Our third sample was composed of individuals from an epidemiologically based cohort from Cambridge aged 84 and older in which cognitive function was assessed using the MMSE 3. Of 229 individuals analysed, 47 had MMSE scores less than 22 and 159 had MMSE scores greater than 23 (used as controls in sample 1, above). The odds ratio reported previously for *A2M* (3.56; ref. 2) is detectable with 37 cases and 74 controls (80% power, 5% significance; ref. 5), thus our combined sample of 230 cases and 218 controls should detect this effect. Our autopsy-confirmed sample alone should detect an odds ratio of 2 (80% power, 5% significance).

We designed new primers to facilitate *A2M* genotyping (AmaF, 5′–TTCTCTCATAAGCTTTATCTGTATG–3′; AmaR, 5′–AAGTACTTTCGTACGGTCTCCG–3′). These gave products of 138 bp (wild-type) and 133 bp (deletion), which corresponded with alleles determined using the previous method[2].

We compared frequencies of carriers for at least one **2* allele with wild-type homozygotes[2] and allele frequencies in cases and controls from our three groups and in the pooled cases with controls (Table 1). We saw no significant influence of *A2M*2* on AD or dementia risk in any of these comparisons ($P>0.05$, χ^2 tests) or on MMSE score (Cambridge cohort; $P>0.05$), even after using a multiple regression approach to account for the effect of age on MMSE score.

*A2M*2* is not strongly associated with AD risk in our UK samples. Our 95% confidence intervals for the odds ratio of AD associated with carriers of *A2M*2* did not overlap with that in the Blacker study[2] (1.80–7.03) when we compared autopsy-confirmed cases with all non-demented controls, Cambridge cases with Cambridge controls and all cases with all controls (Table 1). The proportion of *A2M*2* carriers was 32.6% in our elderly, non-demented controls versus 29.5% in the Blacker *et al.* cases, and the *A2M*2* allele frequencies were 17.9% and 16.4% in our controls and the Blacker *et al.*[2] cases, respectively. Our data are unlikely to be confounded by population stratification, because they include two epidemiologically based cohort studies from confined regions including almost exclusively English individuals of European descent.

The discrepancies between our data and those reported previously[2] may be due to regional population differences in AD susceptibility associated with this polymorphism, or the gene may be associated with AD confined to particular ages and the UK and USA samples may have different age distributions. There may be another functional variant closely linked to *A2M*2* conferring AD risk, which is in linkage disequilibrium with the deletion allele in the USA sample but not in the UK samples. Family based association studies[2] may give different odds ratios from population-based case-control studies, either because these are families with a high density of disease, or because affected and unaffected individuals in family based studies are more likely to share other familial factors than population-based cases and controls. We have looked at autopsy-confirmed cases, however, avoiding the diagnostic uncertainties associated with clinical assessments and at epidemiologically based samples, which reflect the impact of this gene in the general population, as opposed to highly selected families.

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α-2 macroglobulin gene and Alzheimer disease

Blacker *et al.*[1] reported an association between a deletion in exon 18 of the α-2 macroglobulin (*A2M*) gene and Alzheimer disease (AD) in a sample of affected and unaffected siblings from families segregating AD. They observed that the degree of conferred risk for AD in *A2M* allele 2 (*A2M*2*) carriers was similar in magnitude to that for carriers of the apolipoprotein (*APOE*) *ε4* allele (*APOE*E4*). We set out to test for a similar association in a powerful, case-control sample composed of 2,616 individuals taken from populations from Europe and the United States. In addition, we extended our analysis of the National Institute of Mental Health (NIMH) family samples studied by Blacker *et al.*[1] using linkage and association approaches. We pooled this NIMH series (270 sibpairs) with a similar series of 125 sibpairs collected through the auspices of the National Institution of Aging (NIA) at the Indiana Alzheimer cell repository to increase the power of our analysis.

We studied four independent association samples of unrelated AD patients with onset over 50 years and relevant controls (Table 1) and diagnosed AD patients according to NINCDS-ADRDA (ref. 2) criteria with either probable or definite AD. We calculated that this sample had over 99% power to detect an effect of equivalent size to that reported by Blacker *et al.*[1] in their familial sample (odds ratio (OR)=3.56), assuming a stringent α level of 0.001. Furthermore, we calculated that our sample was sufficiently powerful (80%) to detect an extremely small effect (OR≥1.2) assuming an α level of 0.05.

We detected the exon 18 mutation using standard PCR and RFLP methods (available on request). We used the Woolf method[3] to test for association in the combined data set and MAPMAKER/SIBS (ref. 4) to compute single-point maximum lod scores.

The genotype distributions of *A2M*2* in relation to *APOE* status are shown

Table 1 • Association between *A2M* and AD

Research centre	Sample descriptions	Summary statistics			
		Whole samples **2/*2* and **1/*2* versus **1/*1*	*APOE*E4–* **2/*2* and **1/*2* versus **1/*1*	Whole sample allele **2/*1*	*APOE*E4–* allele **2/*1*
UK:Cardiff	152 AD patients: 63% F, AO 71.9±8.13	1.5 (0.8–2.8)	1.3 (0.6–2.8)	1.4 (0.8–2.4)	1.2 (0.6–2.5)
	99 controls: 54% F, age 72.6±6.55				
France:Lille	616 AD patients: 63% F, AO 69.4±8.6	1.1 (0.9–1.5)	1.1 (0.8–1.6)	1.1 (0.9–1.4)	1.1 (0.8–1.4)
	648 controls: 63% F, age 73.1±8.5				
US:Wash U	143 AD patients: 62.4% F, AO 73.7±9.51	1 (0.6–1.6)	1.7 (0.9–3.3)	1 (0.6–1.5)	1.6 (0.9–2.7)
	144 controls: 65.2% F, age 77±9.2				
US:Mayo	327 AD patients: 65.5% F, AO 75.5±9.7	0.8 (0.6–1.1)	0.8 (0.5–1.2)	0.8 (0.6–1.1)	0.9 (0.6–1.2)
	487 controls: 67.1% F, age 82.7±7.5				
Combined Sample	1,238 AD patients	1 (0.9–1.2)	1.1 (0.9–1.4)	1 (0.9–1.2)	1.1 (0.9–1.3)
	1,378 controls				

AO, age at onset.

(Table 2), as well as the summary statistics (Table 1). We did not observe an association between AD and possession of at least one copy of the deletion (P=0.7) in the sample overall. We also failed to detect an allelic association (P=0.4), although the combined sample showed the expected association between AD and *APOE*E4* ($P<10^{-10}$, OR=4.6, 95% confidence interval (CI)=3.9–5.5).

As Blacker *et al.*[1] observed a stronger effect in individuals without an *APOE*E4* allele, we stratified our sample according to *APOE*E4* status. We found no evidence of either a genotypic (*A2M*2/*2* and *A2M*2/*1* versus *A2M*1/*1*) or allelic association in individuals with no *APOE*E4* alleles (P=0.5 and P=0.4). All samples of cases and controls were in Hardy-Weinberg equilibrium.

We speculated that the difference between affected and unaffected siblings observed by Blacker *et al.*[1] may reflect the action of a confound, such as age-related differences in exon 18 frequencies between groups. We tested this in the NIMH sample used by Blacker and colleagues[1] and found some evidence of age differences in affected (mean age=75.7±10.9) versus unaffected siblings (mean age=82.5±7.2). We then tested for age differences (50–79 versus 80+ years) in proportions of individuals with the exon 18 deletion in our control samples. We found no association between the *A2M*1/*1* genotype and age (P=0.2). Indeed, we found that the trend was slightly in the opposite direction (data not shown), suggesting that age did not act as a confound.

Our collaborative group recently obtained evidence[5] consistent with linkage (MLS=1.91) to polymorphic markers on chromosome 12 in a region encompassing the *A2M* locus in the same NIMH sample studied by Blacker *et al.*[1]. This did not meet Lander and Kruglyak's criterion[4] for suggestive linkage but was the second highest score obtained in our genome scan and was observed in 68 *APOE*E4*-negative families[6]. We therefore tested for linkage between AD and the exon 18 polymorphism analysed in the NIMH/NIA sample using the exon 18 polymorphism and obtained a lod score of 0.7 in *APOE*E4*-negative affected sibling pairs and a lod score of 0.17 for the sample overall. We thus observed a similar trend towards excess allele sharing in *APOE*E4*-negative siblings, but with a reduced level of significance that might reflect a lack of informativity of the polymorphism.

Finally, we were concerned that the tests used by Blacker *et al.*, which included all members of sibships, may have overestimated the size of the association in their sample. We tested for association using one pair of affected/unaffected siblings chosen at random from each family. We found significant but weaker genotypic (*A2M*2/*2* and **1/*2* versus **1/*1*; P=0.04) and allelic (*A2M*2*; P=0.01) associations in the NIMH sample than those observed by Blacker

Table 2 • Genotype distributions of *A2M* as a function of *APOE*E4* status

	Whole sample			**E4+*			**E4–*		
UK: Cardiff	**1/*1*	**1/*2*	**2/*2*	**1/*1*	**1/*2*	**2/*2*	**1/*1*	**1/*2*	**2/*2*
AD	112	37	3	68	23	2	44	14	1
Control	80	17	2	21	2	1	59	15	1
France: Lille									
AD	434	165	17	245	99	12	189	66	5
Control	474	158	16	88	42	2	386	116	14
US: Wash U									
AD	102	37	4	57	14	0	45	23	4
Control	102	37	5	25	14	1	77	23	4
US: Mayo									
AD	242	78	7	137	45	3	105	33	4
Control	340	132	15	80	25	6	260	107	9
Total									
AD	890	317	31	507	181	17	383	136	14
%	(72%)	(25.5%)	(2.5%)	(72%)	(25.6%)	(2.4%)	(72%)	(25.5%)	(2.5%)
Control	996	344	38	214	83	10	782	261	28
%	(72%)	(25%)	(3%)	(69%)	(27%)	(3%)	(73%)	(24.4%)	(2.6%)

et al.[1], showing ORs of 2.13 and 2.17, respectively. Similar though non-significant trends were also observed in the NIA sample ($P = 0.1$; $P = 0.1$). Combined analyses of the NIMH and NIA samples showed significant genotypic ($P = 0.007$, OR=2.13, CI=1.22–3.7) and allelic ($P=0.003$, OR=2.02, CI=1.3–3.2) associations with AD.

We did not find associations between AD and *A2M*2*, or genotypes containing *A2M**2, in a powerful, case-control sample. We did, however, detect significant but weaker associations in the NIMH sample than those quoted by Blacker *et al.*[1] using the same sample, together with similar but non-significant trends in an independent family sample (NIA). We believe the ORs we observed provide more accurate estimates of the effect sizes than those quoted originally[1]. We conclude that if these data are not due to chance, *A2M*, or a gene in linkage disequilibrium with it, may be weakly associated with AD in some samples and may reflect either an interaction with other susceptibility gene/s segregating in these families or a stronger genetic effect in some of these families. Either case would limit the power of conventional case-control samples to detect association.

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An α-2-macroglobulin insertion-deletion polymorphism in Alzheimer disease

Blacker *et al.*[1] reported an association between Alzheimer disease (AD) and the deletion allele (*A2M*2*) of an intronic polymorphism in the α-2-macroglobulin gene (*A2M*; ref. 2), which confers a risk for AD (OR=3.55, 95% CI=1.90–7.03) comparable with that of *APOE*E4* (OR=3.54, 95% CI=1.76–7.12). We analysed two independent sets of AD families[3,4] using the same family-based association (sibship disequilibrium test[1] (SDT) and sib transmission-disequilibrium test[5] (S-TDT)) methods. Following the scheme of Blacker *et al.*[1], we limited these analyses to nuclear families of European descent in which all affected individuals had AD onset over 50 years, marker information was available for at least one unaffected sib and, in the case of the S-TDT, two or more distinct genotypes were present in the sibship. We averaged *P* values for the S-TDT over 100 iterations (10,000 replicates per iteration) using a Monte-Carlo method[6]. Both data sets demonstrated the association of AD and *APOE*E4* (SDT, Duke, $P=0.000007$; Toronto, $P=0.0009$), indicating sufficient power to detect associations of the magnitude reported for *A2M*2* (ref. 1). Although prior evidence shows that these pedigrees are enriched for an AD locus on chromosome 12 near *A2M* (refs 3,4,7), we were unable to detect an association with the *A2M*2* polymorphism using either the SDT or S-TDT, even when the "stringent unaffecteds" method[1] was applied (SDT, Duke, n = 60, $P = 0.80$, Toronto, n = 45, $P = 0.82$; S-TDT, Duke, n = 17, $P = 0.64$, Toronto, n = 21, $P = 0.75$). Furthermore, we did not detect an association in two independent series of sporadic AD cases of European descent after adjustment for *APOE*E4* status (Table 1), despite the fact that each data set had more than 80% power to detect an odds ratio as low as 1.87 (an effect much smaller than reported for *A2M*2*; ref. 1).

Blacker *et al.* analysed 104 sibships from the NIMH data set[1], but we investigated an overlapping set of 143 NIMH sibships that met the same selection criteria[1]. Analysis of this larger data set generated marginal or non-significant results (SDT, n=143, $P=0.08$; S-TDT, n=59, $P=0.05$). Furthermore, these results derive almost entirely from the subset of sibships collected at a single institution, NIMH site 3 (SDT, n=56, $P=0.03$; S-TDT, n=20, $P=0.04$). The lack of a trend towards an association in sibships from NIMH sites 1 (SDT, n=46, $P=1.0$; S-TDT, n=19, $P=0.85$) and 2 (SDT, n=41, $P=0.65$; S-TDT, n=20, $P=0.53$), together with the robust association with *APOE*E4* ($P<0.0009$) in all three NIMH sub-data sets, indicates that the results for sites 1 and 2 are not due to reduced statistical power.

The *A2M* polymorphism involves nt –7 to –2 upstream of the AG splice sequence of exon 18. The 5-bp insertion/deletion does not affect either the upstream 15-nt imperfect (or the internal 11-nt perfect) polypyrimidine tract or putative hnRNA stem–loop structures (G=–7.1 kCal/mol for the insertion, –8.8 kCal/mol for the deletion) thought to be important for splicing[8]. In agreement with this, RT-PCR studies, including studies using primers specific for a fusion of exons 17 and 19, did not detect aberrant *A2M* splice products in brain (12 deletion carriers versus 6 insertion homozygotes) or liver (3 deletion carriers versus 7 insertion homozygotes; Fig. 1*a*).

Western-blot analysis of A2M in biological samples is difficult because denaturation of A2M tetramers often generates artefactual fragments (for example, heat-induced cleavage at the thiolester site/proteolytic cleavage of the bait region[9,10]). Claims that such fragments are anything other than artefacts will require both precise correlation with *A2M*2* genotype and direct carboxy-terminal protein sequencing. We tested several different antibodies, including monoclonal antibodies raised to epitopes encoded by regions upstream of exon 18, and found no correlation between