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

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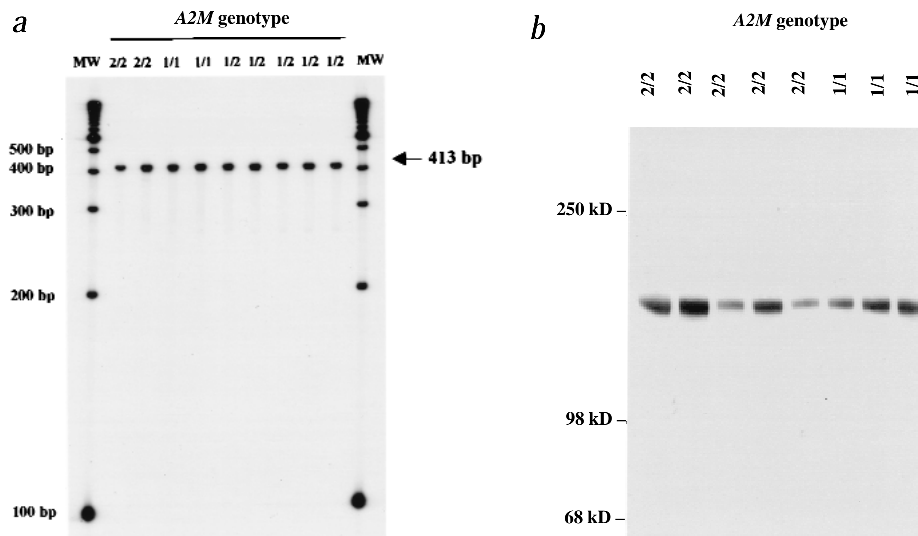

Fig. 1 No difference in molecular weight or abundance of *A2M* transcript and A2M protein monomer in *A2M*2* carriers. ***a***, Overexposed autoradiogram of radiolabelled RT-PCR of brain mRNA from subjects homozygous for the insertion (1/1) and heterozygous (1/2) and homozygous (2/2) for the deletion *A2M* alleles. The analysis generated exclusively the 413-bp product containing exon 18, and did not generate transcripts of 293 bp corresponding to the putative exons 17–19 product. Identical results were obtained with liver mRNA using a single round of long-range PCR and deletion-specific primers (data not shown). ***b***, Western blot of plasma from subjects homozygous for either the insertion (1/1) or the deletion (2/2) *A2M* alleles. Plasma was diluted in ammonia and kept for 30 min at RT to inactivate the internal thiolesters in A2M to prevent heat-induced fragmentation during subsequent denaturation[9,10]. Samples were denatured at 95 °C for 5 min after addition of 1% SDS and 2% 2-mercaptoethanol (final concentrations) in the presence of a protease-inhibitor cocktail[13]. Proteins were separated by 6% Tris-glycine PAGE, transferred to nitrocellulose membrane and detected with monoclonal antibody F45DA5 directed against the N-terminal domain of A2M (refs 9,10). 'Exon 18 skipping' would give rise to truncated fragments of A2M migrating with an expected Mr of approximately 95 kD (refs 9,10). Identical results were obtained with brain homogenates.

truncated A2M fragments and *A2M* genotype (plasma/serum, 10 deletion carriers versus 21 insertion homozygotes; brain homogenates, 8 deletion carriers versus 3 insertion homozygotes; Fig. 1*b*). Plasma A2M levels measured by immuno-electrophoresis[10] and nephelometry, which are known to be stable in a given individual[11], were similar between 7 insertion homozygotes (mean±s.d.=1.80±0.36 mg/ml) and 7 deletion homozygotes (1.61±0.37 mg/ml).

Three possible explanations are envisioned for the disparity between these and previous results[1]. First, the association is restricted to a small subset of cases (for example NIMH site 3). Similar to all other data sets in this and the previous report, however, the NIMH 3 site subset was collected from a mixed population of European descent. Second, population stratification or a systematic diagnostic difference might have occurred in NIMH site 3, but the NIMH collection employed validated standard procedures similar to our own. Third, the reported association may be spurious. This would be concordant with: (i) the reduction in strength of association when more samples from the original NIMH data set are added; (ii) the biological implausibility of results in the Blacker *et al.* study showing random transmission of *A2M* alleles among sibs destined to be affected yet a tendency toward non-sharing among sibs destined to be discordant for AD; (iii) the failure to replicate the association in any of the four independent data sets examined here or elsewhere[12]; and (iv) the absence of a biochemical defect in A2M protein. The prior genetic evidence for an AD susceptibility locus on chromosome 12 (refs 3,4,7) would therefore seem most likely to arise from genetic variations other than in *A2M*2* alleles.

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Table 1 • *A2M* allele and genotype frequencies among sporadic AD cases and controls

	Duke						Toronto					
		Allele frequency (%)		Genotype frequency (%)				Allele frequency (%)		Genotype frequency (%)		
Group	N	*1	*2	*1/*1	*1/*2	*2/*2	N	*1	*2	*1/*1	*1/*2	*2/*2
AD	185	83.0	17.0	68.6	28.7	2.7	207	81.2	18.8	65.7	30.9	3.4
Controls	156	81.4	18.6	66.0	30.8	3.2	164	86.9	13.1	74.4	25.0	0.6
						*APOE*E4* (+) subjects						
AD	127	83.1	16.9	68.5	29.1	2.4	108	77.8	22.2	61.1	33.3	5.6
Controls	45	84.4	15.6	68.9	31.1	0.0	43	83.7	16.3	67.4	32.6	0.0
						*APOE*E4* (–) subjects						
AD	56	82.2	17.8	67.8	28.6	3.6	97	85.1	14.9	71.1	27.8	1.0
Controls	111	80.2	19.8	64.9	30.6	4.5	97	85.6	14.4	72.2	26.8	1.1

AD cases were diagnosed using standard consensus criteria[14]. Clinical characteristics and ascertainment procedures have been reported[15]. *A2M* genotypes were as previously determined[1], and corroborated in a subset of samples using an allele-specific oligonucleotide method. Odds ratios for *A2M*2* on risk of AD computed using logistic regression procedures[16] and adjusted for age, sex and *APOE*E4* were not significant (Duke, OR = 0.92, 95% CI = 0.56–1.53; Toronto, OR = 1.24, 95% CI = 0.69–2.21).

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In reply—We are pleased by Rudrasingham and colleagues'[1] confirmation in the NIA family sample of our finding of a genetic association between the gene encoding α-2-macroglobulin (*A2M*) and Alzheimer disease (AD) in the NIMH sample[2]. We also do not find it surprising that the case-control studies conducted by Rudrasingham *et al.*, Rogaeva *et al.* and Dow *et al.* did not find an association[1,3,4].

The findings above highlight some differences between family based and case-control association studies. First, many but not all family based studies are free of biases resulting from population admixture[5–7]. Second, as Rudrasingham *et al.* point out, the family based design estimates the magnitude of the effect in the context of other shared familial factors (genetic or environmental). Because family based association studies estimate an odds ratio conditional on being from the same family, which is expected to be larger[8], they cannot be used to estimate power for case-control design (as all three papers here have done[1,3,4]).

The statistical methods needed to properly analyse family data when unaffected siblings serve as controls[5,6,9,10] are new and unfamiliar to many geneticists. This is probably responsible for some of the apparent discrepancies among the analyses of the three overlapping samples from the NIMH set[1–3]. Differences are also due to the distribution of somewhat different NIMH samples to each group. In response, we present the results of both proper and improper statistical analyses using 120 NIMH families, an expansion of our original 104 (ref. 1).

Rudrasingham *et al.*[1] estimate the effect of the *A2M*2* allele on AD using the crude odds ratio (OR) in discordant NIMH and NIA sibpairs without taking family relationship into account, yielding estimates that are biased downwards[8]. To obtain an unbiased estimate, the Mantel-Haenszel OR (ref. 11) or equivalently conditional logistic regression[10,12] should be used. Contrary to the assertion of Rudrasingham *et al.*, these methods yield unbiased estimates irrespective of sibship size[10] (but estimates of statistical significance may be somewhat inflated[11,12], especially if large families are genetically linked to one allele). Using our 120 families from the NIMH sample, the crude OR in discordant sibpairs is 1.68 (95% CI, 0.94, 2.99; P=0.13); however, the more accurate OR based on conditional logistic regression using all siblings is 2.31 (1.27, 4.19; P=0.006), and using only sibpairs yields a similar OR of 2.50 (1.10, 5.68; P=0.029).

The sibship disequilibrium test[6] (SDT) in the enlarged NIMH sample is shown (Table 1). Unlike Rogaeva *et al.*'s analysis in 143 NIMH families[3], but consistent with Rudrasingham's analysis of their overlapping NIMH subset[1], our 120 families continue to show highly significant evidence of association of *A2M*2* with AD. When we stratified the sample by site[3], we observed a consistent trend at all three sites, but only two remained statistically significant, as expected with reduced power. If the sample is limited to discordant sibpairs, however, power is substantially reduced, especially in the stratified analysis, leading to reduced significance. Such restriction is unnecessary because the SDT remains valid in sibships of arbitrary size[6].

Notwithstanding the disparate findings contained in these reports, the weakly positive signal from the NIA family based study[1], along with our extended analyses in the NIMH sample, lend further support to a genetic association between *A2M*2* and AD. Moreover, the biological plausibility of this association remains high: three linkage studies have observed peaks in this region[13–15] and there is evidence implicating *A2M* in AD pathogenesis (for example, Aβ clearance[2]). Additional studies will be

Table 1 • Sibship disequilibrium test on an enlarged NIMH sample

All eligible families	No. families	No. subjects	B, C	*P* value
	120	437	12, 34	0.0016
Site 1	45	145	6, 9	0.61
Site 2	34	119	4, 14	0.031
Site 3	41	173	2, 11	0.022

B, number of families favouring transmission of **2* to unaffecteds; C, number of families favouring transmission of **2* to affecteds.