TOMM40 and *APOE*: Requirements for replication studies of association with age of disease onset and enrichment of a clinical trial

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Abstract
A number of recent studies have not replicated the association of the translocase of the outer mitochondrial membrane pore subunit (*TOMM40*) rs10524523 polymorphism, which is in linkage disequilibrium with apolipoprotein E (*APOE*), with age of onset of Alzheimer's disease (AD). This perspective describes the differences between these later studies and the original experiments. We highlight the necessity for using standardized and informative assessment tools and processes when determining the age of development of AD or AD symptoms, and also stress that this clinical phenotype is best measured reliably in prospective studies during which subjects are monitored over time. This is true when assessing potential biomarkers for age of onset and when assessing the therapeutic potential of medicines that may delay the onset or progression of this disease.
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To replicate an experiment in human biology and undeniably confirm or, in particular, refute a previous result is challenging. The checkered history of whole-genome association studies of complex human phenotypes attests to the veracity of this statement. Unrecognized selection bias can confound the replication of experimental results. Within an experiment, measurement of variation must be both accurate and consistently reproducible to be useful. For comparison across experiments, the same measurements should be conducted with the same level of precision and reproducibility. This is true for both the definition and measurement of the phenotype, and of the genetic locus or loci of interest. Measurement of variation can be relatively straightforward—measuring someone's height, for example—or very complex. Alzheimer's disease (AD) research is proving to be a challenge in measurement and recognition of possibly confounding heterogeneity. Here, we describe where some of those challenges lie in the research of AD genetics, address the minimal requirements for replication of results,

and also describe one example of an experiment to validate prospectively the contribution of a genetic locus to a quantitative trait—in this case, age of onset (AOO) of AD. We also address the pragmatic use of the translocase of the outer mitochondrial membrane pore subunit (*TOMM40*) rs10524523 (523) and apolipoprotein E (*APOE*) genotypes for enrichment of a clinical trial.

Twenty years ago, *APOE* was identified as a significant risk factor for development of late-onset AD. Subsequent studies have confirmed the relationship between this locus and AD, which is not challenging using today's technologies and considering the large effect size of this locus for disease risk. The risk is highest for those who are homozygous for the *APOE* ε4 allele, or about 2% of whites. In addition, the only *APOE* genotype agreed by the field to be useful for the prediction of AOO of late-onset AD is *APOE* ε4/4. The mean age of AD onset in *APOE* ε4/4 whites is approximately 70 years. Indeed, it is difficult to find cognitively normal individuals with the *APOE* ε4/4 genotype who are older than 90 years of age. Arguably, *APOE* ε2/2 is also highly informative because the occurrence of AD in people with this genotype is rare or nonexistent. However, only ∼1% of whites have an *APOE* ε2/2 genotype. Therefore, the two potentially

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informative *APOE* genotypes are rare. The development of an extensive body of clinical information and molecular studies added weight to the genetic findings and led to acceptance by the AD research field that carriage of *APOE* ε4, in *APOE* ε4/4 and *APOE* ε3/4 genotypes, is deleterious. Individuals with *APOE* ε3/3, the most common genotype, and also *APOE* ε2/3, are assumed to be unaffected by whatever *APOE* ε4 does to increase disease risk and decrease the AOO of AD. These assumptions subsequently led to broad comparisons of *APOE* ε4-positive and *APOE* ε4-negative groups in many clinical and pharmacological studies.

The genomic region surrounding the *APOE* gene is characterized by strong linkage disequilibrium—different alleles at polymorphic loci in the region tend to be co-inherited faithfully from generation to generation. A number of other genes are encoded within the *APOE* region, including *TOMM40*, which codes for the protein that forms the channel subunit of the multisubunit complex in the outer mitochondrial membrane through which nuclear-encoded proteins enter mitochondria. The association between the 523 polymorphism and age of AD onset was first reported by Roses and colleagues [1]. Fig. 1 illustrates the type of genetic data, based on DNA sequencing of the polymorphic poly-T, that describes the 523 locus. The sequencing identified three categorical length alleles for 523, and a two-haplotype system for *APOE* ε3–523. Attached to *APOE* ε3, and also to *APOE* ε2, which is thought to have arisen later in human history, is either a short (S; ≤19 T residues) form or a very long (VL; >29 T residues) form of 523. In contrast, 98% of the time, *APOE* ε4 is linked to, and thus inherited with, the long (L; 19–29 T residues in length) 523 poly-T allele.

The publication by Roses and colleagues [1] demonstrated the power of phylogenetic mapping for post-genomewide association study analysis of regions of high
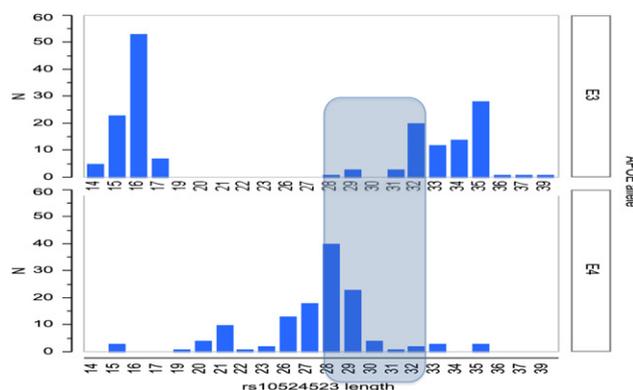

Fig. 1. Histograms of 523 poly-T lengths and allele frequencies linked to specific *APOE* alleles. The data were obtained from Sanger sequencing of 23-Kb DNA that contained both the 523 poly-T variant and the *APOE* epsilon single nucleotide polymorphisms, so linkage between the 523 and *APOE* alleles was unambiguous. The data were obtained from a white cohort of 83 patients with Alzheimer's disease and 67 age-matched control subjects, and included subjects with *APOE* ε3/3, ε3/4, and ε4/4 genotypes (no ε2 alleles; details of the cohort are given in Li and colleagues [2]). The overlap region for the long (L) and very long (VL) categories is shown in the gray area.
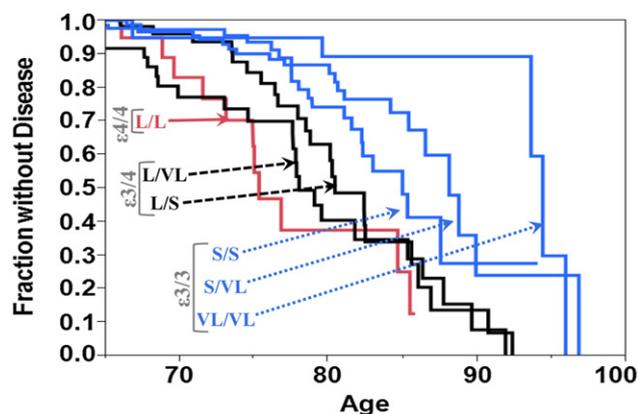

Fig. 2. Age at onset of cognitive impairment curves for *TOMM40* 523 genotypes. Data were obtained from The Joseph and Kathleen Bryan Alzheimer's Disease Research Center (Bryan ADRC), Memory, Health & Aging cohort [3] (n = 508, 106 conversion events) and were monitored prospectively at the Bryan ADRC at Duke University. Age at which cognitive impairment occurred was stratified by *TOMM40* genotype, and Kaplan-Meier curves were constructed. L, long; VL, very long; S, short.

linkage disequilibrium, showing that haplotypes composed of the 523 S allele and *APOE* ε3 have a different inferred evolutionary history than haplotypes containing *APOE* ε3 linked to 523 VL. That is, *APOE* ε3–523 S and *APOE* ε3–523 VL haplotypes segregate to different clades on the phylogenetic tree constructed from DNA sequences of the *APOE*–523 region for AD patients and control subjects. Similarly, 523 L, which is almost always linked to *APOE* ε4, occurs in distinct clades, and the tree structure suggests that *APOE* ε4–523 L haplotype has an evolutionary history that is more similar to *APOE* ε3–523 VL than to *APOE* ε3–523 S haplotypes. The branch of the tree that contains the *APOE* ε3–523 VL and *APOE* ε4–523 L haplotypes is enriched for AD patients, whereas the branch containing *APOE* ε3–523 S is relatively enriched for the control subjects from the study group analyzed. The coincidence of distinct *APOE* ε3 haplotypes with *APOE* ε4 haplotypes on the tree suggested that these *APOE* ε3 haplotypes might also be associated with higher disease risk. In fact, the relationship is stronger with age of disease onset than with lifetime risk. Thus, by using phylogenetic mapping, resolution of the contribution of different alleles/haplotypes within this highly correlated region was discerned. There is a very large risk signal that is undeniably associated with the *APOE* ε4–523 L haplotype, but age-dependent risk can also now be assigned to haplotypes containing *APOE* ε3.

Fig. 2 illustrates stratification of the age of cognitive impairment by 523 genotype in which case status is determined clinically during an ongoing prospective study conducted by the Joseph and Kathleen Bryan Alzheimer's Disease Research Center using carefully applied, standardized, and validated neuropsychological tests [4,5]. The six Kaplan-Meier curves illustrated in Fig. 2 map a single *APOE* ε4/4 AOO distribution, two distinct distributions for *APOE* ε3/4, and three distributions for haplotypes containing *APOE* ε3/3 or *APOE*

ε2/3. Note that the three distinct AOO distributions corresponding to *APOE* ε3/3, now distinguished by the 523 genotypes—S/S, S/VL, and VL/VL—provide risk estimates for ∼40% of whites. No curves are provided for *APOE* ε2/4 or *APOE* ε2/2 separately (which together account for only 3% of the white population) because of insufficient numbers of disease onset events for *APOE* ε2 carriers. Thus, the 523 genetic locus, discovered by phylogenetic mapping, is more informative for AOO distributions than the two categories provided by *APOE* ε3/4 and *APOE* ε3/3 (523 L/L and *APOE* ε4/4 genotypes are almost perfect surrogates). By using 523 genotypes to stratify age-dependent disease risk, risk predictions can be made for >97% of the population sample.

Some recent experiments have not replicated the association of *TOMM40* 523 with AOO of AD, most notably reports from Jun and associates [6] and Cruchaga and coworkers [7]. An editorial that accompanied the article by Jun and associates [6] asserted, based on the conditional analysis presented by Jun and associates [6], that there was no independent genetic association between *TOMM40* 523, nor any other single nucleotide polymorphism in the *TOMM40–APOE* genomic region other than the two single nucleotide polymorphisms that define the *APOE* genotype, with AD risk [8]. Guerreiro and Hardy [8] (page 1243) state that "The golden rule to consider a gene as a true risk factor for a determined disease has been the ability to replicate the original association." This is a true statement; however, there are several aspects to consider when asking why the meta-analysis by Jun and associates [6] and the study by Cruchaga and coworkers [7], which Roses has previously commented on in Alzforum [9], do not replicate the association between 523 and AOO of AD as reported by Roses and colleagues [1]. Box 1 lists the requirements for replication of the findings by Roses and colleagues [1].

A number of methodological aspects may account for the differences observed between the survival (age of AD onset) analyses conducted by Jun and associates [6] compared with the data shown in Fig. 2. The article by Jun and associates [6] presents the results of a meta-analysis

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Box 1: Outline of steps necessary to corroborate the association of *TOMM40* 523 polymorphism and age of onset of Alzheimer's disease onset

- Accurate, standardized definition of clinical age of onset from prospectively monitored clinical cohorts
- An analytically validated assay for accurate *TOMM40*-523 poly-T sizing
- Differentiation of the poly-T overlap region so that L, VL, and S alleles can be assigned accurately to each individual
- Age of onset distributions for each 523 genotype (ie, not grouping three distinct 523 genotypes into a single *APOE* ε3/3 risk group using a proportion of a population)
- Accurate means of accounting for the presence of one or more *APOE* ε4 or *APOE* ε2 alleles in an individual genotype
- Determination of whether an individual's S or VL alleles are linked to an *APOE* ε2 or *APOE* ε3 allele to provide an accurate prognostic haplotype

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of 15 data sets comprised of 11,840 AD patients and 10,931 cognitively normal elderly control subjects assembled by the Alzheimer's Disease Genetic Consortium for genomewide association study research. Of all the larger data sets, only 1256 patients and 1605 control subjects were genotyped for the *TOMM40* poly-T marker. Of the complex assemblage of studies that yielded cases for the analyses by Jun and associates [6], only some were prospective, where incident cases were captured during the course of the study. Other studies were cross-sectional, or had a cross-sectional component, and would have contributed patients with retrospectively reported AOO of AD in which recall and survivor biases are likely to undermine the accuracy of the AOO data. Only one of the three studies that were genotyped for *TOMM40* by Jun and associates [6] and used in their survival analyses was strictly prospective. The Adult Changes in Thought Study (ACT) began with cognitively normal subjects and incident cases of AD were captured during the course of the study. The other two studies—Alzheimer's Disease Neuroimaging Initiative (ADNI) and the Aging Alzheimer's Disease Centers (ADC) studies—are composed of both prevalent and incident cases. It is not clear whether the ADNI or ADC data that were used by Jun and associates [6] and Cruchaga and coworkers [7] were only the incident cases. If prevalent cases were included in the AOO analyses, AOO would be reported retrospectively, and recall and survivor biases and variously defined clinical presentations would confound the interpretation of the results. Moreover, even prospective studies may introduce methodological challenges resulting from the use of different instruments or clinical staging when cross-study comparisons are made. Within-study reconciliation may also be problematic when a large number of sites are involved, as with ADNI, because there is increased risk for variability in application of the assessment instruments and thus in ascertaining AOO. Unfortunately, neither the uniformity, or lack thereof, of definitions of AD diagnosis nor an assessment of accuracy or within- and cross-study consistency for ascertaining AOO are detailed in the article. For example, among the three series used in the survival analyses [6], either the subject samples analyzed from each study are very different, or case diagnosis is very different because (*i*) the average AOO for the ACT sample is 12 years older than for ADNI or ADC; (*ii*) the AOO distribution for *APOE* ε4-plus subjects in the ACT study is pushed to later ages than the *APOE* ε4-minus distribution, and both occur at later ages than seen for the ADC and ADNI cases; and (*iii*) *APOE* ε4-plus and ε4-minus AOO distributions are very similar within ADNI and ADC samples. The critical measure for a delay of disease onset clinical trial is the age at which symptoms appear. Introducing error by allowing variability in the definition of the end point or the measurement of that end point will ensure the failure of the study.

A second potential source of measurement bias in this research is a result of the technical difficulty of measuring

accurately the length of the 523 poly-T tract and assigning it correctly to an allele category: S, L, or VL. To reproduce the original association study it would have been necessary for Jun and associates [6] to use the polymerase chain reaction (PCR)-plus-sequencing poly-T assay performed by Polymorphic DNA Technologies, Inc (Alameda, CA, USA), in the original experiments by Roses and colleagues [1], because different assays will have unique measurement biases. Approximately 9500 assays of 523 length have now been performed by Polymorphic DNA Technologies for research purposes, with continuous quality assurance monitoring. Substantial data now exist for the reproducibility and robustness of Polymorphic's assay. The assay performed by Polymorphic DNA Technologies is available for fee-for-service use at very low cost. Although some major research institutions are employing this vendor to genotype the 523 locus and *APOE*, others have developed assays de novo. These other tests are unlikely to have the analytical validation of the Polymorphic DNA Technologies assay.

The Polymorphic DNA Technologies assay uses Sanger sequencing, whereas Jun and associates [6] and Cruchaga and coworkers [7] used gel filtration sizing of PCR products of the locus. After extensive analyses, Polymorphic DNA Technologies found that the sequencing-based assay had a standard deviation of ± 1–2 T as a consequence of PCR slippage. For the longer 523 alleles, L and VL, for which the length distributions overlap, the PCR slippage may introduce error in the call of the categorical allele designation (L or VL). Fig. 1 illustrates that the boundaries of the peaks for allele lengths for L and VL are immediately adjacent so different methodologies for determining poly-T length will potentially result in calling different categorical 523 alleles. Fig. 1 further illustrates that as an internal check of the 523 allele calls at these boundaries, one can use a subject's *APOE* genotype to distinguish between L and VL, since L is (almost without exception) linked to *APOE* ε4 and VL is (almost without exception) not linked to *APOE* ε4 in whites. For example, if all poly-T lengths >30 T (ie, the gray overlap in Fig. 1) were labeled VL regardless of *APOE* genotype, these could be miscalls because some may be L alleles. Using *APOE* genotypes informs accurate calls for 523 allele lengths in this overlap region.

It is necessary to test whether allele calls are consistent among studies to know that findings in different studies are comparable. Unfortunately, although Jun and associates [6] had the means to do so, they did not report whether the results of their 523 poly-T assay were concordant with allele calls made using the sequencing-based assay performed by Polymorphic DNA Technologies. This would have been possible with the ADNI cohort because Zinfandel Pharmaceuticals, Inc (Durham, NC), sponsored the 523 and *APOE* genotype tests for a subset of the ADNI cohort, and the data were transferred to ADNI researchers. Earlier, Roses and colleagues were interested in providing 523 genotypes to the ADNI database and in co-authoring with ADNI collaborators an analysis of AOO stratified by 523 genotype.

The genotype data were provided to ADNI collaborators before the AOO data were accessed and reviewed with them. When Roses and colleagues examined how the AOOs were determined for the ADNI series, it became clear that there was insufficient standardization across sites for determining AOO, nor was there any way to verify the AOO of each case. The best estimate of year of onset of AD symptoms was recorded; however, this was not defined uniformly as the date of initial symptoms, diagnosis of mild cognitive impairment (MCI) by stated criteria, or diagnosis of AD by a clinician using standard diagnostic criteria of AD or MCI. Because of the uncertainty of the AOO data, Roses and colleagues determined that a clinically useful article or a true replication of the original association study could not be produced, and they chose not to participate in publication of an AOO analysis of this data set.

The accuracy of the Kaplan-Meier curves of AOO stratified by 523 genotype reported by Jun and associates [6] and also by Cruchaga and coworkers [7] can reasonably be questioned considering the ambiguity introduced by the nonstandardized determination of AOO, in which at least some of the determinations are retrospective, and the potential for miscalling of the 523 alleles in the area of overlap. Consistent 523 genotype calls and accurately recorded and standardized determination of age of AD onset (within a year or two in prospective clinical follow-up) should be the minimum requirement for a replication study (Box 1).

The *APOE* and *TOMM40* 523 genotype combination is informative of age-dependent risk of AD for the white population. The importance of the relationship between *APOE* and *TOMM40* 523 is not the independent statistical association with AOO for AD. Rather, the use of the 523 poly-T genotype increases the proportion of the at-risk population for which genetics may provide an estimate of age-dependent risk, and also improves resolution of age-dependent risk for individual subjects, to support clinical research and development of much-needed therapies. Although the *APOE* ε4/4 genotype alone is informative of age-dependent risk of disease for 2% of the white population, *APOE* and 523 genotype information, when combined, is informative of age-dependent AD risk for >97% of whites.

Conducting clinical trial practically to test a therapy for delay AD onset requires a means to identify those at increased risk of developing disease within the expected duration of a trial. Without a risk enrichment strategy, prevention trials will likely be much larger in size and/or take longer to accrue sufficient numbers of end point conversions to demonstrate statistical differences between treated and untreated groups. The *APOE* and *TOMM40* 523 genotype combination appears to provide the means to do this. The genotype combination as a predictor of age-dependent risk of cognitive decline will be validated in a prospective study, using standardized clinical assessments for identifying onset of MCI resulting from AD [10], uniformly conducted by trained clinicians at defined time intervals, and using a technically validated assay for calling genotypes. If, as a result of this study, it is demonstrated

that the age of AD onset can be delayed by a therapeutic, then the statistical significance of *TOMM40* relative to that of *APOE* becomes a relatively trivial discussion.

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