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C9ORF72 hexanucleotide repeat expansions in clinical Alzheimer's disease

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Abstract

Objective—Hexanucleotide repeat expansions in *C9ORF72* underlie a significant fraction of frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS). This study investigates the frequency of *C9ORF72* repeat expansions in clinically diagnosed late-onset Alzheimer's disease (AD).

Design, setting and patients—This case-control study genotyped the *C9ORF72* repeat expansion in 872 unrelated familial AD cases and 888 controls recruited as part of the NIA-LOAD cohort, a multi-site collaboration studying 1000 families with two or more individuals clinically diagnosed with late-onset-AD.

Main Outcome Measure—We determined the presence or absence of the *C9ORF72* repeat expansion by repeat-primed PCR, the length of the longest non-expanded allele, segregation of the genotype with disease, and clinical features of repeat expansion carriers.

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Results—Three families showed large *C9ORF72* hexanucleotide repeat expansions. Two additional families carried more than 30 repeats. Segregation with disease could be demonstrated in 3 families. One affected expansion carrier had neuropathology compatible with AD. In the NIA-LOAD series, the *C9ORF72* repeat expansions constituted the second most common pathogenic mutation, just behind the *PSEN1* A79V mutation, highlighting the heterogeneity of clinical presentations associated with repeat expansions.

Interpretation—*C9ORF72* repeat expansions explain a small proportion of patients with a clinical presentation indistinguishable from AD, and highlight the necessity of screening "FTD genes" in clinical AD cases with strong family history.

Introduction

Alzheimer's Disease (AD) constitutes the most common cause of dementia in clinical practice[1]. AD manifests clinically with progressive cognitive dysfunction with prominent memory loss, while its neuropathology is characterized by the presence of extracellular neuritic plaques and intracellular neurofibrillary tangles[1]. Familial AD can be caused by mutations in amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*)[2–6]. Interestingly, mutations in genes typically associated with frontotemporal dementia (FTD), including microtubule associated protein tau (*MAPT*) and granulin (*GRN*), have also been reported in clinical AD cases[6–13], highlighting the overlapping phenotypes of AD and amnestic-FTD.

Recently, an intronic hexanucleotide repeat (GGGGCC) in the *C9ORF72* gene was identified as a frequent cause of sporadic and familial FTD and amyotrophic lateral sclerosis (ALS)[14, 15]. Because some individuals with clinical AD carry mutations in FTD genes[7–13, 16–18], two studies investigated the role of *C9ORF72* expansions in AD, with conflicting results. The first report identified *C9ORF72* repeat expansions in 3 of 342 cases with familial AD and 6 of 711 cases with sporadic AD. Interestingly, reanalysis of autopsy material from two expansion carriers showed FTD pathology[19], suggesting that both patients had amnestic presentations of FTD rather than AD. The second study identified no repeat expansions in 568 patients with probable AD by clinical criteria and concluded that *C9ORF72* repeats are specific for FTD[20].

To clarify the role of *C9ORF72* hexanucleotide repeat expansions in familial late-onset AD, this study screens 872 unrelated AD cases and 888 unrelated controls and investigates whether expanded repeats are associated with age at dementia onset. Furthermore, we address whether a larger, but non-expanded, GGGGCC allele is associated with risk for AD or age at onset.

Material and Methods

Patients

Individuals from 872 unrelated families with at least two individuals affected by AD and 888 unrelated unaffected controls from the National Institute of Aging Late Onset Alzheimer Disease Family Study (NIA-LOAD Family Study) were included. All AD cases had been diagnosed with dementia of the Alzheimer's type using criteria equivalent to the National Institute of Neurological and Communication Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) for probable AD[21, 22]. All patients had a family history of Alzheimer's disease, but not other types of dementia or other neurodegenerative disease. Probands were required to have a diagnosis of definite or probable late-onset AD (onset >60) and a sibling with definite, probable or possible late-onset AD with a similar age at onset. A third biologically-related family member (first,

second or third degree) was also required, regardless of affected status. If unaffected, this individual had to be ≥60 years of age, but ≥50 years of age if diagnosed with LOAD or mild cognitive impairment[23]. Within each pedigree, we selected a single individual to screen by identifying the youngest affected family member with the most definitive diagnosis (i.e. individuals with autopsy confirmation were chosen over those with clinical diagnosis only). A summary of the demographics of all subjects is shown in Table 1. Written informed consent was obtained from all participants, and the study was approved by local IRB committees.

Genetic analysis

Repeat-primed PCR—The presence of the expanded hexanucleotide repeat and the number of repeat units in the longest allele was determined using previously reported methods for repeat-primed PCR and fluorescence-based fragment size analysis[14]. Briefly, repeat-primed PCR was performed in a total reaction volume of 28 μl containing 100 ng genomic DNA, 1× FastStart PCR Master Mix (Roche Applied Science, Indianapolis, IN, USA), 3.5% DMSO, 1× Q solution (Quiagen, Valencia, CA) and 0.18 mM of deazaGTP (NEB, Ipswich, MA). Primer concentrations and sequences (chr9:27563580F and chr9:27563465R) were the same as previously reported[14]. PCR products were run on an ABI® 3130×l Genetic Analyzer (Applied Biosystems) and analyzed using GeneMapper®. Consistent with standards used in prior studies, a sample was considered to have a repeat expansion when assay replicates demonstrated >30 peaks and a decrementing saw-tooth pattern with 6 base-pair periodicity (Supplementary Figure 1). A normal repeat alleles was defined as 30 or fewer peaks[14, 15, 19, 20].

Cross-repeat PCR—In all cases, repeat expansions identified with repeat-primed PCR were confirmed by attempting to PCR across the GGGGCC repeat using fluorescence-based fragment size analysis ("cross-repeat PCR") as reported with minor modifications[14]. Briefly, using previously reported primers with fluorescent labels, PCR was performed on 100 ng genomic DNA with 1× Phusion High-Fidelity DNA Polymerase Master mix (Thermo Fisher Scientific, Lafayette, CO), 5% DMSO, 1.25M betaine, and 0.2 mM deazaGTP. Products were run on a 2% agarose gel for visual inspection and then analyzed for fragment size determination as described for repeat-primed PCR. Based on our analysis, cross-repeat PCR can amplify alleles with ≤35 GGGGCC repeats but not the 700–1600 repeats reported for pathological expansions[14]. To investigate repeat expansion segregation, we also genotyped all family members (n=491) from every pedigree with ≥10 hexanucleotide repeats.

Southern Blot—Southern blot hybridization analysis was conducted using previously described methods and probe sequences[14] to estimate the number of repeat units in expansion carriers.

Risk Haplotype Genotyping—We also analyzed all repeat expansion carriers for the 24 SNP "at-risk" haplotype that has been associated with pathologically expanded *C9ORF72* repeats[24]. All samples were genotyped with the Illumina Human 610 Beadchip with direct genotyping of all analyzed SNPs. Stringent quality control criteria was applied to remove low-quality SNPs[23]. We used the entire NIA-LOAD GWAS dataset and the HapMap CEU population as reference populations. MACH[25] software was used to phase the 24 SNPs.

Statistical and Bioinformatic Analyses

A linear regression model (SAS) was used to test whether the number of GGGGCC repeats was associated with risk for AD, including age, gender and *A*POE genotype as covariates. Association with age at onset (AAO) was carried out using the Kaplan-Meier method and

tested for significant differences using a Cox proportional hazards model (proc PHREG, SAS) that included gender and *APOE*.

RESULTS

C9ORF72 repeat expansion frequency and segregation in AD families

We screened 872 unrelated familial AD cases and 888 unrelated controls for expansions of the hexanucleotide repeat in *C9ORF72*. Five Caucasian individuals with a clinical diagnosis of AD (0.57%) and 1 normal control (age 73) showed an abnormal expansion with repeat-primed PCR, defined as more than 30 repeats with 6 base-pair periodicity and the expected decrementing saw-tooth pattern (Supplementary Figure 1). To better clarify the number of repeats in these patients, we attempted to PCR across the GGGGCC repeat. However, in all 6 individuals, only a single peak representing the normal size allele was obtained. Southern-blot of AD probands showed expansions of 1200–1300 GGGGCC repeats for Families 1, 2 and 3, but only 35–100 repeat units in Families 4 and 5 (data not shown). Regardless of repeat size, all 5 AD cases were found to carry the "at-risk" haplotype associated with pathological repeat expansions in all FTD and ALS patients reported to date (Supplementary Table 1)[24]. All 5 of these patients had also tested negative for the most common mutations in *APP, PSEN1, PSEN2, GRN* and *MAPT*[16].

Pedigrees for the 5 families with abnormal (>30) *C9ORF72* repeats are shown in the Figure and clinically summarized in Table 2. All additional affected individuals had been diagnosed with probable AD based on the NINCDS-ADRDA criteria. DNA for additional family members was available for all but Family 1. Family 2 showed complete segregation of the large repeat expansion and risk haplotype with disease status (Figure, Supplementary Table 2). Family 3, in which all the affected individuals are APOE 3/3 homozygous, showed segregation of the expanded repeat and risk haplotype except for a single individual who developed dementia with only 7 repeat units. Additionally, this individual did not carry the risk haplotype and had dementia onset at a later age than relatives with repeat expansions. In Families 4 and 5 (repeat expansions between 35 and 100 repeats), the characteristic repeat-primed chromatogram pattern and the risk haplotype segregated perfectly with disease status. Overall, in these five families, 10 of 11 affected individuals for whom DNA was available carried abnormal GGGGCC expansions and the risk haplotype. It is important to note that all the individuals with more than 30 repeats also carried the risk haplotype.

The age at onset for individuals with more than 30 repeats was earlier than cases with normal repeat alleles (65.6 ± 5.5 vs 71.4 ± 6.8; p=0.04). This association retained statistical significance even after APOE genotypes were included in the model (p=0.03).

Clinical case and neuropathology descriptions

The proband of Family 1 (1200–1300 repeats), an APOE 2/3 carrier, was diagnosed with dementia, Alzheimer type, at age 73. Formal neurocognitive testing was not available, but prominent memory loss with repetitive questioning and wandering behavior had developed by age 78. Upon death 12 years after diagnosis, a brain autopsy was carried out by the Brain Bank at McLean Hospital (Belmont, MA) in 1999. Microscopic examination found extensive plaque and tangle pathology. Neurofibrillary tangles and neuritic plaques (>40 plaques per 100× field) were present throughout the neocortex, hippocampus, amygdala and nucleus basalis of Meynert. Scattered neocortical Lewy bodies were seen. Severe neuronal loss in the substantia nigra pars compacta was accompanied by a few Lewy bodies. This case would be diagnosed as having NIA-Reagan high likelihood criteria for AD and coexisting neocortical-predominant LBD using current criteria[26, 27]. Tissue was no longer

available to carry out additional relevant staining (e.g. tau, ubiquitin, TDP-43, or p62), preventing an assessment for possible FTD pathology.

Aside from age at symptom onset and meeting clinical criteria for probable AD, limited clinical information was available for the remaining index cases. The proband of Family 2 (1200–1300 repeats) was diagnosed clinically with probable AD at the age of 71, three years after disorientation and memory loss began. By the time of study inclusion at age 74, the patient was non-verbal and too impaired to participate in cognitive testing. The proband of Family 3 (1200–1300 repeats) was diagnosed with probable AD at the age of 60. Three years later, the Clinical Dementia Rating (CDR) was 0.5, which progressed over three years to CDR=1.0. Review of limited caregiver reports suggested difficulties with disinhibition and anxiety were out-of-proportion to memory impairment. No additional clinical information was available for members of Families 4 and 5 (35–100 repeats in each).

Associations of *C9ORF72* repeat length in AD

Previous studies in ALS and FTD have clearly demonstrated that *C9ORF72* repeat expansions are causative for disease[14]. However, the minimum repeat number required for disease has not been established. Furthermore, it is not known if higher repeat numbers (but still within the normal range) are associated with risk for ALS or FTD. To address this question in AD, we compared the longest non-expanded allele in cases and controls. The average longest allele was not statistically different from controls (6.5± 4.1 repeat units vs 4.48 ± 3.7, p=0.10), and the distribution of longest allele lengths were similar. Thus within the normal range, higher repeat numbers do not appear to be a risk factor for AD in this population. Furthermore, we found no association between the length of the longest non-expanded allele and age at onset (p=0.52), nor evidence for an interaction with APOE genotype.

Comparison of the *C9ORF72* repeat expansion frequency with other pathogenic gene mutations within the same cohort

In a previous study, we sequenced *APP, PSEN1, PSEN2, MAPT and GRN* genes in a discovery series comprised of 439 cases included in this study[16]. The most common pathogenic mutation identified by sequencing in the discovery series (*PSEN1*, A79V), was then genotyped in the entire cohort (follow-up series). Overall the A79V mutation was found in 4 of the 872 cases (0.48%)[16] compared to the 5 pedigrees where abnormal *C9ORF72* repeat expansions were found. Furthermore, we analyzed the overall frequency of "AD gene" mutations (*APP, PSEN1,* and *PSEN2*), vs. FTD gene mutations (*MAPT, GRN* and *C9ORF72*), and found that 1.82% of probands carry a pathogenic, or very likely pathogenic, mutation in *APP, PSEN1 and PSEN2*, while a slightly large number (1.94%) have mutations in *MAPT, GRN*, or *C9ORF72*.

DISCUSSION

This study assessed *C9ORF72* hexanucleotide expansions in familial late-onset AD cases and normal controls, identifying 5 AD families carrying abnormal *C9ORF72* hexanucleotide repeat expansions. This frequency is very similar to that found in an independent AD series[15], but significantly lower than in FTD or ALS.

Three families with clinical AD (0.34%) were found to have repeat expansions in the range reported for FTD and ALS (>1000). In a previous report documenting expansions in clinically diagnosed AD, re-evaluation of autopsy material demonstrated FTD pathology and suggested that AD cases with *C9ORF72* repeat expansions represent amnestic variants of FTD[9, 14, 15, 28, 29]. We were unable to perform an equivalent analysis because autopsies had

not been performed or tissue was no longer available. Therefore, even in the proband from Family 1, where the neuropathology showed coexisting AD and LBD pathology, we cannot rule out the possibility that the families identified in this study also represent amnestic presentations of FTD rather than AD. It is notable that several *C9ORF72* FTD cases reported, several have shown concurrent AD pathology[9, 14, 15, 28, 29] and an additional expansion carrier with FTD+ALS demonstrated enough plaque and tangle pathology to meet diagnostic criteria for AD[28]. These cases suggest that some individuals with *C9ORF72* repeat expansions could present with clinical symptoms of AD and have a high enough burden of AD neuropathology that biomarker analysis (cerebrospinal tau or Aβ, and/or PIB-PET neuroimaging) would also support an AD diagnosis. This hypothesis is supported three recently reported individuals with early onset AD, CSF profiles typical of AD[30], who were found to carry *C9ORF72* repeat expansions. In this setting, the correct diagnosis (amnestic FTD) would presumably only be reached by neuropathologic studies or genetic testing. Our cases and previous studies reinforce the heterogeneous clinical and neuropathological presentations of *C9ORF72* repeat expansions (ALS, FTLD, FTLD-ALS, and clinical AD).

We also identified two families carrying smaller, but abnormal repeats (>35, but smaller than 100 units). Despite segregating with disease status, it remains unclear whether these smaller repeat expansions cause disease, increase risk for dementia, or are incidental. Future studies correlating quantified repeat sizes with disease status will be required to answer this question.

Although the frequency of large *C9ORF72* repeat expansions is low in our cohort, it is the second most common pathogenic mutation (3/872), just behind *PSEN1* A79V (4/872). In addition, mutations in “FTD genes” were as common as mutations in “AD genes” (1.94% compared to 1.87%). Our results confirm that the clinical phenotype of mutations in “FTD genes”, including *GRN, MAPT* and *C9ORF72*, can be clinically indistinguishable from typical AD. This fact has important implications for clinicians, who should consider both “FTD” and “AD” genes when evaluating families with strong histories of AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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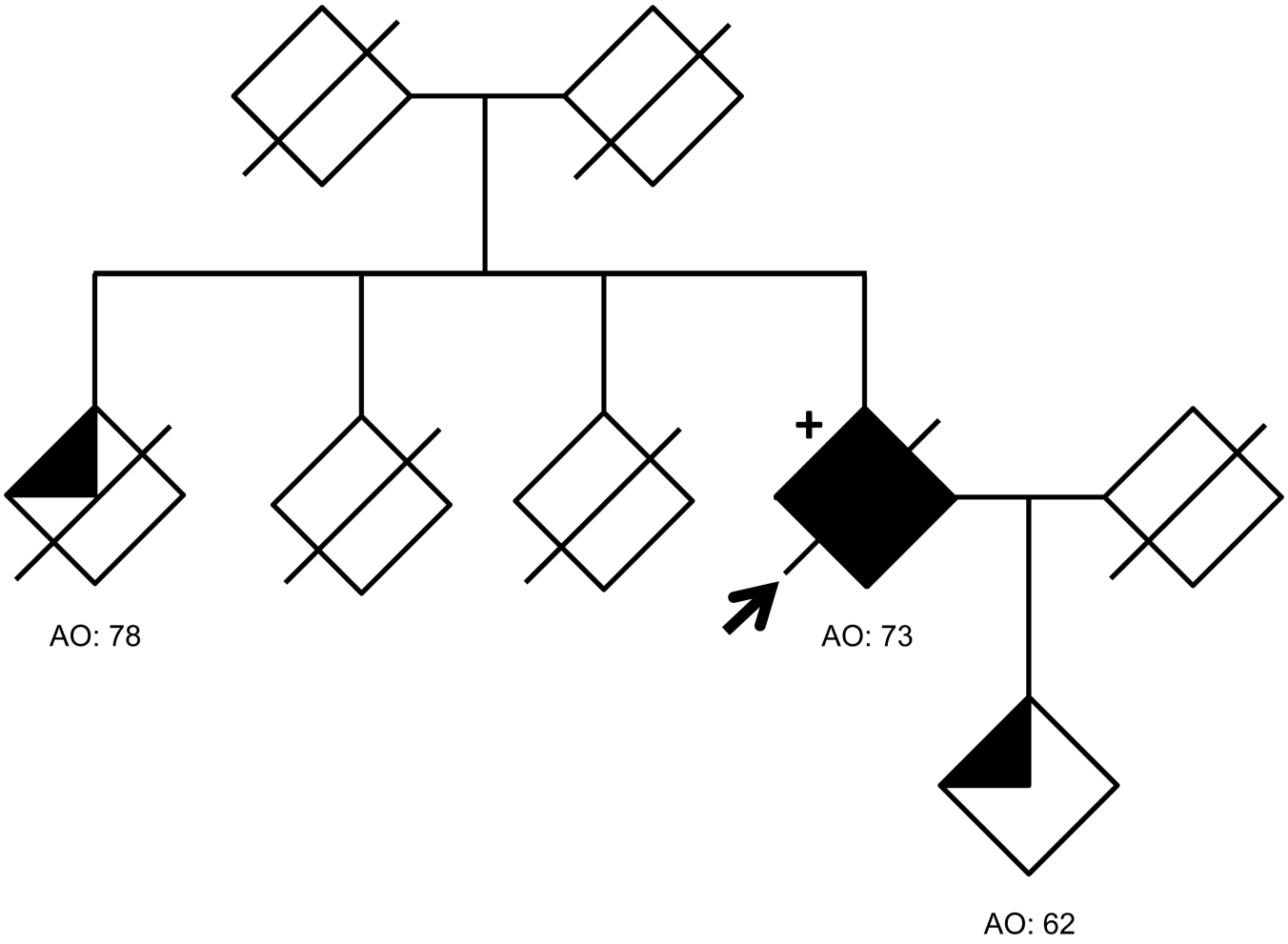

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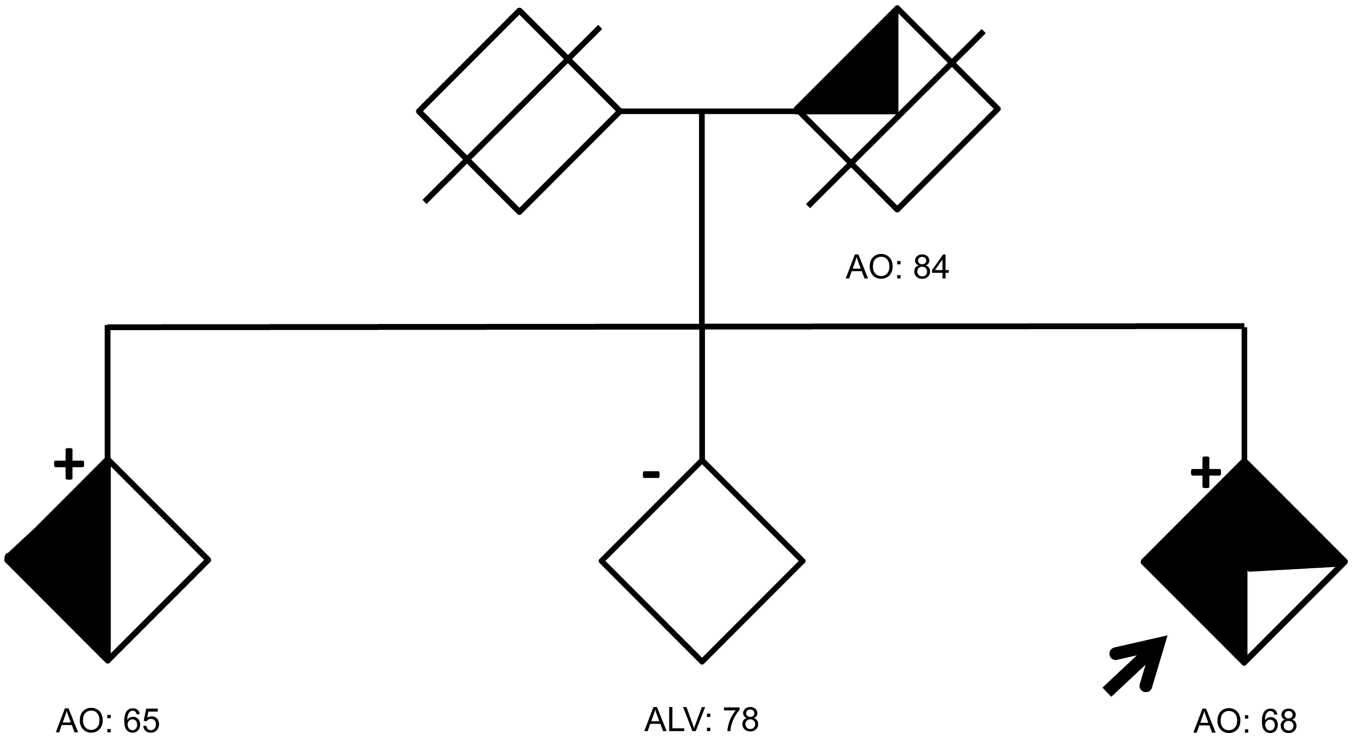

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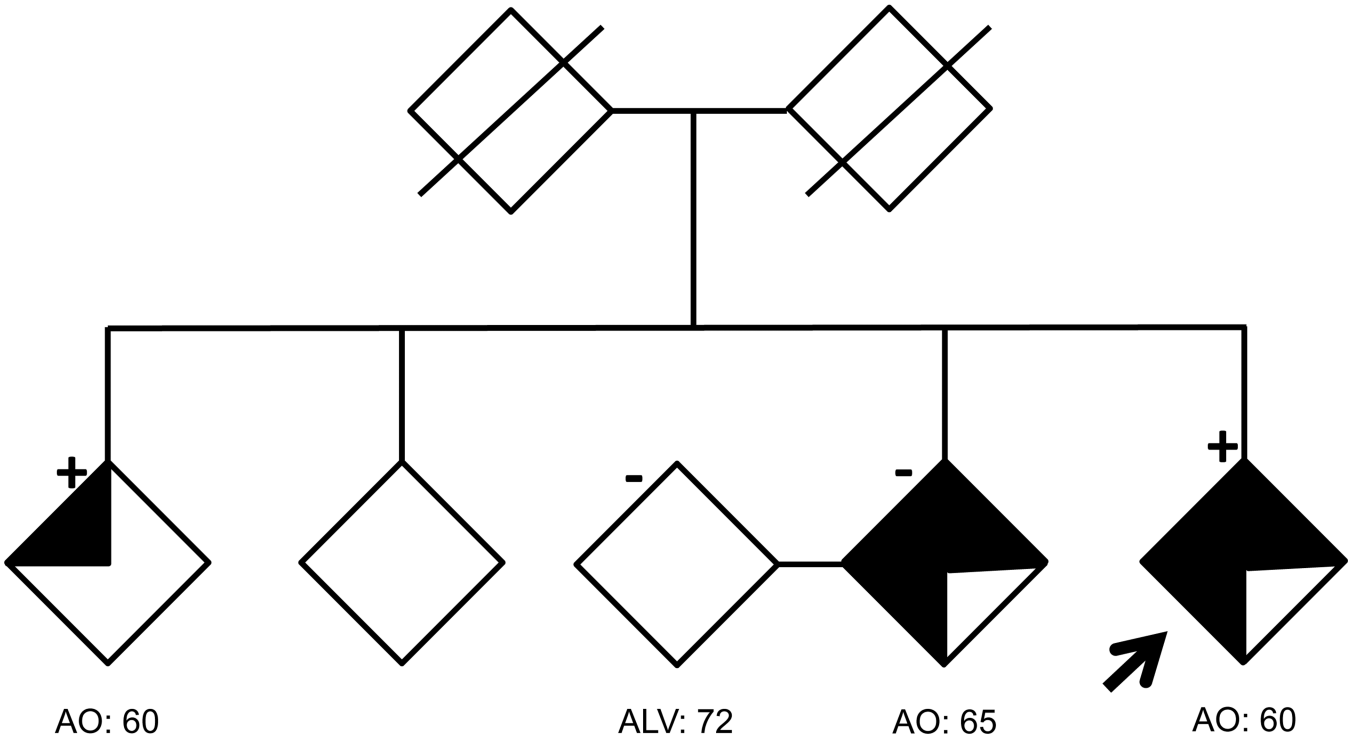

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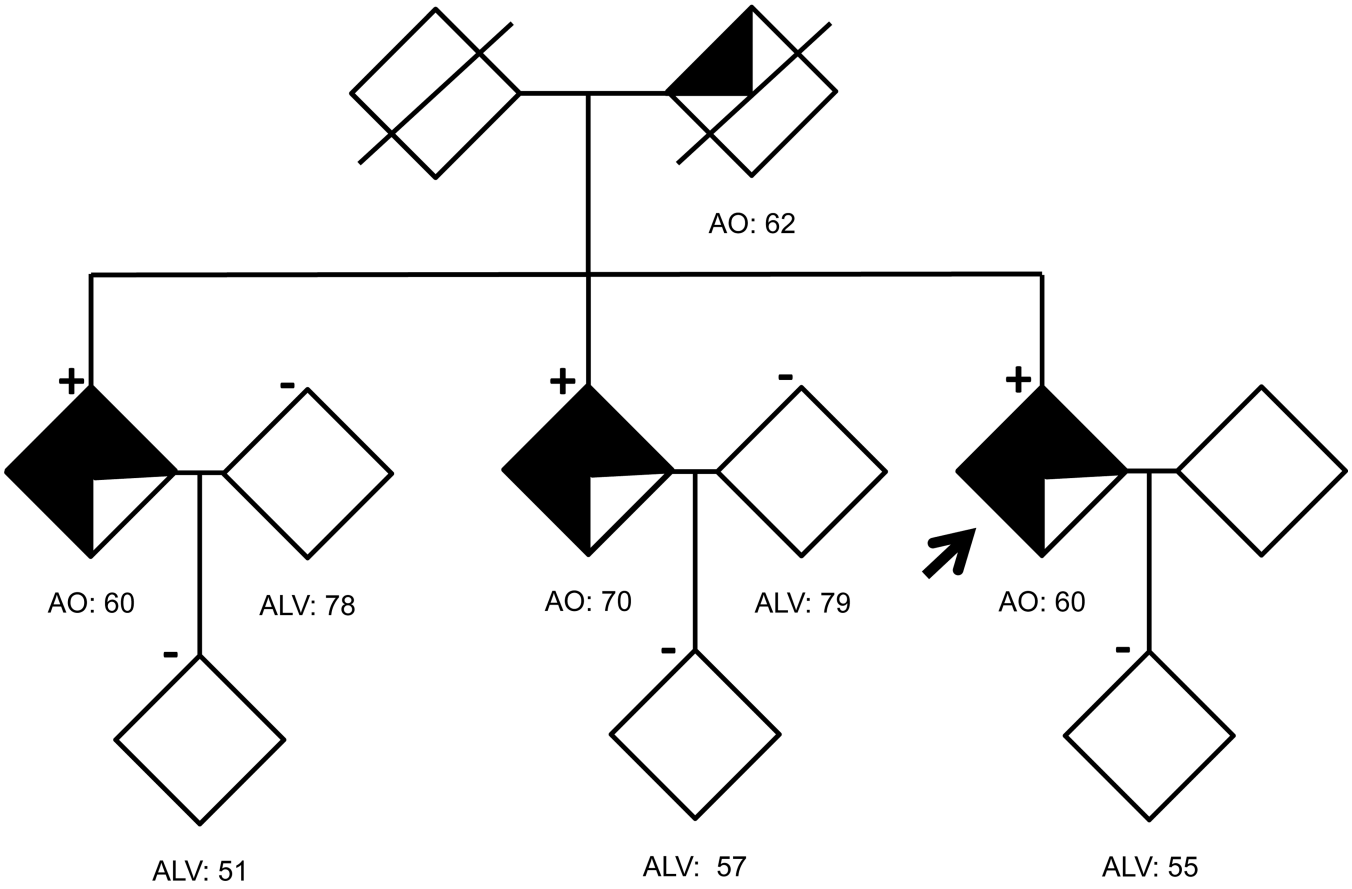

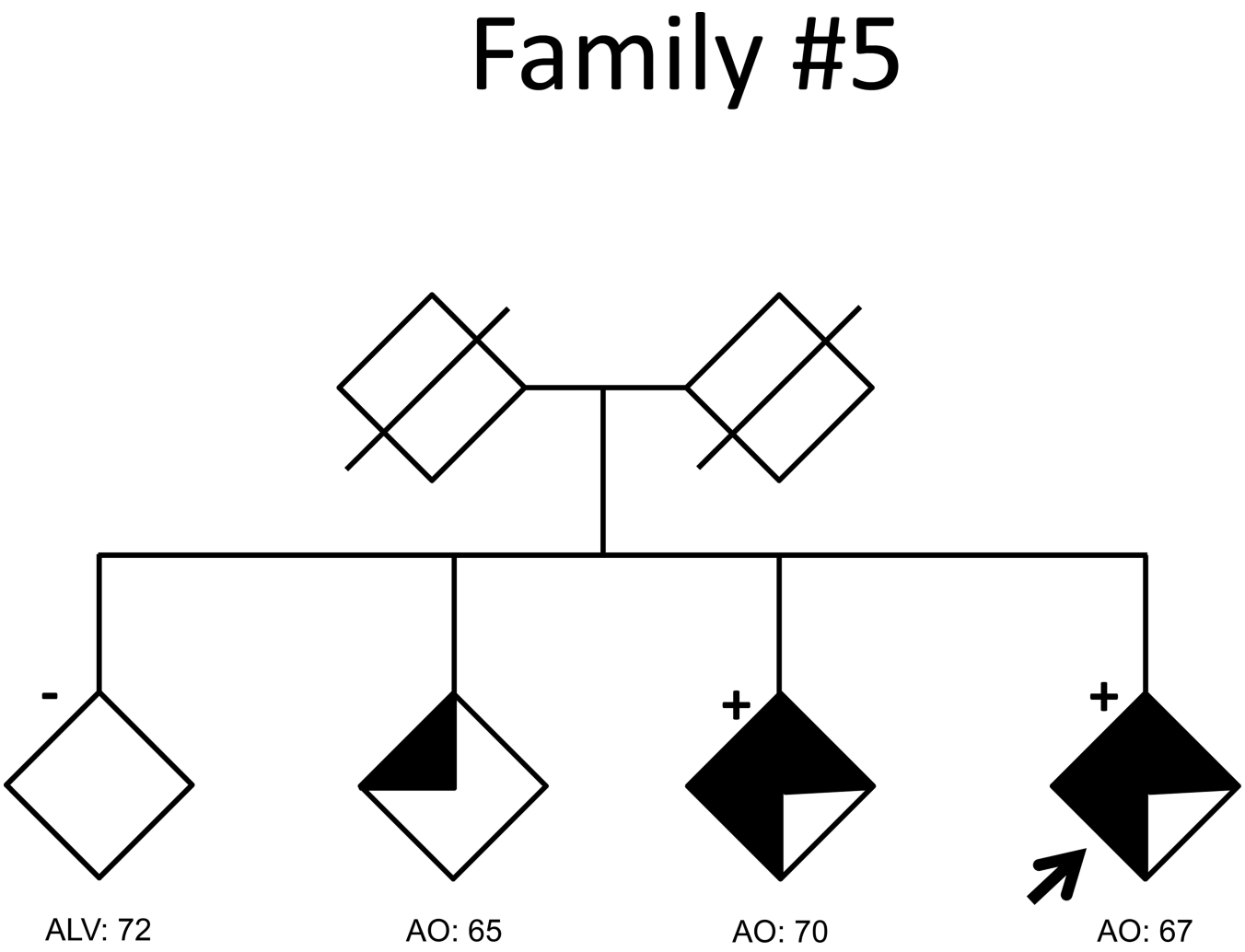

Figure 1. AD pedigrees carrying abnormal *C9ORF72* repeat expansions

Fully shaded symbols indicate autopsy-confirmed AD. Clinical diagnoses were made using NINCDS-ADRDA criteria, with three-quarters and one-half shading indicating probable and possible AD, respectively. Repeat expansion genotypes are indicated by '+' when present, and '−' when absent. The absence of a genotype symbol indicates that DNA was not available for analysis. An arrow marks the proband of each pedigree.

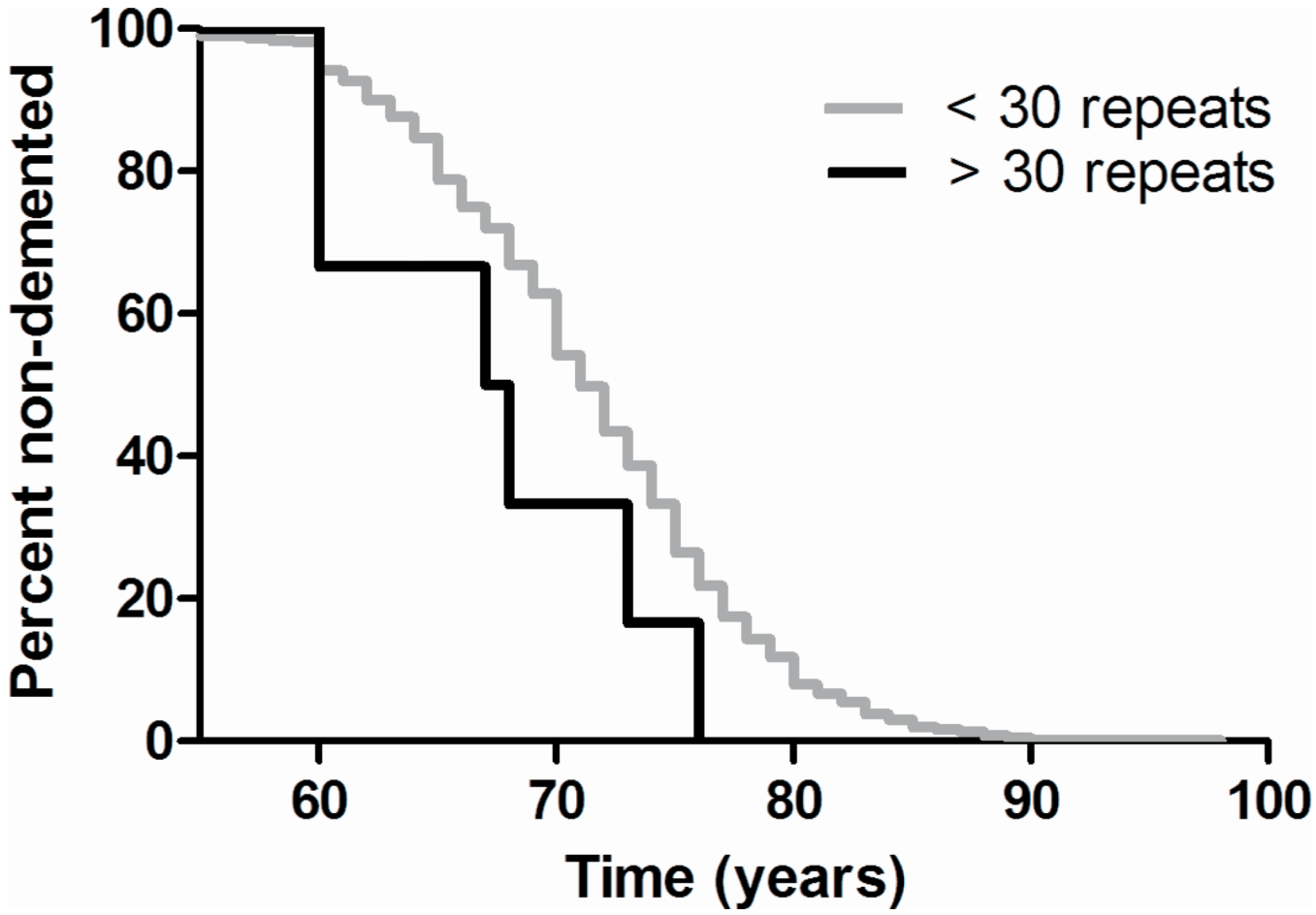

Figure 2. C9ORF72 repeat expansions correlate with an earlier age at onset

Age at onset (AAO) was analyzed for association with repeat expansion genotypes by the Kaplan-Meier method and tested for significant differences using a proportional hazards model (proc PHREG, SAS). Repeat expansion carriers show an earlier AAO than non-carriers (68.25 ± 5.8 vs 71.4 ± 6.8; p=0.04).

Table 1

Cohort Demographics

	AD Cases	Controls
Number	872	888
Age (years)[a]	75.8 ± 8.9	71.41 ± 6.7
Age range (years)	42–101	48–98
Male (%)	33.9	39.7
APOE 4+ (%)[b]	76.2	30.6
No. C9ORF72 (%)	5 (0.57)	1 (0.11)

[a] For AD cases, age indicates onset of symptoms but refers to age at last assessment for controls.

[b] APOE 4+ refers to those carrying at least 1 APOE 4 allele.

Table 2

Segregation analysis of pedigrees with repeat expansions

		Affected with AD		Unaffected	
C9ORF72 repeat expansion		+	−	+	−
Overall	# of Individuals	10	1	0	8
	Mean Age ± SD[a]	65.3 ± 4.9	65	60	69.2 ± 11.3
	Age range	60–73			55–79
Family 1	# of Individuals	1	0	0	0
	Ages	73	-	-	-
Family 2	# of Individuals	2	0	0	1
	Ages	65,68	-	-	78
Family 3	# of Individuals	2	1	0	1
	Ages	60	65	-	72
Family 4	# of Individuals	3	0	0	5
	Ages	60,60,70	-	-	55–79
Family 5	# of Individuals	2	0	0	1
	Ages	67,70	-	-	72

[a]For AD patients, age refers to age at symptom onset, while for unaffected individuals it refers to the age at last assessment.