*-independent association of the *TOMM40* poly-T with LOAD related phenotypes by including *APOE* genotypes as a covariate in the statistical models, or by designing the study to include only individuals with the same *APOE* genotypes (*e.g.* analyzing the association in the *APOE* ε3/3 stratum). It is important to note that other studies have not supported an *APOE*-independent association of the *TOMM40* poly-T variant with LOAD risk or age of onset [33, 34] [35] (Table 2). These studies included all *APOE* genotypes and adjusted for the *APOE* ε4 allele counts as a covariate in the statistical models, and some repeated the analyses with the *APOE* ε3/3 stratum. Amongst the two largest size analyses performed for the *APOE* ε3/3 subsets, Jun *et al.* reported no statistically-significant associations of the *TOMM40* poly-T with LOAD risk or AAO[33], while Cruchaga *et al.* observed statistically-significant association with LOAD risk and a trend towards association with AAO[36]. A number of methodological aspects that may account for the contradictory results and requirements for replication studies of genetic associations with AAO were discussed previously[37], however, there is an

interpretation of prior studies that *TOMM40* poly-T associations with LOAD-related phenotypes are simply a consequence of LD with the *APOE* coding SNPs[38].

Three allele groups were defined for the *TOMM40* poly-T polymorphism, based on the modes of the distributions of the number of 'T'-residues: 'Short' (S, T = 19), 'Long' (L, 20–29) and 'Very Long' (VL, T = 30)[21]. The VL allele appears to have different effects on LOAD-related phenotypes, depending on whether it forms a haplotype with *APOE* ϵ 3/3 or *APOE* ϵ 3/4. Kaplan-Meier age of onset (AOO) curves showed that in *APOE* ϵ 3/3 individuals the VL allele was associated with a later AOO of MCI due to AD than S, whereas in *APOE* ϵ 3/4 it was associated with earlier AAO[27], consistent with the original discovery[21]. These observations suggested that although the *TOMM40* poly-T has autonomous associations with LOAD related phenotypes, as discussed above, the direction of the effects are influenced by interactions with *APOE*.

We hypothesized that the identity of the *TOMM40* poly-T risk allele depends on the phenotype being evaluated and the ages at assessment of the study subjects. To gain insights into the nature of the *TOMM40* poly-T allele risk pattern, we compared a number of features across several studies that identified significant associations with the *TOMM40* poly-T alleles (Table 1), including: study design, the phenotypes evaluated, and the demographic characterization of the study sample. In these studies, the analyses were constrained to *APOE* ϵ 3/3 subjects only and/or adjusted for *APOE* genotypes, so that the *APOE* status did not contribute to the direction of the *TOMM40* poly-T effect. A recent study showed that the VL allele was significantly associated with increased hippocampal thinning relative to the S allele, specifically reduced thickness of the entorhinal cortex [23]. An earlier study found that the VL allele was associated, in a dose-dependent manner, with decreased gray matter volume in regions of the brain affected early in the development of AD in *APOE* ϵ 3/3, cognitively normal, middle-age people [24]. Together, these studies suggest that the VL allele is deleterious regarding the structural integrity of brain regions affected early in the course of AD. Furthermore, the latter study also reported that the VL/VL group showed lower performance on primacy retrieval from a verbal list learning task, a cognitive deficiency noted in the early manifestation of AD [24]. The VL allele also was associated with lower cognitive measures in several additional community dwelling studies [28–30] [32]. Two cross-sectional design studies in cognitively healthy elderly reported that S homozygotes performed better on measures of memory and executive function, domains that are preferentially affected in early-stage AD[29, 30]. Another longitudinal study, in a larger cohort of cognitively normal individuals, found a significant effect of the VL on flattened test-retest improvement only in individuals <60 years [28]. The VL allele was also associated with faster rate of cognitive decline, which was significant for vocabulary ability, suggesting that the VL has a risk effect [32]. However, the same study also reported that the S correlated with reduced vocabulary ability [32]. An association analysis using a large *APOE* ϵ 3/3 case-control series found that the VL allele was underrepresented in LOAD cases compared to controls, supporting the opposite direction, *i.e.* the VL exhibited a protective role[36]. The protective effect of the VL allele was further demonstrated in a recent large study of *APOE* ϵ 3/3 elderly individuals that reported statistically significant association of the S allele with faster decline in global cognition, primarily in the domains of episodic and semantic memory [31]. Similarly, in the PREPARE data, the S allele was also

associated with faster decline in MoCA scores in both *APOE* $\epsilon 3/4$ individuals and in *APOE* $\epsilon 3/3$ individuals. These differences were not statistically significant; however, the trend is suggestive of a protective effect of the VL allele. Collectively, while several studies have suggested that the VL allele has risk effect other studies have suggested a beneficial effect. Studies that evaluated neuroimaging phenotypes were performed inclusively in middle-aged adults and consistently showed that the VL is deleterious (Table 1). Longitudinal studies that assessed the rate of cognitive decline suggest that the mean age of the study subjects influenced the direction of the *TOMM40* poly-T effects. While the VL had an adverse effect in cohorts with mean age of 57.8 and 65, it showed a beneficial effect in an older cohort (mean age 78.5). However, the influence of the subjects' age on the direction of the associations with cognitive performance in cross sectional (single time-point) evaluations is not conclusive (Table 1). A recent study[39] addressed the question of whether VL was a risk or protective allele by examining two cohorts: the Wisconsin Registry for Alzheimer's Prevention and the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.usc.edu). The primary result of this study was that parental history of LOAD (FH) was the critical factor that determines whether the *TOMM40* poly-T VL allele is risk or protective. For FH negative participants, gene-dose preservation of memory and global cognition was seen for VL vs. S carriers. For FH positive, an opposite gene-dose decline was seen for VL vs. S carriers. However, we cannot exclude the possibility that FH is a surrogate for parental carriers vs. non-carriers of the *APOE* $\epsilon 4$.

Another example of alleles that display both risk and protective effects that depend on the evaluated phenotypes and/or age is represented by the *APOE* genotype. In the context of neurodegenerative disorders, *APOE* $\epsilon 4$ is associated with higher risk for LOAD and *APOE* $\epsilon 2$ is considered as a protective allele, but with respect to the lipoprotein disorder type III HLP (dysbetalipoproteinemia) homozygosity for *APOE* $\epsilon 2$ is the primary causal genetic factor[50]. Noteworthy, it was also suggested that the *APOE* $\epsilon 4$ has beneficial early life effects on cognition and other traits (reviewed in[51, 52]). This evidence exemplified the antagonistic pleiotropic effects of *APOE*. Other examples include mutations in the CFTR and the HBB genes, whereas homozygotes individuals suffer from Cystic Fibrosis and Sickle-cell anemia, respectively; individuals with heterozygous mutated alleles of CFTR and HBB showed advantage in protection against cholera and malaria, respectively[53, 54].

Biomarkers and neuropathological features that track the progression of LOAD or correlate with changes in cognition assist with development of testable hypotheses to elucidate the biological mechanisms that underlie genetic associations. To interpret the autonomous association of the *TOMM40* poly-T with LOAD, recently, Yu *et al* investigated the neuropathologic features associated with *TOMM40*-*APOE* haplotypes in a large cohort of community-dwelling older persons who had annual cognitive assessments and brain autopsies after death[55]. First, they assessed the independent effect of the *TOMM40* poly-T using the *APOE* $\epsilon 3/3$ stratum, and found that individuals carrying the *TOMM40* poly-T S/S genotype had faster cognitive decline relative to the S/VL or VL/VL genotypes, consistent with their previous report [31] and pointing to the S allele as the risk allele for the cognitive decline phenotype[55]. Comparing the effect size of the *TOMM40* poly-T S allele on cognitive decline to that of the *APOE* $\epsilon 4$ //*TOMM40* poly-T L, indicated that the former effect size is smaller by approximately 40%. In further analyses Yu *et. al* also showed that

neuropathologies of amyloid load, phosphorylated paired helical filament tau, macroscopic infarcts, hippocampal sclerosis, TDP-43 and cerebral amyloid angiopathy are associated with the *APOE* ϵ 4- *TOMM40* poly-T L haplotypes[55]. However, these neuropathologies were not associated with the *APOE* ϵ 3- *TOMM40* poly-T S/S haplotype. Collectively, the results of this study suggested that there are two independent effects of *APOE*//*TOMM40* poly-T haplotypes on cognitive decline: a large effect mediated by common neuropathologies for *APOE* ϵ 4//*TOMM40*L and a second, smaller effect for *APOE* ϵ 3-*TOMM40*S that is not mediated by common neuropathologies[55]. Our perspective is that the smaller effect is mediated by and/or associated with changes in gene expression of *APOE* and/or *TOMM40* genes [56].

A relatively modest change in mRNA expression may produce pathology that accumulates over time and is expressed clinically in later age. Structural DNA variations, especially in noncoding intronic or intergenic regions like the *TOMM40* poly-T variant, most likely exert their effects on phenotype by altering gene regulation including transcription efficiency[40–45]. We and others demonstrated that *TOMM40* poly-T acts as a *cis*-regulator of gene expression. We showed that the *TOMM40* poly-T affects the expression levels of *APOE*- and *TOMM40*- mRNAs in the temporal and occipital cortexes of LOAD patients and normal controls. In order to dissect the *APOE*-independent effect of the *TOMM40* poly-T on gene expression we performed follow-up analyses constrained to *APOE* ϵ 3/3 donors, and demonstrated that the VL allele is associated with a significant increase in *TOMM40*- and *APOE*- mRNA expression compared to the S allele in brain samples from *APOE* ϵ 3/3 individuals[46]. We evaluated the direct effect of the *TOMM40* poly-T variation on transcription regulation in a cell-based luciferase reporting system, and showed that the VL allele in its native genomic context elicited significantly higher reporter expression than the S, recapitulating the observations in the human brain study[46]. Moreover, in unpublished studies we further validated the *TOMM40* poly-T regulatory effect and its direction, by measuring the *TOMM40*- and *APOE*- mRNA levels in mESCs humanized for the *TOMM40*-*APOE* genomic region that were generated by targeted replacement for the purposes of creating the humanized mouse models. We found that human *TOMM40*- and *APOE*- mRNAs levels are higher in the *APOE* ϵ 3//*TOMM40* poly-T VL mESCs compared to the mESCs of the *APOE* ϵ 3//*TOMM40* poly-T S transgene. The regulatory nature of the *TOMM40* poly-T has been independently confirmed by other groups[13, 32]. On the contrary, other studies found no associations between the *TOMM40* poly-T variant and either *APOE*- and *TOMM40*- mRNA levels in cortex samples from cognitively normal and AD donors, [36] or TOM40 and APOE protein expression levels in fibroblast cell cultures [47].

We suggest that, in some cases, the vulnerability to LOAD phenotypes is mediated by alteration in *APOE*- and/or *TOMM40*- expression levels. Here we propose two hypotheses based on the interplay between age, *TOMM40* poly-T variant, and gene expression that could orchestrate the direction of the pathogenic effect on LOAD phenotypes. First, changes in the regulation of gene expression may have a differential impact on clinical and pathological outcomes depending on the phenotypes being evaluated, age and sex of the subjects as well as the *APOE* background and other factors. For example, while increased expression in young subjects could be deleterious, later in life the elevated expression levels

may have a bona fide protective effect, and vice versa. Alternatively, genotype-by-age interaction (and/or genotype-by-sex) may determine the ultimate expression levels in concert, wherein the threshold of physiological vs. pathological expression levels is steady. The premise for the latter is supported by the observations that age affects gene expression in a tissue specific manner[48], and genetic variants associated with age-related gene expression patterns, termed temporal expression quantitative trait loci (teQTL) were found to be enriched in human brain and associated with neurodegenerative diseases such as LOAD[49]. These scenarios could potentially provide, at least in part, the molecular mechanisms underlying the opposite direction of the *TOMM40* poly-T alleles discussed in this review.

The identity of the *TOMM40* poly-T risk allele/s, the factors leading to the distinct deleterious effects, and the underlying molecular mechanisms warrant further investigations. Additional large well-characterized longitudinal cohorts across a wide range of ages from which different phenotypic outcomes have been collected will further advance the current knowledge. These population based studies will have the power to confirm the reported associations of the *TOMM40* poly-T with LOAD related phenotypes, however, there is an unmet need to take the genetic associations to the next level and translate these findings to biological mechanism. Understanding, in depth, the mechanistic role of the *TOMM40* poly-T will provide insights into the interplay between the *TOMM40* poly-T, *APOE* SNPs, and age that drives the direction of the effect on the phenotypic outcomes. Toward this goal current research directions should focus on developing strategies, including *in vitro* and *in vivo* models, that will enable us to decode genetic association signals into direct functional effect. Human Induced Pluripotent Stem Cells (hiPSC)-derived brain cell types represent a powerful tool to model *in vitro* molecular and cellular aspects of human neurodegenerative diseases including LOAD, and enable deeper exploration of key molecular mechanisms underlying the disease in pathology-relevant neuronal/glia cell types otherwise inaccessible[57, 58]. Genome editing technology, such as CRISPR/Cas9[59], leverages the application of iPSC-based models for functional genetic investigations as it allows the generation of *isogenic* iPSC lines in which a specific variant is precisely modified but otherwise are genetically identical. *Isogenic* iPSC-derived models represent a compelling strategy to dissect the mechanistic role of the interaction between *TOMM40* poly-T and *APOE* variants. Several strategies have been developed for inducing aging-like features in hiPSC-derived neurons[60] that can be applied to introduce the age factor to the crosstalk between the genetic variants. An alternative approach to evaluate the contribution of age, using cell-based systems derived from patients, is the directed conversion of fibroblasts into iNeurons (iNs) that retain the aging-signature of the donors[61]. With respect to *in vivo* models, humanized mouse models created by targeted replacement of the mouse genomic region with the homologous human region that contains *TOMM40-APOE* cassette are highly valuable to explore the interactions among the *TOMM40* poly-T, *APOE* SNPs and age and characterize their direct effects on tissue and whole animal levels. The integration of human population studies, *in vitro* and *in vivo* genetic models will provide an overall robust strategy to resolve the enigma regarding the contribution of *TOMM40* poly-T to LOAD related phenotypes and to determine the nature of its role in relation to other factors, *APOE* and age, that have been identified as the major risk factors in the etiology of LOAD.

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RESEARCH IN CONTEXT

Systematic review: The authors reviewed the Literature using Pubmed, meeting abstracts and presentations. Conflicting results for an *APOE*-independent association of the *TOMM40* poly-T with late-onset Alzheimer's disease (LOAD) related phenotypes have been reported. Moreover, the specific *TOMM40* poly-T risk allele is not consistent between studies and between different LOAD-related phenotypes. Interpretation: Our findings generated the hypothesis that the nature of the beneficial vs risk *TOMM40* poly-T is influenced by the phenotype being evaluated, the ages of the study subjects, and the context of the *APOE*-SNPs. Future directions: the manuscript proposes a framework for in-depth investigation of the genetic variation in the *TOMM40-APOE* genomic region in the following directions: (a) Further evaluations of the *TOMM40* poly-T association with LOAD phenotypes using additional large well-characterized longitudinal cohorts from which different phenotypic outcomes have been collected; (b) Performing the genetic associations stratified by age groups; (c) Studying the *TOMM40* poly-T-by-age interaction in relation to expression of genes in the *TOMM40-APOE* region in brain tissue vulnerable to LOAD. (d) Establishing *in vitro* and *in vivo* systems to model the effect of the *TOMM40* poly-T on gene regulation for in depth exploration of its mechanistic role.

HIGHLIGHTS

- *TOMM40* poly-T has autonomous associations with LOAD related phenotypes.
- The direction of the *TOMM40* poly-T effects are influenced by interactions with *APOE*.
- The identity of the *TOMM40* poly-T risk allele depends on the phenotype being evaluated and the ages of the study subjects.

Table 1Positive associations of the *TOMM40* poly-T with LOAD related phenotypes

Ref	Method of phenotype assessment	Associated phenotype	Sample size	Age yrs. mean± SD; range	Male (%)	APOE	TOMM40 poly-T Risk allele
Burggren [23]	MRI	Hippocampal thickness	41	63.5±9.2	29.2	3/3	VL
Johnson [24]	MRI	gray matter volume	117	55.5±6.0	37.4	3/3	VL
Johnson [24]	cognitive tests	primacy retrieval from a verbal list	117	55.5±6.0	37.4	3/3	VL
Caselli [28]	cognitive tests	Test-retest flattening	336	57.8±12.1; 21–97	28.8	3/3	VL
Greenbaum [29]	cognitive tests	memory and executive function	331	72.0±4.9; 64–88	65.6	3/3	VL
Hayden [30]	cognitive tests	memory and executive function	101	80.6±6.0; 64–93	31.5	3/3	VL
Payton [32]	cognitive tests	Rate of Vocabulary decline	1613	65; 44–93	30.0	all	VL
Payton [32]	cognitive tests	Vocabulary – single time point	1613	65; 44–93	30.0	all	S
Yu [31]	cognitive tests	Rate of cognitive decline	1,170	78.5±7.4; 54–98	29.5	3/3	S
Cruchaga [36]	case- control	AAO	495	75.0±8.5; 44–103	42.5	3/3	S
Crenshaw [27]	case- control	AAO	232	74.8±9.6; 55–104	34.5	3/3	S
Crenshaw [27]	case- control	AAO	161	71.4±8.7; 52–92	42.9	3/4	VL
Roses [21]	case- control	AAO	34	71.5±6.7	32.4	3/4	VL

* AAO, Age at onset

Table 2
Negative *APOE*-independent associations of the *TOMM40* poly-T with LOAD related phenotypes

Ref	Study Sample	Evaluated phenotypes	Sample size	Age yrs. mean ± SD	Male (%)	<i>APOE</i>	Comment
Jun[33]	case-control	AD Risk AAO	2973	79.8±6.3	47.1	all	No significant associations also in <i>APOE</i> 33 subgroup.
Cruchaga[36]	case-control	AD Risk AAO CSF biomarkers: tau, Aβ42	2784	74±7.7	39.3	all	Significant association (p=0.004) with LOAD risk when analysis was restricted to <i>APOE</i> 33 individuals (n=1175)
Chu[35]	probable or possible LOAD cases	AAO	892	84±6.7	33.2	all	
Maruszak[62]	case-control	AAO	719	72.5±5.8	68.6	all	Significantly lower frequency of VL allele in LOAD cases compared to controls and centenarians (p<0.0001)
Helisalmi[34]	LOAD cases	AAO CSF biomarkers: Aβ42, T-tau, P-tau	336	71.1±8.3	35.4	all	No significant effect on AAO or CSF biomarker levels was found in <i>APOE</i> 33 patients subgroup
Pomara[63]	Cognitively normal elderly	CSF biomarkers: Aβ, tau	47	67.1±6.2	U	all	
Lyall[64]	community -dwelling older adults	Hippocampal volume (MRI)	655	72.7± 0.7	52.4	all	No effect also within the <i>APOE</i> 33 or <i>APOE</i> 34 subgroups.

* AAO, Age at onset