Human Molecular Genetics, 2003, Vol. 12, No. 21 **2765–2776**
DOI: 10.1093/hmg/ddg310

Genetic association of Alzheimer's disease with multiple polymorphisms in alpha-2-macroglobulin

Aleister J. Saunders[1,†,‡], Lars Bertram[1,†], Kristina Mullin[1], Andrew J. Sampson[1], Khushal Latifzai[1], Sanjay Basu[1], Jennifer Jones[1], Devon Kinney[1], Laura MacKenzie-Ingano[1], Stephen Yu[1], Marilyn S. Albert[2,3], Thomas J. Moscarillo[3], Rodney C.P. Go[4], Susan S. Bassett[5], Mark J. Daly[6], Nan M. Laird[7], Xin Wang[8], Gonul Velicelebi[8], Steven L. Wagner[8], David K. Becker[8], Rudolph E. Tanzi[1,*] and Deborah Blacker[3,9]

[1]Genetics and Aging Research Unit, Center for Aging, Genetics, and Neurodegeneration, Department of Neurology, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA, USA, [2]Department of Neurology, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA, USA, [3]Gerontology Research Unit, Department of Psychiatry, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA, USA, [4]Department of Epidemiology, School of Public Health, University of Alabama, Birmingham, AL, USA, [5]Department of Psychiatry and Behavioral Sciences, Johns Hopkins University Medical Institutions, Baltimore, MD, USA, [6]Whitehead Institute for Biomedical Research, Cambridge, MA, USA, [7]Department of Biostatistics, Harvard School of Public Health, Boston, MA, USA, [8]Neurogenetics Inc., La Jolla, CA, USA and [9]Department of Epidemiology, Harvard School of Public Health, Boston, MA, USA

Received June 12, 2003; Revised and Accepted August 28, 2003

Alpha-2-Macroglobulin (*A2M*) is a highly plausible candidate gene for Alzheimer's disease (AD) in a region of chromosome 12 that has numerous independent reports of genetic linkage. We previously reported that a 5 bp deletion in *A2M* was associated with AD in a subset of the National Institute of Health (NIMH) Genetics Initiative AD family sample. Efforts to replicate this association finding in case–control samples have been largely negative, while those in family samples have been more positive. We hypothesized that variable findings regarding this deletion, along with variable reports of association with V1000I, another polymorphism in the gene, result from linkage disequilibrium in the area as well as ascertainment differences between family-based and case–control studies. Thus, we resequenced the *A2M* locus to identify novel polymorphisms to test for genetic association with AD. We identified seven novel polymorphisms and tested them in the full NIMH sample of 1439 individuals in 437 families. We found significant genetic association of the 5 bp deletion and two novel polymorphisms with AD. Substantial linkage disequilibrium was detected across the gene as a whole, and haplotype analysis also showed significant association between AD and groups of *A2M* polymorphisms. Several of these polymorphisms and haplotypes remain significantly associated with AD even after correction for multiple testing. Taken together, these findings, and the positive reports in other family-based studies, continue to support a potential role for *A2M* or a nearby gene in AD. However, the negative case–control studies suggest that any underlying pathogenic polymorphisms have a modest effect, and may operate primarily among individuals with a family history of AD.

*To whom correspondence should be addressed at: Genetics and Aging Research Unit, C3809, 114 16th St, Charlestown, MA 02129, USA. Tel: +1 617 726 6845; Fax: +1 617 724 1823; Email: tanzi@helix.mgh.harvard.edu
†The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.
‡Present address:
Department of Bioscience and Biotechnology, Drexel University, 3141 Chestnut St, Philadelphia, PA 19104, USA.

INTRODUCTION

Alzheimer's disease (AD) is one of the greatest public health problems in the USA, and its impact will only increase with demographic changes anticipated in the coming decades. Our growing understanding of AD genetics has been central to the explosion in knowledge of AD biology. Although *APOE*-ε4 is well established as a potent susceptibility gene for late-onset AD, a recent segregation analysis estimated that ~90% of the total genetic variance for the disease remains unknown, and that four to seven additional AD genes of at least moderate effect remain to be discovered (1). In an effort to identify these unknown AD susceptibility genes, the authors and others are looking for candidate genes to test for association with the disease. Genetic, neuropathological and biochemical evidence indicates that excessive amyloidogenic processing of the β-amyloid precursor protein, generating excessive β-amyloid (Aβ) peptide, plays a fundamental role in the pathogenesis of AD, so genes coding for proteins that are involved in Aβ production or degradation pathways are compelling biological candidates for AD. One of the most studied candidate genes is alpha-2-macroglobulin (*A2M*), which is supported both by genetic evidence of linkage to chromosome 12p (2–9) and biological evidence for the α2-M protein's role in Aβ catabolism, as detailed below. We initially reported significant genetic association of a splice site deletion in exon 18 of *A2M* with AD (10) in 104 families of the NIMH Genetics Initiative sample. Here we describe the results of testing this polymorphism and an additional seven *A2M* polymorphisms in the complete NIMH Genetics Initiative sample of 437 families.

α2-M and its potential role in AD pathogenesis

α2-M is a 718 kDa glycoprotein found at high concentrations in the serum (11) and cerebrospinal fluid. The best-studied function of α2-M is its pan-protease inhibitory activity (12). A variety of proteases cleave any of a number of susceptible peptide bonds in the bait region (residues 666–706) of the α2-M tetramer (12–14). Cleavage triggers a large conformational change in the α2-M–protease complex, referred to as activation, which results in entrapment of the protease within the tetramer (11). Unable to dissociate from α2-M, proteases are still able to cleave small peptide substrates (15,16). Protease-mediated activation also results in exposure of the α2-M receptor–LRP binding domain (17). Exposure of this LRP binding domain is a prerequisite for LRP-mediated endocytosis of α2-M/ligand complexes (11). Internalization of α2-M–ligand complexes can be targeted for degradation in the lysosome.

In addition to inhibiting a variety of proteases, α2-M binds the Aβ peptide specifically and tightly (18,19). There are three biological manifestations of the Aβ–α2-M interactions that may be directly relevant to the etiology of AD. First, the interaction between α2M and Aβ prevents Aβ fibril formation and fibril associated neurotoxicity (18,19). Second, protease activation of α2-M/Aβ complexes or protease activation of α2-M followed by Aβ binding can promote the protease-mediated degradation of α2-M-bound Aβ (15). Because of its small size, Aβ is accessible to the encaged protease, permitting proteolysis. Third, protease activated α2m/Aβ complexes may undergo LRP-mediated endocytosis followed by trafficking of Aβ to the lysosome for degradation (20). The established late-onset risk factor ApoE-ε4 accelerates Aβ deposition (21), and ApoE is found in a complex with α2-M in plasma (22). Taken together, these findings indicate that *A2M* is a superb biological candidate gene for AD.

Genetic linkage findings in the vicinity of *A2M*

There have been numerous reports of genetic linkage of AD to loci on chromosome 12 (2–9). The reported linkage peaks cluster into two distinct regions. One region is located at the p-ter, from ~6 to 30 cM, in the same vicinity as the *A2M* gene (located at ~20 cM) (2,3,6,9). The other region is peri-centromeric, from ~48 to 68 cM, in the same vicinity as the *LRP* gene (~68 cM) (4,6–8). In general, evidence for linkage increases slightly in all studies when analyses are limited to families without an *APOE*-ε4 allele. Of note, although earlier reports (3,9) using a subset of the NIMH data report linkage in the vicinity of *A2M*, our recent genome screen of the full NIMH sample does not show a signal in this region (23).

Prior association findings for *A2M*

Based on the biological and genetic data above, we and others have tested polymorphisms in *A2M* for genetic association with AD (Table 1). Family-based association studies have been largely supportive of genetic association between *A2M* and AD. Our group initially reported an association between AD and an intronic deletion polymorphism adjacent to exon 18, *A2M*-18i (referred to as *A2M*-2 in our original report) (10), in a sample of 104 discordant sibships ascertained through the NIMH Genetics Initiative, and this finding held up in an enlarged 120-sibship subset from the NIMH sample as well (24). As can be seen in Table 1, three of the remaining four reported family-based samples also gave some evidence for association (25–28). On the other hand, case–control association studies of AD and this deletion polymorphism have been largely negative. As can be seen in Table 1, of the 32 published case–control studies, only five report positive association findings (29–33), a figure only slightly larger than the number expected by chance (even without accounting for publication bias).

A second polymorphism in *A2M*, a non-synonymous SNP in exon 24 (*A2M*-24e) that results in Val to Ile substitution at amino acid position 1000, has also been widely investigated in association studies (Table 1). Six case–control studies report some evidence of association between AD and *A2M*-24e (25,29,34–37). Ten other case–control studies did not find significant association, nor did three family-based association studies (25,27,38). Finally, one report analyzed both the *A2M*-18i and -24e polymorphisms together as a haplotype and found significant haplotypic association (39).

For both case–control and family-based studies, and for both the 18i deletion and the 24e substitution, stratification on *APOE* status, age and neuropathological confirmation of AD do not generally affect the evidence for association.

In an effort to further clarify these inconsistent association findings, and to identify a possible pathogenic polymorphism underlying the association signal, we resequenced the *A2M* locus and then tested six novel polymorphisms, the 24e

Table 1. Reported *A2M* association finding

	Authors	*n*	*n*	*P*-value	Odds ratio[m]
A2M-18i	*Family-based studies*	Families	Individuals		
1	Blacker *et al.* (10)	104	398	0.0003[a]	3.56 (1.80,7.03)[a]
2	Poduslo *et al.* (25)	89	276	0.0519[a,c]	NR
3	Rogaeva *et al.* (28)	105	NR	>0.6[b,c]	NR
4	Romas *et al.* (27)	51	147	0.04[b]	3.4 (1.1,10.7)[b]
5	Rudrasingham *et al.* (26)	395	790	0.003[b]	2.13 (1.22,3.7)[a]
	Case–control studies	Cases	Controls		
6	Alvarez *et al.* (33)	190	400	0.001[b,d]	0.36 (0.21,0.64)[b]
7	Bagli *et al.* (58)	102	351	0.26[a]	1.1 (0.64,.89)[a]
8	Blennow *et al.* (59)	449	349	0.26[b]	0.95 (0.69,1.30)[a]
9	Bullido *et al.* (60)	154	217	0.16[a]	1.40 (0.87,2.23)[a]
10	Chen *et al.* (61)	196	180	0.67[b]	0.72 (0.24,2.09)[b]
11	Crawford *et al.* (62)	177	113	0.63[a]	1.13 (0.64,2.00)[a]
12	Dodel *et al.* (32)	309	281	0.025[b]	1.5 (1.1,2.2)[b]
13	Dow *et al.* (63)	225	218	>0.05[b]	0.81 (0.54,1.21)[b]
14	Gibson *et al.* (64)	195	107	>0.05[a]	0.80 (0.47,1.36)[a]
15	Halimi *et al.* (65)	281	84	0.52[b]	1.19 (0.68,2.06)[b]
16	Higuchi *et al.* (66)	426	382	0.21[b]	1.13 (0.64,2.02)[b]
17	Hu *et al.* (67)	82	110	>0.1[b]	0.89 (0.37,2.12)[b]
18	Hu *et al.* (68)	65	84	>0.1[b]	0.59 (0.25,1.40)[b]
19	Jhoo *et al.* (31)	62	169	0.01[b,e,f]	5.97 (1.53,23.34)[b,e,f]
20	Ki *et al.* (69)	89	50	0.61[a]	1.29 (0.39,4.30)[a]
21	Korovaitseva *et al.* (70)	76	89	0.41[a,f]	0.76 (0.44,1.34)[b]
22	Koster *et al.* (71)	356	242	0.53[b]	0.91 (0.69,1.21)[b]
23	Kovacs *et al.* (72)	96	64	0.49[a]	1.21 (0.70,2.11)[a]
24	McIlroy *et al.* (73)	219	237	0.60[a]	0.91 (0.61,1.37)[a]
25	Myllykangas *et al.* (35)	77	52	0.67[a,e,g]	0.78 (0.25,2.41)[a,e,g,h]
26	Nacmias *et al.* (30)	90	98	0.001[b]	2.52 (1.45,4.41)[b]
27	Perry *et al.* (74)	111	78	>0.05[b]	0.9 (0.5,1.7)[b]
28	Poduslo *et al.* (25)	386	216	0.099[a]	0.9 (0.72,1.2)[a]
29	Rogaeva *et al.* (28)	207	164	NR	1.24 (0.69,2.21)[b,i]
30	Rogaeva *et al.* (28)	185	156	NR	0.92 (0.56,1.53)[b,j]
31	Rudrasingham *et al.* (26)	1238	1378	0.4[b]	1 (0.9,1.2)[b]
32	Shibata *et al.* (75)	55	69	0.38[b]	1.53 (0.59,3.96)[b]
33	Singleton *et al.* (76)	209	105	0.24[b]	0.76 (0,48,1.21)[b]
34	Sodeyama *et al.* (77)	62	90	0.35[b]	1.89 (0.50,7.10)[b]
35	Wang *et al.* (37)	555	446	0.12[b]	0.64 (0.37,1.13)[b]
36	Zappia *et al.* (29)	132	184	0.019[a]	1.93 (1.2,3.1)[a]
37	Zill *et al.* (78)	88	118	0.29[a]	1.39 (0.76,2.54)[a]
A2M-24e	*Family-based studies*	Families	Individuals		
1	Poduslo *et al.* (25)	89	276	0.33[a,c]	NR
2	Wavrant-DeVrieze *et al.* (38)	133	274	0.1[a]	NR
3	Romas *et al.* (27)	51	147	0.5[a]	1.8 (0.3,12.2)[a]
	Case–control studies	Cases	Controls		
4	Gibson *et al.* (64)	195	107	0.76[b]	0.95 (0.67,1.34)[b]
5	Higuchi *et al.* (66)	426	382	0.21[b]	1.13 (0.64,2.02)[b]
6	Koster *et al.* (71)	356	242	0.64[b]	0.95 (0.77,1.17)[b]
7	Liao *et al.* (34)	737	449	0.01[a]	1.77 (1.16,2.70)[a]
8	McIlroy *et al.* (73)	219	237	0.79[a]	1.14 (0.79,1.64)[a]
9	Myllykangas *et al.* (35)	77	52	0.004[b,e,g]	6.86 (1.63,28.91)[a,e,g,h]
10	Nacmias *et al.* (30)	90	98	0.89[b]	0.97 (0.61,1.53)[b]
11	Poduslo *et al.* (25)	398	184	0.054[a]	1.1 (0.7,1.9)[a]
12	Shibata *et al.* (79)	111	95	0.81[b]	0.91 (0.43,1.94)[b]
13	Singleton *et al.* (76)	209	105	0.66[b]	1.08 (0.77,1.52)[b]
14	Styczyncka *et al.* (80)	100	100	0.34[a]	1.4 (0.7,3.1)[a]
15	Tang *et al.* (36)	114	190	0.02[a]	2.1 (1.1,4.1)[a]
16	Wang *et al.* (37)	555	446	0.04[b,k]	0.80 (0.64,0.99)[b,k]
17	Wavrant-DeVrieze *et al.* (38)	1200	1346	0.21[b]	1.08 (0.96,1.21)[b]
18	Zappia *et al.* (29)	264	368	0.001[a]	4.1 (1.86,9.04)[a]
19	Zill *et al.* (78)	88	118	0.60[b]	0.89 (0.58,1.36)[b]
A2M-18i/24e	*Case–control studies*	Cases	Controls		
1	Verpillat *et al.* (39)	303	343	0.001[b,e,l]	4.97 (1.03,24.05)[b,e]

[a]Genotypic/carrier. [b]Allelic. [c]SDT results for non-NIMH samples. [d]>81 years. [e]Reported as *APOE-ε4* negative. [f]>65 years. [g]Neuropathologically confirmed AD. [h]>85 years. [i]Toronto sample. [j]Duke sample. [k]≤80 years. [l]>70 years.
[m]In cases where no OR was reported, a crude OR (unadjusted for age, sex, APOE, or other covariates) was calculated from the reported data.

Table 2. Sequence location, context, and primer sequences used in polymorphism genotyping

SNP	Location	Sequence context	PCR primers (sense, antisense)	SBE primer
5U	5′-UTR 28 bp 5′ of start codon	GTACAATACAGTCTG **[G/T]** TCTCCTCCAGCTCCT	CGCCAGGAATTAACCTTGAC, AGGTTCATGCTTCACGCTCT	GAAAGAAGGAGCTGGAGGAGA
7i	174 bp 3′ of exon 6	CTGTGAATAGGACTT **[C/A]** ACATAGCTTCGTTAT	CGTGGAGGAATTTGGTATGG, GGGCTCTGAAAAGGTAGCTC	CTACAGAAAATAACGAAGCTATGT
12e	Synonymous (Y432Y)	TCGTAGTCCCTGTTA **[C/T]** GGCTACCAGTGGGTG	GGGTCCTTCTCCCACTTTTC, TGTCTGAGTATGGCCACAGG	CAAGGATCGTAGTCCCTGTTA
15i	3 bp insertion/deletion 132 bp 3′ of exon 14	CAGCAAGGTAGTGGCA **[AAG]** GAGTCACACTAAGAA	FAM-CACATGTGAACATTAGGGCC, CCCAACGTTTCATCATAGAAG	NA
18i	5 bp insertion/deletion at the exon 18 5′ splice site	CTTCTTCCTCACTCA **[CCATA]** GAGTCAGATGTAATG	FAM-CTTTCCTTGATGACCCAAGCGCC, GTTGAAAATAGTCAGCGACCTC	NA
20e	Non-synonymous (A844V)	AGAAGGAACAAG **[C/T]** GCCTCACTGCATCTG	CTTTAATGTGAGGCAACCTG, AATCGCAGCATCTAGTGG	NA
21i	4 bp 5′ of exon 21	TACTGGCCTTTTTTTT **[T/A]** CAGGAAATGTGAATT	TTGCATGTTTCCGTTC, CCAACAGAGGCTTGAT	CAGTGAAATTCACATTTCCTG
24e	Non-synonymous (V1000I)	CAGCTTACTCCAGAG **[A/G]** TCAAGTCCAAGGCCA	GACTTCCCATCTCTCCCATTC, TGAAACCTACTGGGAAATCCA	AGCCAATGGCCTTGGACTTGA
28i	97 bp 5′ of exon 28	TCGTTGGAAAAATGC **[G/A]** TTTGAAAAACTTCTG	GGCTATTTACCACCACACTC, GTCTTCCAGGCTGACTCCAG	TCACAGCACAGAAGTTTTTCAAA

NA, not applicable.

polymorphism, and the original 18i deletion polymorphism for association with AD in the full NIMH AD Genetics Initiative sample of 437 families (40). To obtain maximum information from the data, we use family-based association tests, haplotype analyses and conditional logistic regression.

RESULTS

Polymorphism discovery

We resequenced the *A2M* locus in 29 individuals that carry at least one copy of the A2M-18i deletion polymorphism. Seven novel SNPs were identified and genotyped (Table 2). None of these novel SNPs code for amino acid changes: two are synonymous SNPs, and five are in the non-coding region of the gene. Interestingly, the SNP in intron 21 (*A2M*-21i) is found within the 5′ splice site of exon 21. One of these, a synonymous SNP in exon 20, *A2M*-20e, was genotyped in approximately half of the full NIMH sample (∼800 subjects) and found to have a minor allele frequency of only ∼4%, so we elected not to pursue it further.

Association of individual *A2M* SNPs with AD

The six novel polymorphisms and the two previously identified polymorphisms (18i and 24e) were genotyped in the full NIMH Genetics Initiative sample. Association results, reported in Table 3, were generated using the Family Based Association Test Package (FBAT) using phenotype information from affected subjects only or from both affected and unaffected subjects (see Methods). Analyses including phenotype information on unaffecteds generally gave greater evidence of association than the affecteds only analyses, as would be expected given the use of more of information. In general, the late-onset stratum (all sampled affecteds with onset ages $\geq$65, see Methods) displayed increased evidence for association. We also tested for differences by *APOE*-ε4, stratifying based on whether a family has at least one *APOE*-ε44 *homozygote* with AD, and then on whether a family has at least one *APOE*-ε4 *carrier* with AD (see Methods). In both cases the larger stratum [*APOE*-ε44-negative (67.5% of the sample) and *APOE*-ε4 positive (84.4% of the sample)] gave results similar to those in the total sample (although with somewhat lower levels of statistical significance given the reduction in sample size), and the smaller stratum [*APOE*-ε44 positive (32.5% of the sample) and *APOE*-ε4 negative (15.6% of the sample)] was entirely negative: $P > 0.25$ for all individual polymorphisms (data not shown). Thus, the apparent discrepancies are likely to be due to the increased power of the larger subsamples, and not to the *APOE-ε4* genotype *per se.* This is in agreement with the results of conditional logistic regression analyses that gave no evidence for interactions between *APOE-ε4* and any of the associated polymorphisms in *A2M* (see below).

Consistent with our previous report, the 18i deletion polymorphism is associated in the total sample ($P_{\text{nominal}} = 0.02$ for affecteds only, and 0.0059 with unaffected phenotypes included) and more strongly associated in the late-onset sample ($P_{\text{nominal}} = 0.0033$ for affecteds only, and 0.0023 with unaffected phenotypes included). The previously reported exon 24

Table 3. Results of association analyses of individual polymorphisms[a]

Polymorphism	Frequency[d]	Affecteds only				Unaffected phenotypes included[b]			
		Total sample		Late[c] stratum		Total sample		Late[c] stratum	
		Families[e]	P[f]	Families[e]	P[f]	Families[e]	P[f]	Families[e]	P[f]
5U	0.49	119	0.77	78	0.37	122	0.72	80	0.49
7i	0.32	102	0.45	65	0.21	103	0.25	67	0.067
12e	0.07	39	0.0018	31	0.0031	40	0.00080	32	0.0012
15i	0.33	105	0.28	67	0.15	107	0.17	69	0.043
18i	0.18	74	0.020	53	0.0033	77	0.0059	54	0.0023
21i	0.17	71	0.041	50	0.0039	74	0.019	51	0.0031
24e	0.33	105	0.093	66	0.10	105	0.058	66	0.037
28i	0.34	100	0.14	62	0.094	101	0.099	63	0.037

[a]All analyses are performed in FBAT using the empirical variance function.
[b]These analyses used the offset function to include phenotypic information from unaffected subjects.
[c]All sampled affecteds onset ≥65 years old.
[d]Allele frequency of the minor allele; the minor alleles are: 5U (T), 7i (A), 12e (T), 15i (deletion), 18i (deletion), 21i (T), 24e (G), and 28i (A).
[e]Number of informative pedigrees.
[f]Nominal P-value.

non-synonymous SNP (24e; Val 1000 Ile) displays a trend towards association in most analyses, and reaches significance in the late-onset stratum when unaffected phenotypes are included in the analysis ($P_{\text{nominal}} = 0.037$).

Association analyses of the six novel polymorphisms extend our original association findings. Depending on the sample used and whether unaffected phenotype information is included, from two to four of these give significant evidence for association. Strongly significant nominal association results were obtained for the synonymous SNP found in exon 12 (12e) in the total sample ($P_{\text{nominal}} = 0.0018$ for affecteds, only and 0.0008 with unaffected phenotypes included), with slightly less significant results in the late-onset stratum. Second, the SNP in the intron immediately adjacent to exon 21 (21i) was significantly associated in the total sample ($P_{\text{nominal}} = 0.041$ for affecteds only, and 0.019 with unaffected phenotypes included), with more significant results in the late-onset stratum. The polymorphisms in intron 15 (15i) and intron 28 (28i) only gave significant evidence of association in the late-onset stratum when unaffected phenotypes were included ($P_{\text{nominal}} = 0.043$ for 15i, and 0.037 for 28i), and the polymorphism in intron 7 (7i) displayed only a trend towards association in the same setting ($P_{\text{nominal}} = 0.067$).

For the polymorphisms showing at least a trend toward association in FBAT (see Table 3), odds ratios (ORs) for their effect on AD risk were calculated using conditional logistic regression, and are given in Table 4. The 95% confidence intervals (CIs) are provided to give an idea of the precision of these estimates, but it should be noted that these CIs are slightly too narrow (see Methods). Carriers of the 12e 'T' allele have a 3-fold increase in risk (OR = 3.27, 95% CI = 1.74, 6.16). For the 18i 'deletion' and the 21i 'A' allele, the increase in risk is less than 2-fold (for 18i OR = 1.79, 95% CI = 1.21, 2.63; for 21i OR = 1.73, 95% CI = 1.17, 2.56). Looking at the genotypic ORs, the point estimates for 12e and 21i suggest a dose–response relationship, but these values are not stably estimated. Interestingly, two copies of the 15i 'insertion' or 24e 'A' or 28i 'G' allele might be protective, or, viewed alternatively, being a carrier of the other allele could actually increase risk for AD. None of the associated polymorphisms in *A2M* gave any evidence for an interaction with the ε4-allele in *APOE* (data not shown) when included in the CLR analyses.

Table 4. Odds ratio from conditional logistic regression

Polymorphism	OR (95% CI[a])		
	Carrier	Genotypic	
	Any 2[b]	12[b]	22[b]
7i	1.61 (0.94, 2.75)	1.68 (0.97, 2.90)	1.43 (0.78, 2.61)
12e	3.48 (1.82, 6.67)	3.38 (1.76, 6.74)	12.21 (0.91, 164)
15i	1.85 (1.08, 3.15)	1.92 (1.11, 3.31)	1.64 (0.89, 3.00)
18i	1.86 (1.24, 2.79)	1.82 (1.21, 2.74)	3.07 (0.98, 9.60)
21i	1.78 (1.19, 2.70)	1.78 (1.18, 2.69)	1.86 (0.56, 6.22)
24e	1.97 (1.16, 3.35)	2.02 (1.18, 3.47)	1.81 (0.99, 3.31)
28i	1.81 (1.05, 3.15)	1.85 (1.06, 3.23)	1.72 (0.92, 3.21)

[a]These confidence intervals provide a rough estimate of the precision of each estimate, but they may be slightly too narrow because standard errors are slightly underestimated in this setting (see text).
[b]Here we define '2' as the risk allele. The risk alleles, relative to the sequence context in Table 2, are: 7i (C), 12e (T), 15i (insertion), 18i (deletion), 21i (T), 24e (A), 28i (G).

Because of a concern that a few larger families could be driving the association results, we re-ran the association analyses in FBAT and the conditional logistic regression analyses including only one nuclear family per pedigree (see Methods). None of these analyses resulted in appreciable differences in the results, although P-values were sometimes slightly larger, and confidence intervals slightly wider, in the reduced sample (data not shown).

Association of *A2M* haplotypes with AD

To gain insight into the haplotype structure underlying the association results in Table 3, we grouped polymorphisms into haplotypes for further analysis in HBAT, the new haplotype program available in the FBAT package (see Methods). Combining all eight polymorphisms in one analysis revealed a trend for association in the total sample ($P_{\text{global,nominal}} = 0.08$) and nominally significant association in the late-onset families ($P_{\text{global,nominal}} = 0.015$). To explore which part of the gene

Table 5. Results of haplotype analysis for *A2M* polymorphism[a]

A2M polymorphisms[b]									Haplotype statistics			Global haplotype statistics			
5U	7i	12e	15i	18i	21i	24e	28i		Strata	Frequency	*P*-value	Strata	χ^2	d.f.	*P*-value
T	C	C	ins	ins	*(P)*				Total	0.48	0.28				
									Late	0.49	0.26				
G	A	C	del	ins	*(P)*				Total	0.31	0.31	Total	9.36	5	0.096
									Late	0.30	0.064				
G	***C***	**C**	***ins***	***del***	*(R)*				Total	0.12	0.19				
									Late	0.12	**0.031**				
G	***C***	***T***	***ins***	***del***	*(R)*				Total	0.04	0.062	**Late**	**13.60**	**5**	**0.018**
									Late	0.046	**0.037**				
T	***C***	***T***	***ins***	**ins**	*(R)*				**Total**	0.007	**0.043**				
									Late	0.008	0.055				
	C	C	ins	ins	A	*(P)*			Total	0.50	0.38				
									Late	0.51	0.45				
	A	C	del	ins	A	*(P)*			Total	0.30	0.34	Total	8.02	5	0.15
									Late	0.29	0.11				
	C	C	ins	del	T	*(R)*			Total	0.11	0.28				
									Late	0.11	0.13				
	C	T	ins	del	T	*(R)*			Total	0.038	0.34	Late	9.98	5	0.076
									Late	0.040	0.14				
	C	T	ins	ins	A	*(R)*			Total	0.009	0.035				
									Late	0.010	0.045				
		C	ins	ins	A	A	*(P)*		Total	0.50	0.39				
									Late	0.51	0.36				
		C	del	ins	A	G	*(P)*		Total	0.30	0.16	**Total**	**11.28**	**5**	**0.046**
									Late	0.29	0.053				
		C	ins	del	T	A	*(R)*		Total	0.11	0.21				
									Late	0.11	0.15				
		T	ins	del	T	A	*(R)*		Total	0.038	0.21	**Late**	**14.93**	**5**	**0.011**
									Late	0.040	0.079				
		T	***ins***	**ins**	**A**	***A***	*(R)*		**Total**	**0.007**	**0.050**				
									Late	0.009	0.070				
			ins	ins	A	A	G	*(P)*	Total	0.50	0.47				
									Late	0.52	0.34				
			del	**ins**	**A**	**G**	**A**	*(P)*	Total	0.30	0.12	**Total**	**9.12**	**3**	**0.028**
									Late	0.29	**0.039**				
			ins	***del***	***T***	***A***	***G***	*(R)*	**Total**	0.15	**0.036**	**Late**	**13.56**	**3**	**0.0036**
									Late	0.15	**0.010**				

[a]Results are based on analyses of using either the total sample or only the late-onset stratum. Only haplotypes for which there are at least 10 informative pedigrees are included. Bold indicates significantly associated haplotypes and statistics.
[b]Specific bases for each polymorphism are given relative to the sequence context given in Table 2. Ins = insertion, del = deletion. '*(R)*' adjacent to a haplotype indicates that it appears to confer risk, i.e. observed transmissions exceed expected transmissions; '*(P)*' indicates protective, i.e. observed transmissions are less than expected. In windows with global haplotype association, the risk allele for each individually associated polymorphism is in italics within each individually associated haplotype.

contributes most to this overall association, we employed a 'sliding window' approach, where each set of five consecutive polymorphisms was tested for association with AD (Table 5). In these analyses, the strongest association signals were observed in the 3′ portion of the gene, i.e. in the last two adjacent windows: [(12e, 15i, 18i, 21i, 24e), $P_{\text{global,nominal}} = 0.046$ (total) and 0.011 (late); and (15i, 18i, 21i, 24e, 28i), $P_{\text{global, nominal}} = 0.028$ (total) and 0.0036 (late)]. These windows also contain, respectively, three (12e, 18i and 21i) and two (18i and 21i) of the individually most significantly associated polymorphisms, and the results for specific haplotype alleles are consistent with this (Table 5). Interestingly, it was also across the 3′ portion of *A2M* that two-locus LD assessment showed the highest D' values, although evidence for LD between polymorphisms was generally high across the entire gene (Table 6). As with the individual polymorphisms, we re-ran the haplotype analyses in HBAT including only one nuclear family per pedigree, again with no appreciable differences in the results, but with slightly larger *P*-values consistent with the reduced sample size (data not shown).

DISCUSSION

Findings in the full NIMH sample

We originally reported a significant association of an intronic deletion polymorphism (18i) in a subset (104 families) from the NIMH Genetics Initiative sample (10). The results and analyses presented here, using the complete NIMH Genetics Initiative sample of 437 families, confirm and extend our initial findings. Three polymorphisms (12e, 18i and 21i) display nominally significant association in the affecteds only analysis, and three additional polymorphisms [15i (late-onset stratum only), 24e and 28i] are nominally significant when unaffected phenotypes are

Table 6. Pairwise disequilibrium coefficients (D') between *A2M* polymorphisms

	5′U	7i	12e	15i	18i	21i	24e	28i
5′U	—	0.89	0.70	0.99	0.96	0.96	0.96	0.97
7i	<0.000001	—	0.92	0.99	0.98	0.95	0.96	0.97
12e	<0.000001	<0.000001	—	0.88	0.77	0.74	0.88	0.88
15i	<0.000001	<0.000001	<0.000001	—	1	0.96	0.96	0.96
18i	<0.000001	<0.000001	<0.000001	<0.000001	—	1	0.98	0.98
21i	<0.000001	<0.000001	<0.000001	<0.000001	<0.000001	—	0.99	0.98
24e	<0.000001	<0.000001	<0.000001	<0.000001	<0.000001	<0.000001	—	0.99
28i	<0.000001	<0.000001	<0.000001	<0.000001	<0.000001	<0.000001	<0.000001	—

Upper cells = D' (LD-parameter, i.e. 0 = no LD, 1 = perfect LD). Lower cells = P-value associated with D' [Fisher's exact two-sided, based on the average number of affecteds (= 883)].

included (Table 3). Even conservatively accounting for multiple tests using a Bonferroni correction (see Methods), the observed association of AD with *A2M*-12e remains statistically significant in the total sample ($P_{\text{corrected}} = 0.043$ in the affecteds only analysis, $P_{\text{corrected}} = 0.019$ when unaffected phenotypes are used), and association findings for 12e, 18i and 21i in the late-onset sample are consistent with a trend for association ($P_{\text{corrected}} = 0.07$, 0.08, and 0.09, respectively, in the affecteds-only analyses; and $P_{\text{corrected}} = 0.029$, 0.055, and 0.074 when unaffected phenotypes are used). Two adjacent five-locus haplotypes give strong evidence for nominal association ($P_{\text{nominal}} = 0.0036$), and even after correcting for multiple tests there is a trend towards association ($P_{\text{corrected}} = 0.086$). Examining the individual and haplotype results overall suggests that the stronger LD signal is in the 3′ portion of the gene. The most strongly associated individual SNPs are found in this region, which also includes the bait region and the β-Cys-γ-Glu thiol ester bond between Cys-949 and Glu-952. The strength and consistency of this evidence suggests that this association is genuine, but there are several important caveats.

Comparison with prior linkage and association findings

Our findings are consistent with the generally positive prior findings of family-based association studies of *A2M*, but not with the primarily negative case–control studies. Non-replication of association findings can be due to chance, particularly where there is inadequate power to detect effects of small or moderate magnitude, or, in case–control studies, due to bias due to population stratification, but the large number of negative studies argues against both of these possibilities. The more likely cause of the discrepancy between the family-based and case–control studies is differences in ascertainment. Family-based samples frequently include only families with two or more affected individuals, and thus may identify a more genetically determined form of AD. Case–control samples may have fewer genetic determinants to be identified (although they may be more typical of AD in general—unless the case ascertainment *excludes* individuals with a family history, which leads to an underestimation of family history). Thus, the observed difference between case–control studies and family-based studies is consistent with the idea that *A2M* is predominantly a risk factor for late-onset AD *with a family history.*

Although other groups, including ones using portions of the NIMH sample, have reported evidence for linkage in this region, we have not been able to detect significant linkage in this region (23), even to marker *D12S1695*, which is only ~65 kb from *A2M* (data not shown). The reason for the discrepancy between our linkage findings in the NIMH sample and those of other groups is unclear, but may reflect the specific sampling scheme for the subsample, analytic strategies, or marker choice. We were also unable to detect evidence of *association* between *D12S1695* and AD (data not shown). The lack of association across a gap of 65 kb was not unexpected, but the lack of linkage findings in an area with a strong association signal in the same sample is more puzzling. Although it is unusual to detect strong association in the absence of linkage, it is not impossible. Risch (41) suggests that association analysis can be locally more powerful for detecting small or moderate gene effects than linkage analysis. A similar scenario with the insulin gene and diabetes (42) was part of the motivation for developing the TDT.

Conclusions

Using a large uniformly ascertained and evaluated family-based sample, we report significant individual and haplotypic association between several *A2M* polymorphisms and AD, including some that remain significant when applying an overlying conservative Bonferoni correction. While it seems unlikely that any of these associated polymorphisms and/or haplotypes are pathogenic, we are currently exploring the possibility. A more likely explanation is that these associated polymorphisms are in linkage disequilibrium with a pathogenic polymorphism either in *A2M* itself or in a nearby gene. The relatively modest association findings, the lack of confirmation in case–control studies, and the inconsistent evidence for linkage suggest that the underlying defect may have only a modest effect. In particular, the discrepancy between family-based association findings and those in case–control studies suggests that the gene may be a risk factor primarily in individuals with a family history, and could either be a gene of modest effect or perhaps a modifier gene. In any case, these new findings, along with prior positive results in family-based studies, suggest that *A2M* and nearby genes merit further testing in other family-based datasets, and further exploration of its potential biological role in AD.

MATERIALS AND METHODS

Subjects

The ascertainment and assessment of the AD families collected under the NIMH AD Genetics Initiative Sample are described

elsewhere (40). Briefly, participants were evaluated following a standardized protocol to ensure that they met NINCDS/ADRDA criteria for AD (43,44). Of the sampled affected subjects, 29% had a diagnosis of definite (autopsy-confirmed) AD, 64.5% probable AD, and 6.5% possible AD. Sixty-eight percent of the sample was female. Family structure varied widely. Most (79%) of the families were nuclear (about half affected sibpairs and half larger sibships of varying sizes), but there were nearly 100 families with more complex structure. Forty percent of families included an unaffected member. Families with more complex structure were broken into nuclear families (see below): there were a total of 586 nuclear families when the larger pedigrees were broken down in this way, but not all of them are informative.

Only families in which all sampled affecteds had onset ages $\geq$50 years were included in the present analyses ($n = 437$ families, $n = 1439$ individuals; mean age of onset 72.5, SD 7.7); 73% of families were in our late-onset stratum (all sampled affecteds showed an onset at or beyond age 65) and 27% of families were in our early/mixed stratum (at least one sampled affected showed an onset before age 65, but none under age 50). *APOE* genotypes were determined for 99.5% of the sample. We stratified families on *APOE* status in two ways. First, we stratified based on whether or not a family included an *APOE*-ε44 *homozygote* with AD : 120 families (including 832 individuals, 32.5% of the sample) were in the *APOE*-ε44-positive stratum (at least one *APOE*-ε44 homozygote with AD in the family), and 317 families (1725 individuals, 67.5% of the sample) were in the *APOE*-ε44-negative stratum (no *APOE*-ε44 homozygotes with AD in the family). This stratification, which has been used consistently in prior work in the NIMH sample, makes sense because in this sample the *APOE*-ε44 genotype has a much stronger impact on disease risk and age of onset (40), and results in more balanced groups. For comparison purposes, we repeated our analyses using the more commonly used stratification based on *APOE*-ε4 *carrier* status: 358 families (2159 individuals, 84.4% of the sample) were in our *APOE*-ε4-positive stratum (at least one *APOE*-ε4 carrier with AD) and 79 families (398 individuals, 15.6% of the sample) were in our *APOE*-ε4-negative stratum (no *APOE*-ε4 carriers with AD).

Polymorphism discovery

Querying public databases and direct DNA sequencing were used to identify polymorphisms within the *A2M* locus. Specifically we mined for SNPs using three public databases, dbSNP (NCBI), Human SNP database (Whitehead Institute), and the Genetic Annotation Initiative (National Cancer Institute). Using these sources we identified three non-synonymous SNPs in *A2M* [rs2277413 (A807V), rs1800434 (H704R), rs1802965 (L1431F)]. However, when genotyped in a test sample of ~200 subjects, these SNPs were not found to be polymorphic and were therefore excluded from further genotyping efforts.

In direct resequencing of the *A2M* locus, we sequenced all exons and ~150 bp of the adjacent introns, as well as 1000 bp of the promoter and the 3′-UTR. These resequencing reactions were performed on DNA from 29 subjects who carried at least one copy of the A2M-18i deletion allele, which we previously reported to be associated with AD (10). Sequencing was performed and analyzed on an ABI3700 (Perkin-Elmer).

Genotyping

The 5 bp deletion polymorphism found in intron 18 was newly genotyped using a fluorescence-based detection method, as opposed to the radio-isotope method that we originally reported (10). A 321 or 326 bp PCR product was amplified, using an annealing temperature of 64°C, with one fluorescently labeled PCR primer and one unlabeled PCR primer (Table 2). Fluorescently labeled insertion (326 bp) and deletion (321 bp) products were separated and detected using a MegaBACE 1000 (Amersham-Pharmacia). Allele calling was performed using Genetic Profiler 1.0 (Amersham-Pharmacia) with visual reinspection of all genotype calls. The 3 bp insertion/deletion polymorphism in intron 15 was genotyped in a similar fashion. The 194 or 197 bp PCR product was amplified at an annealing temperature of 55°C.

SNPs found in 5′-UTR, intron 7, exon 12, intron 21, exon 24 and intron 28 were genotyped using fluorescent polarization detected single base extension (FP-SBE) (45). PCR primers (Table 2) were designed to yield products between 200 and 400 bp in length, and were used at a final concentration of 100–300 nM (InVitrogen) along with *Taq* polymerase (0.25 U/reaction; Qiagen) and dNTPs (2.5 μM/rxn; Amersham-Pharmacia). Human genomic DNA, 10 ng, was used as the template for PCR reactions. General PCR cycling conditions were as follows: initial denaturation of 3 min at 94°C; 30 cycles of 94°C for 45 s; primer set specific annealing temperature (see below) for 45 s, and 72°C for 1 min; finally, 6 min at 72°C. All PCR producs were visualized on 2% agarose gels to confirm a single product of the correct size. PCR primers and unincorporated dNTPs were degraded by the direct addition of exonuclease I (0.1–0.15 U/reaction; New England Biolabs) and shrimp alkaline phosphatase (1 U/reaction; Roche) to the PCR reactions and incubated for 1 h at 37°C, then for 15 min at 95°C to inactivate the enzymes. The single base extension step was carried out using Thermosequenase (0.4 U/reaction; Amersham-Pharmacia) and the appropriate mixture (LJL Biosystems Application Note) of R110-ddNTP, TAMRA-ddNTP (3 mM; NEN), and all four unlabeled ddNTPs (22 or 25 μM; Amersham-Pharmacia) to the ExoI/SAP treated PCR product. Incorporation of the SNP specific fluorescent base was achieved by subjecting samples to 35 cycles of 94°C for 15 s and 55°C for 30 s. SBE primers (Table 2) were designed to yield a T_m of 62–64°C. Fluorescent base incorporation was detected using an LJL Biosystems Analyst AD (Sunnyvale, CA, USA) and measuring fluorescent polarization for R110 (excitation 490 nm/emission 520 nm) and TAMRA (excitation 550 nm/emission 580 nm). Genotypes were called manually. All genotyping experiments contained at least three samples whose genotypes were known from direct sequencing.

SNP specific annealing temperature, PCR product lengths, and SBE dye mixtures are given below. For the 5′-UTR polymorphism, PCR primers yielded a 344 bp product using a 61°C annealing temperature, and an R110-ddATP/TAMRA-ddCTP dye mixture was used in the SBE step. For the intron 7 polymorphism, PCR primers yielded a 380 bp product using a 61°C annealing temperature, and an R110-ddUTP/TAMRA-ddGTP

dye mixture was used in the SBE step. For the exon 12 polymorphism, PCR primers yielded a 304 bp product using a 64°C annealing temperature, and an R110-ddUTP/TAMRA-ddCTP dye mixture was used in the SBE step. For the intron 21 polymorphism, PCR primers yielded a 264 bp product using a 57.2°C annealing temperature, and an R110-ddUTP/TAMRA-ddATP dye mixture was used in the SBE step. For the exon 24 polymorphism, PCR primers yielded a 272 bp product using a 54°C annealing temperature, and an R110-ddUTP/TAMRA-ddCTP dye mixture was used in the SBE step. For the intron 28 polymorphism, PCR primers yielded a 266 bp product using a 61°C annealing temperature, and an R110-ddUTP/TAMRA-ddCTP dye mixture was used in the SBE step.

The polymorphism in exon 20 was genotyped by restriction fragment length polymorphism. PCR primers (Table 2) were used with an annealing temperature of 64°C. The PCR amplifications were performed in the presence of [α-^{33}P]dATP (1 μCi). This polymorphism, a C/T transition, removes a *HhaI* restriction site. PCR products were digested with 5 U of HhaI (New England Biolabs) for 8 h at 37°C in the appropriate buffer. Following digestion, products were denatured for 3 min at 94°C and then and separated by polyacrylamide gel electrophoresis (Ultra Pure SequaGel-6, National Diagnostics) for 1 h at 60 W and exposed to film for 24 h.

Genotyping quality control measures

To ensure consistent and high-quality genotyping data, the same three control samples of known genotype were included in every genotyping experiment. In addition, 10% of the samples were genotyped in duplicate. The genotypes of the duplicate samples were checked for consistency. We found discrepancy rate >0% for only two polymorphisms: 21i (0.63%) and 24e (1.27%). In addition, we have very little missing data: we have obtained genotypes for >95% of the samples (Table 3) for all polymorphisms.

Statistical analysis

Tests of individual polymorphisms for association with AD were performed in the Family-Based Association Test package (46,47) (FBAT; version 1.2), which puts tests of different models, different sampling designs, different disease phenotypes, missing parents and different null hypotheses all in the same framework. Like the transmission-disequilibrium test (TDT) and other family-based association tests, this method is not susceptible to bias due to population admixture. FBAT uses a generalized score statistic to perform a variety of TDT-type tests (46). We used an additive model which performs well under a variety of true genetic models (48). We also used the empirical variance option in FBAT (EV-FBAT), which is valid in the presence of linkage (even when extended families are broken into component nuclear families) (49), because of the reports of linkage in this vicinity (2–9), although linkage in this area was not seen by our group (23). We performed both the default affecteds-only analyses in FBAT, which uses information from unaffected siblings only to provide information about possible parental genotypes, and analyses using an offset to include phenotypic information from unaffected siblings. The latter approach makes maximal use of the available data. We set the offset equal to 0.5 for the affecteds and unaffecteds, which gives equal weight to each group. In principle, the offset should be equal to the prevalence, but in highly selected samples such as this one this approach works well (50–52).

For the nominally associated polymorphisms, we also performed conditional logistic regression (CLR) stratified on family to assess the magnitude of any effect on disease risk (53). We include confidence intervals to provide a rough idea of the precision of these estimates. However, it should be noted that these intervals may be slightly too narrow because CLR may slightly underestimate the standard errors when multiple affected and unaffected subjects are included in each family. The magnitude of this effect is expected to be small unless genetic effects are very large (54).

To gain insight into the underlying haplotype structure of *A2M*, we combined individual polymorphisms into multilocus haplotypes, assuming no recombination between SNPs. Haplotype analysis is especially useful when the true risk modifying polymorphism has not been identified (which could be expected here owing to conflicting findings from independent studies), as this method should be able to extract more inheritance information from any given set of loci than can be accomplished by testing polymorphisms individually (55,56). Here, five-locus haplotypes were reconstructed from the eight genotyped polymorphisms in *A2M* and tested for excess transmission in affected individuals using the HBAT option (55,57) within the FBAT package. We chose five-locus haplotypes because in our experience a window of this size offers maximal informativeness within a reasonable computation time. Results of preliminary analyses using smaller and larger SNP windows were not appreciably different. The HBAT option in FBAT extends the conditioning algorithm of Rabinowitz and Laird to account for missing phase. As in FBAT, many pedigrees will be non-informative, because the conditioning places positive probability on only one outcome. That is, families with only one or two affected offspring, no unaffected offspring and no parental genotype data will not contribute to the test statistic. In addition, it uses weights to increase information in the presence of uncertainty about phase. The resulting test statistic is unbiased for testing association even in the presence of admixture and/or population stratification, and under the alternative hypothesis that association is present, it provides good power relative to a setting where phase is known (57). As described above for FBAT, we used the empirical variance option to account for the reported the reported linkage in the region, which also ensures a valid test when extended pedigrees are broken into nuclear families (49). As in FBAT, we used an offset of 0.5 to include phenotypic information from unaffected subjects. Nominal global *P*-values are reported, along with *P*-values for individual haplotype alleles with more than 10 informative pedigrees. The haplotype frequencies are obtained from the estimated phased genotype frequencies of the founders. These latter frequencies maximize the likelihood based on all the genotype data, assuming the null hypothesis of no linkage and no association, and assuming random mating. Because we estimate phased genotype frequencies rather than haplotype frequencies, Hardy–Weinberg equilibrium is not assumed. These phased genotype

frequencies are used to attach weights to any outcomes with ambiguous phase. For example if one unphased genotype is compatible with two phased genotypes, then weights summing to one are assigned to each phased genotype, where the weight equals the probability of the phased genotype given the unphased genotype, based on estimated phased genotype probabilities.

Because of a concern that a few larger families could be driving the association findings, we re-ran the individual polymorphism association analyses in FBAT, the conditional logistic regression analyses and the haplotype analyses in HBAT including only one nuclear family per pedigree. This resulted in a reduction of the sample to 2156 individuals (84.3% of the total sample).

We tested eight polymorphisms, and analyzed the sample as a whole, two strata based on age of onset and two strata based on *APOE-ε4* status, as described above. To account for multiple testing, we used the Bonferroni method, and corrected for 24 tests (i.e. eight polymorphisms tested in the full sample and in strata based on onset age and *APOE-ε4* status). We only counted each stratification once because each pair together comprises the total sample. Similarly, we did not consider haplotypes in calculating the number of tests because the haplotypes are composed of the individual polymorphisms. Because of the extensive correlations among these polymorphisms due to LD, this is a particularly conservative choice, but in effect it sets a lower bound on the statistical significance for these findings.

ACKNOWLEDGEMENTS

This work was sponsored by grants from the NIMH, NIA (ADRC) and the Alzheimer's Association. A.J.S. was supported by a National Research Service Award from NIA and the Harvard Center for Neurodegeneration and Repair (HCNR). L.B. was supported by Deutsche Forschungsgemeinschaft (DFG) and is now a recipient of a translational research fellowship from the Harvard Center for Neurodegeneration and Repair (HCNR). Data and biomaterials were collected in three projects that participated in the National Institute of Mental Health (NIMH) Alzheimer Disease Genetics Initiative. From 1991 to 1998, the Principal Investigators and Co-Investigators were: Massachusetts General Hospital, Boston, MA, Marilyn S. Albert and Deborah Blacker; Johns Hopkins University, Baltimore, MD, Susan S. Bassett, Gary A. Chase and Marshal F. Folstein; University of Alabama, Birmingham, AL, Rodney C.P. Go and Lindy E. Harrell.

REFERENCES

1. Daw, E.W., Payami, H., Nemens, E.J., Nochlin, D., Bird, T.D., Schellenberg, G.D. and Wijsman, E.M. (2000) The number of trait loci in late-onset Alzheimer disease. *Am. J. Hum. Genet.*, **66**, 196–204.
2. Mayeux, R., Lee, J.H., Romas, S.N., Mayo, D., Santana, V., Williamson, J., Ciappa, A., Rondon, H.Z., Estevez, P., Lantigua, R. *et al.* (2002) Chromosome-12 mapping of late-onset Alzheimer disease among Caribbean Hispanics. *Am. J. Hum. Genet.*, **70**, 237–243.
3. Myers, A., Wavrant De-Vrieze, F., Holmans, P., Hamshere, M., Crook, R., Compton, D., Marshall, H., Meyer, D., Shears, S., Booth, J. *et al.* (2002) Full genome screen for Alzheimer disease: Stage II analysis. *Am. J. Hum. Genet.*, **114**, 235–244.
4. Pericak-Vance, M.A., Bass, M.P., Yamaoka, L.H., Gaskell, P.C., Scott, W.K., Terwedow, H.A., Menold, M.M., Conneally, P.M., Small, G.W., Vance, J.M. *et al.* (1997) Complete genomic screen in late-onset familial Alzheimer disease. Evidence for a new locus on chromosome 12. *JAMA*, **278**, 1237–1241.
5. Poduslo, S.E. and Yin, X. (2001) Chromosome 12 and late-onset Alzheimer's disease. *Neurosci. Lett.*, **310**, 188–190.
6. Rogaeva, E., Premkumar, S., Song, Y., Sorbi, S., Brindle, N., Paterson, A., Duara, R., Levesque, G., Yu, G., Nishimura, M. *et al.* (1998) Evidence for an Alzheimer disease susceptibility locus on chromosome 12 and for further locus heterogeneity. *JAMA*, **280**, 614–618.
7. Scott, W.K., Grubber, J.M., Abou-Donia, S.M., Church, T.D., Saunders, A.M., Roses, A.D., Pericak-Vance, M.A., Conneally, P.M., Small, G.W. and Haines, J.L. (1999) Further evidence linking late-onset Alzheimer disease with chromosome 12. *JAMA*, **281**, 513–514.
8. Scott, W.K., Grubber, J.M., Conneally, P.M., Small, G.W., Hulette, C.M., Rosenberg, C.K., Saunders, A.M., Roses, A.D., Haines, J.L. and Pericak-Vance, M.A. (2000) Fine mapping of the chromosome 12 late-onset Alzheimer disease locus: potential genetic and phenotypic heterogeneity. *Am. J. Hum. Genet.*, **66**, 922–932.
9. Wu, W.S., Holmans, P., Wavrant-DeVrieze, F., Shears, S., Kehoe, P., Crook, R., Booth, J., Williams, N., Perez-Tur, J., Roehl, K. *et al.* (1998) Genetic studies on chromosome 12 in late-onset Alzheimer disease. *JAMA*, **280**, 619–622.
10. Blacker, D., Wilcox, M.A., Laird, N.M., Rodes, L., Horvath, S.M., Go, R.C., Perry, R., Watson, B., Jr., Bassett, S.S., McInnis, M.G. *et al.* (1998) Alpha-2 macroglobulin is genetically associated with Alzheimer disease. *Nat. Genet.*, **19**, 357–360.
11. Borth, W. (1992) Alpha 2-macroglobulin, a multifunctional binding protein with targeting characteristics. *FASEB J.*, **6**, 3345–3353.
12. Barrett, A.J. and Starkey, P.M. (1973) The interaction of alpha 2-macroglobulin with proteinases. Characteristics and specificity of the reaction, and a hypothesis concerning its molecular mechanism. *Biochem. J.*, **133**, 709–724.
13. Harpel, P.C. (1973) Studies on human plasma alpha 2-macroglobulin-enzyme interactions. Evidence for proteolytic modification of the subunit chain structure. *J. Exp. Med.*, **138**, 508–521.
14. Sottrup-Jensen, L., Sand, O., Kristensen, L. and Fey, G.H. (1989) The alpha-macroglobulin bait region. Sequence diversity and localization of cleavage sites for proteinases in five mammalian alpha- macroglobulins. *J. Biol. Chem.*, **264**, 15781–15789.
15. Lauer, D., Reichenbach, A. and Birkenmeier, G. (2001) Alpha 2-macroglobulin-mediated degradation of amyloid beta 1–42: a mechanism to enhance amyloid beta catabolism. *Exp. Neurol.*, **167**, 385–392.
16. Qiu, W.Q., Borth, W., Ye, Z., Haass, C., Teplow, D.B. and Selkoe, D.J. (1996) Degradation of amyloid beta-protein by a serine protease-alpha2–macroglobulin complex. *J. Biol. Chem.*, **271**, 8443–8451.
17. Strickland, D.K., Ashcom, J.D., Williams, S., Burgess, W.H., Migliorini, M. and Argraves, W.S. (1990) Sequence identity between the alpha 2-macroglobulin receptor and low density lipoprotein receptor-related protein suggests that this molecule is a multifunctional receptor. *J. Biol. Chem.*, **265**, 17401–17404.
18. Du, Y., Bales, K.R., Dodel, R.C., Liu, X., Glinn, M.A., Horn, J.W., Little, S.P. and Paul, S.M. (1998) Alpha2-macroglobulin attenuates beta-amyloid peptide 1–40 fibril formation and associated neurotoxicity of cultured fetal rat cortical neurons. *J. Neurochem.*, **70**, 1182–1188.
19. Hughes, S.R., Khorkova, O., Goyal, S., Knaeblein, J., Heroux, J., Riedel, N.G. and Sahasrabudhe, S. (1998) Alpha2-macroglobulin associates with beta-amyloid peptide and prevents fibril formation. *Proc. Natl Acad. Sci. USA*, **95**, 3275–3280.
20. Narita, M., Holtzman, D.M., Schwartz, A.L. and Bu, G. (1997) Alpha2-macroglobulin complexes with and mediates the endocytosis of beta-amyloid peptide via cell surface low-density lipoprotein receptor-related protein. *J. Neurochem.*, **69**, 1904–1911.
21. Carter, D.B., Dunn, E., McKinley, D.D., Stratman, N.C., Boyle, T.P., Kuiper, S.L., Oostveen, J.A., Weaver, R.J., Boller, J.A. and Gurney, M.E. (2001) Human apolipoprotein E4 accelerates beta-amyloid deposition in APPsw transgenic mouse brain. *Ann. Neurol.*, **50**, 468–475.
22. Krimbou, L., Tremblay, M., Davignon, J. and Cohn, J.S. (1998) Association of apolipoprotein E with alpha2-macroglobulin in human plasma. *J. Lipid Res.*, **39**, 2373–2386.

23. Blacker, D., Bertram, L., Saunders, A.J., Moscarillo, T.J., Albert, M., Wiener, H., Perry, R., Collins, J.S., Harrell, L.E., Go, R.C.P. *et al.* (2003) Results of a high resolution genome screen of 437 Alzheimer's disease families. *Hum. Mol. Genet.*, **12**, 23–32.
24. Blacker, D., Crystal, A.S., Wilcox, M.A., Laird, N.M. and Tanzi, R.E. (1999) In reply. *Nat. Genet.*, **22**, 21–22.
25. Poduslo, S.E., Shook, B., Drigalenko, E. and Yin, X. (2002) Lack of association of the two polymorphisms in alpha-2 macroglobulin with Alzheimer disease. *Am. J. Med. Genet.*, **110**, 30–35.
26. Rudrasingham, V., Wavrant-De Vrieze, F., Lambert, J.C., Chakraverty, S., Kehoe, P., Crook, R., Amouyel, P., Wu, W., Rice, F., Perez-Tur, J. *et al.* (1999) Alpha-2 macroglobulin gene and Alzheimer disease. *Nat. Genet.*, **22**, 17–19.
27. Romas, S.N., Mayeux, R., Rabinowitz, D., Tang, M.X., Zadroga, H.R., Lantigua, R., Medrano, M., Tycko, B. and Knowles, J.A. (2000) The deletion polymorphism and Val1000Ile in alpha-2-macroglobulin and Alzheimer disease in Caribbean Hispanics. *Neurosci. Lett.*, **279**, 133–136.
28. Rogaeva, E.A., Premkumar, S., Grubber, J., Serneels, L., Scott, W.K., Kawarai, T., Song, Y., Hill, D.L., Abou-Donia, S.M., Martin, E.R. *et al.* (1999) An alpha-2-macroglobulin insertion–deletion polymorphism in Alzheimer disease. *Nat. Genet.*, **22**, 19–22.
29. Zappia, M., Cittadella, R., Manna, I., Nicoletti, G., Andreoli, V., Bonavita, S., Gambardella, A. and Quattrone, A. (2002) Genetic association of alpha-2-macroglobulin polymorphisms with AD in southern Italy. *Neurology*, **59**, 756–758.
30. Nacmias, B., Tedde, A., Cellini, E., Forleo, P., Orlacchio, A., Guarnieri, B.M., Petruzzi, C., D'Andrea, F., Serio, A. and Sorbi, S. (2001) Alpha2-macroglobulin polymorphisms in Italian sporadic and familial Alzheimer's disease. *Neurosci. Lett.*, **299**, 9–12.
31. Jhoo, J.H., Kim, K.W., Lee, D.Y., Lee, K.U., Lee, J.H., Kim, S.Y., Youn, J.Y., Youn, J.C. and Woo, J.I. (2001) Association of alpha-2-macroglobulin deletion polymorphism with sporadic Alzheimer's disease in Koreans. *J. Neurol. Sci.*, **184**, 21–25.
32. Dodel, R.C., Du, Y., Bales, K.R., Gao, F., Eastwood, B., Glazier, B., Zimmer, R., Cordell, B., Hake, A., Evans, R. *et al.* (2000) Alpha2 macroglobulin and the risk of Alzheimer's disease. *Neurology*, **54**, 438–442.
33. Alvarez, V., Alvarez, R., Lahoz, C.H., Martinez, C., Pena, J., Guisasola, L.M., Salas-Puig, J., Moris, G., Uria, D., Menes, B.B. *et al.* (1999) Association between an alpha(2) macroglobulin DNA polymorphism and late- onset Alzheimer's disease. *Biochem. Biophys. Res. Commun.*, **264**, 48–50.
34. Liao, A., Nitsch, R.M., Greenberg, S.M., Finckh, U., Blacker, D., Albert, M., Rebeck, G.W., Gomez-Isla, T., Clatworthy, A., Binetti, G. *et al.* (1998) Genetic association of an alpha2-macroglobulin (Val1000lle) polymorphism and Alzheimer's disease. *Hum. Mol. Genet.*, **7**, 1953–1956.
35. Myllykangas, L., Polvikoski, T., Sulkava, R., Verkkoniemi, A., Crook, R., Tienari, P.J., Pusa, A.K., Niinisto, L., O'Brien, P., Kontula, K. *et al.* (1999) Genetic association of alpha2-macroglobulin with Alzheimer's disease in a Finnish elderly population. *Ann. Neurol.*, **46**, 382–390.
36. Tang, G., Zhang, M., Xie, H., Jiang, S., Wang, Z., Xu, L., Hao, Y., Lin, D., Lan, H., Wang, Y. *et al*. (2002) Aplha-2 macroglobulin I1000 V polymorphism in Chinese sporadic Alzheimer's disease and Parkinson's disease. *Neurosci. Lett.*, **328**, 195–197.
37. Wang, X., Luedecking, E.K., Minster, R.L., Ganguli, M., DeKosky, S.T. and Kamboh, M.I. (2001) Lack of association between alpha2-macroglobulin polymorphisms and Alzheimer's disease. *Hum. Genet.*, **108**, 105–108.
38. Wavrant-DeVrieze, F., Rudrasingham, V., Lambert, J.C., Chakraverty, S., Kehoe, P., Crook, R., Amouyel, P., Wu, W., Holmans, P., Rice, F. *et al.* (1999) No association between the alpha-2 macroglobulin I1000V polymorphism and Alzheimer's disease. *Neurosci. Lett.*, **262**, 137–139.
39. Verpillat, P., Bouley, S., Hannequin, D., Belliard, S., Puel, M., Thomas-Anterion, C., Dubois, B., Agid, Y., Campion, D., Clerget-Darpoux, F. *et al.* (2000) Alpha2-macroglobulin gene and Alzheimer's disease: confirmation of association by haplotypes analyses. *Ann. Neurol.*, **48**, 400–402.
40. Blacker, D., Haines, J.L., Rodes, L., Terwedow, H., Go, R.C., Harrell, L.E., Perry, R.T., Bassett, S.S., Chase, G., Meyers, D. *et al.* (1997) ApoE-4 and age at onset of Alzheimer's disease: the NIMH genetics initiative. *Neurology*, **48**, 139–147.
41. Risch, N.J. (2000) Searching for genetic determinants in the new millennium. *Nature*, **405**, 847–856.
42. Spielman, R.S., McGinnis, R.E. and Ewens, W.J. (1993) Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am. J. Hum. Genet.*, **52**, 506–516.
43. McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D. and Stadlan, E.M. (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology*, **34**, 939–944.
44. Blacker, D., Albert, M.S., Bassett, S.S., Go, R.C., Harrell, L.E. and Folstein, M.F. (1994) Reliability and validity of NINCDS-ADRDA criteria for Alzheimer's disease. The National Institute of Mental Health Genetics Initiative. *Arch. Neurol.*, **51**, 1198–1204.
45. Chen, X., Levine, L. and Kwok, P.Y. (1999) Fluorescence polarization in homogeneous nucleic acid analysis. *Genome Res.*, **9**, 492–498.
46. Rabinowitz, D. and Laird, N. (2000) A unified approach to adjusting association tests for population admixture with arbitrary pedigree structure and arbitrary missing marker information. *Hum. Hered.*, **50**, 211–223.
47. Laird, N.M., Horvath, S. and Xu, X. (2000) Implementing a unified approach to family-based tests of association. *Genet. Epidemiol.*, **19**, S36–42.
48. Tu, I.P., Balise, R.R. and Whittemore, A.S. (2000) Detection of disease genes by use of family data. II. Application to nuclear families. *Am. J. Hum. Genet.*, **66**, 1341–1350.
49. Lake, S.L., Blacker, D. and Laird, N.M. (2000) Family-based tests of association in the presence of linkage. *Am. J. Hum. Genet.*, **67**, 1515–1525.
50. Whittaker, J.C. and Lewis, C.M. (1998) The effect of family structure on linkage tests using allelic association. *Am. J. Hum. Genet.*, **63**, 889–897.
51. Lunetta, K.L., Faraone, S.V., Biederman, J. and Laird, N.M. (2000) Family-based tests of association and linkage that use unaffected sibs, covariates, and interactions. *Am. J. Hum. Genet.*, **66**, 605–614.
52. Lange, C. and Laird, N.M. (2002) On a general class of conditional tests for family-based association studies in genetics: the asymptotic distribution, the conditional power, and optimality considerations. *Genet. Epidemiol.*, **23**, 165–180.
53. Witte, J.S., Gauderman, W.J. and Thomas, D.C. (1999) Asymptotic bias and efficiency in case–control studies of candidate genes and gene–environment interactions: basic family designs. *Am. J. Epidemiol.*, **149**, 693–705.
54. Siegmund, K.D., Langholz, B., Kraft, P. and Thomas, D.C. (2000) Testing linkage disequilibrium in sibships. *Am. J. Hum. Genet.*, **67**, 244–248.
55. Clayton, D. (1999) A generalization of the transmission/disequilibrium test for uncertain-haplotype transmission. *Am. J. Hum. Genet.*, **65**, 1170–1177.
56. Akey, J., Jin, L. and Xiong, M. (2001) Haplotypes vs single marker linkage disequilibrium tests: what do we gain? *Eur. J. Hum. Genet.*, **9**, 291–300.
57. Horvath, S., Xu, X., Lake, S.L., Silverman, E.K., Weiss, S.T. and Laird, N.M. (2003) Family based tests for associating haplotypes with general phenotype data: application to asthma genetics. *Genet. Epidemiol.*, in press.
58. Bagli, M., Papassotiropoulos, A., Jessen, F., Schmitz, S., Rao, M.L., Maier, W. and Heun, R. (2000) Identical distribution of the alpha 2-macroglobulin pentanucleotide deletion in subjects with Alzheimer disease and controls in a German population. *Am. J. Med. Genet.*, **96**, 775–777.
59. Blennow, K., Ricksten, A., Prince, J.A., Brookes, A.J., Emahazion, T., Wasslavik, C., Bogdanovic, N., Andreasen, N., Batsman, S., Marcusson, J. *et al.* (2000) No association between the alpha2-macroglobulin (A2M) deletion and Alzheimer's disease, and no change in A2M mRNA, protein, or protein expression. *J. Neural. Transm.*, **107**, 1065–1079.
60. Bullido, M.J., Guallar-Castillon, P., Artiga, M.J., Ramos, M.C., Sastre, I., Aldudo, J., Frank, A., Coria, F., Rodriguez-Artalejo, F. and Valdivieso, F. (2000) Alzheimer's risk associated with human apolipoprotein E, alpha-2 macroglobulin and lipoprotein receptor related protein polymorphisms: absence of genetic interactions, and modulation by gender. *Neurosci. Lett.*, **289**, 213–216.

61. Chen, L., Baum, L., Ng, H.K., Chan, L.Y., Sastre, I., Artiga, M.J., Valdivieso, F., Bullido, M.J., Chiu, H.F. and Pang, C.P. (1999) Apolipoprotein E promoter and alpha2-macroglobulin polymorphisms are not genetically associated with Chinese late onset Alzheimer's disease. *Neurosci. Lett.*, **269**, 173–177.
62. Crawford, F., Town, T., Freeman, M., Schinka, J., Gold, M., Duara, R. and Mullan, M. (1999) The alpha-2 macroglobulin gene is not associated with Alzheimer's disease in a case–control sample. *Neurosci. Lett.*, **270**, 133–136.
63. Dow, D.J., Lindsey, N., Cairns, N.J., Brayne, C., Robinson, D., Huppert, F.A., Paykel, E.S., Xuereb, J., Wilcock, G., Whittaker, J.L. *et al.* (1999) Alpha-2 macroglobulin polymorphism and Alzheimer disease risk in the UK. *Nat. Genet.*, **22**, 16–17; discussion 21–12.
64. Gibson, A.M., Singleton, A.B., Smith, G., Woodward, R., McKeith, I.G., Perry, R.H., Ince, P.G., Ballard, C.G., Edwardson, J.A. and Morris, C.M. (2000) Lack of association of the alpha2-macroglobulin locus on chromosome 12 in AD. *Neurology*, **54**, 433–438.
65. Halimi, G., Duplan, L., Bideau, C., Iniesta, D., Berthezene, P., Oddoze, C., Verdier, J.M., Michel, B. and Berge-Lefranc, J.L. (2000) Association of APOE promoter but not A2M polymorphisms with risk of developing Alzheimer's disease. *Neuroreport*, **11**, 3599–3601.
66. Higuchi, S., Matsushita, S., Nakane, J., Arai, H., Matsui, T., Urakami, K., Yuzuriha, T. and Takeda, A. (2000) Alpha2-macroglobulin gene polymorphisms show racial diversity and are not associated with Alzheimer's disease. *Neuroreport*, **11**, 1167–1171.
67. Hu, C.J., Sung, S.M., Liu, H.C., Hsu, W.C., Lee, L.S., Lee, C.C., Tsai, C.H. and Chang, J.G. (2000) Genetic risk factors of sporadic Alzheimer's disease among Chinese in Taiwan. *J. Neurol. Sci.*, **181**, 127–131.
68. Hu, C.J., Sung, S.M., Liu, H.C., Lee, K.Y., Hsu, W.C., Wong, W.K., Lee, C.C., Tsai, C.H. and Chang, J.G. (1999) No association of alpha-2 macroglobulin gene five-nucleotide deletion with AD in Taiwan Chinese. *Neurology*, **53**, 642–643.
69. Ki, C.S., Na, D.L., Kim, H.J. and Kim, J.W. (2001) Alpha-1 anti-chymotrypsin and alpha-2 macroglobulin gene polymorphisms are not associated with Korean late-onset Alzheimer's disease. *Neurosci. Lett.*, **302**, 69–72.
70. Korovaitseva, G.I., Premkumar, S., Grigorenko, A., Molyaka, Y., Galimbet, V., Selezneva, N., Gavrilova, S.I., Farrer, L.A. and Rogaev, E.I. (1999) Alpha-2 macroglobulin gene in early- and late-onset Alzheimer disease. *Neurosci. Lett.*, **271**, 129–131.
71. Koster, M.N., Dermaut, B., Cruts, M., Houwing-Duistermaat, J.J., Roks, G., Tol, J., Ott, A., Hofman, A., Munteanu, G., Breteler, M.M. *et al.* (2000) The alpha2-macroglobulin gene in AD: a population-based study and meta-analysis. *Neurology*, **55**, 678–684.
72. Kovacs, T., Cairns, N.J. and Lantos, P.L. (1999) Alpha-2-macroglobulin intronic polymorphism is not associated with autopsy-confirmed late-onset Alzheimer's disease. *Neurosci. Lett.*, **273**, 61–83.
73. McIlroy, S.P., Dynan, K.B., Vahidassr, D.J., Lawson, J.T., Patterson, C.C. and Passmore, P. (2001) Common polymorphisms in LRP and A2M do not affect genetic risk for Alzheimer disease in Northern Ireland. *Am. J. Med. Genet.*, **105**, 502–506.
74. Perry, R.T., Collins, J.S., Harrell, L.E., Acton, R.T. and Go, R.C. (2001) Investigation of association of 13 polymorphisms in eight genes in southeastern African American Alzheimer disease patients as compared to age-matched controls. *Am. J. Med. Genet.*, **105**, 332–342.
75. Shibata, N., Ohnuma, T., Takahashi, T., Ohtsuka, E., Ueki, A. and Arai, H. (1999) Genetic association between alpha-2 macroglobulin and Japanese sporadic Alzheimer's disease. *Neurosci. Lett.*, **271**, 132–134.
76. Singleton, A.B., Gibson, A.M., McKeith, I.G., Ballard, C.A., Perry, R.H., Ince, P.G., Edwardson, J.A. and Morris, C.M. (1999) Alpha2-macro-globulin polymorphisms in Alzheimer's disease and dementia with Lewy bodies. *Neuroreport*, **10**, 1507–1510.
77. Sodeyama, N., Yamada, M., Itoh, Y., Suematsu, N., Matsushita, M., Otomo, E. and Mizusawa, H. (2000) Alpha2-macroglobulin polymorphism is not associated with AD or AD-type neuropathology in the Japanese. *Neurology*, **54**, 443–446.
78. Zill, P., Burger, K., Behrens, S., Hampel, H., Padberg, F., Boetsch, T., Moller, H.J., Ackenheil, M. and Bondy, B. (2000) Polymorphisms in the alpha-2 macroglobulin gene in psychogeriatric patients. *Neurosci. Lett.*, **294**, 69–72.
79. Shibata, N., Ohnuma, T., Takahashi, T., Ohtsuka, E., Ueki, A., Nagao, M. and Arai, H. (2000) No genetic association between alpha-2 macroglobulin I1000V polymorphism and Japanese sporadic Alzheimer's disease. *Neurosci. Lett.*, **290**, 154–156.
80. Styczynska, M., Religa, D., Pfeffer, A., Luczywek, E., Wasiak, B., Styczynski, G., Peplonska, B., Gabryelewicz, T., Golebiowski, M., Kobrys, M. *et al*. (2003) Simultaneous analysis of five genetic risk factors in Polish patients with Alzheimer's disease. *Neurosci. Lett.*, **344**, 99–102.