Synergy between the C2 allele of transferrin and the C282Y allele of the haemochromatosis gene (HFE) as risk factors for developing Alzheimer’s disease


Background: There is evidence that iron may play a role in the pathology of Alzheimer’s disease (AD). There may be genetic factors that contribute to iron deposition resulting in tissue damage thus exacerbating AD.

Methods: We have genotyped 269 healthy elderly controls, 191 cases with definite or probable AD, and 69 with mild cognitive impairment (MCI) from the OPTIMA cohort.

Results: We have examined the interaction between the C2 variant of the transferrin (TF) gene and the C282Y allele of the haemochromatosis (HFE) gene as risk factors for developing AD. Our results showed that each of the two variants was associated with an increased risk of AD only in the presence of the other. Neither allele alone had any effect. Carriers of both variants were at 5 times greater risk of AD compared with all others. The interaction was significant by logistic regression (p = 0.014) and by synergy factor analysis (p = 0.015, synergy factor = 5.1). Further, carriers of these two alleles plus apolipoprotein E ε4 (APOE4) were at still higher risk of AD: of the 14 tri-carriers of the three variants, identified in this study, 12 had AD and two MCI.

Conclusion: We suggest that the combination of TF C2 and HFE C282Y may lead to an excess of redox-active iron and the induction of oxidative stress in neurones, which is exacerbated in carriers of APOE4. Since 4% of Northern Europeans carry the two iron-related variants and since iron overload is a treatable condition, these results merit replication.

Methods
All 191 cases of AD (108 women), 69 cases of MCI (30 women) as defined by Petersen et al., and 269 controls (139 women) were Caucasians from the OPTIMA cohort. This is a longitudinal, observational cohort of dementia patients and elderly controls, drawn from the Oxford region and followed for up to 15 years. All participants have undergone annual assessments, including a detailed history, physical examination, blood tests, and computerised tomography (CT) and single photon emission tomography (SPECT) scans (for measuring cerebral blood flow), as well as 6-monthly cognitive assessments using CAMDEX. Post mortem examinations are performed, where consent is given, which is normally the case.

Mean age of onset of AD was 70.5 ± 9.2 years and of death by last examination of controls was 76.1 ± 8.9 years. Of the AD cases, 111 were neuropathologically confirmed by CERAD criteria (96 “definite” and 15 “probable”) and 80 were diagnosed “probable AD” by NINCDS-ADRDA criteria. Possible autosomal dominant cases were excluded, based on family history. MCI cases were based on the criteria of Petersen et al. All 269 controls were without cognitive impairment and with CAMCOG scores >80.

Genotyping of TF C2 was performed as previously described with the following modifications. The number of PCR cycles was increased from 35 to 40, BstEII (New England Biolabs) digestion was performed at 60°C and fragments were resolved using 3% NuSieve:1% agarose gels (Flowgen) buffered with Tris borate EDTA pH 8.3.
**RESULTS**

A total of 529 individuals (191 AD, 69 MCI, and 269 controls) were typed for the C282Y and H63D alleles of HFE and the C2 allele of TF.

Table 1 shows the genotypes of TF C2 and of HFE C282Y and H63D in AD, MCI, and controls. HFE C282Y and H63D genotypes were in Hardy-Weinberg equilibrium; TF C2 genotypes were not in Hardy-Weinberg equilibrium in the control population, as may occur with disease-related genes, for example with the DCP1 gene in several AD association studies. Control frequencies of the TF C2 and HFE C282Y and H63D alleles were 21, 6, and 15%, respectively, which are typical of Northern European populations.

We first examined the association with AD of each of TF C2 and HFE C282Y when stratified for the presence of the other. Neither variant was associated with AD without the other, nor overall, but each increased the risk of AD in the presence of the other (tables 2 and 3).

We then compared carriers of both variants with all other subjects. Table 4 shows the unadjusted odds ratios of AD for bi-carriers of TF C282Y and H63D versus all others was 4.8 (95% confidence interval (CI) 1.7 to 13.4, p = 0.001) and, after adjusting for age, gender, systolic blood pressure, and for years of education, it was 5.4 (CI 1.5 to 19.9, p = 0.01). There was a trend, not statistically significant, for an association of MCI with bi-carriers.

Formal tests of interactions are logistic regression and synergy factor analysis. The former gave p = 0.014 for the interaction in AD risk between HFE C282Y and TF C2, controlling for age and for gender, and p = 0.047, when also controlling for systolic blood pressure and for years of education. The synergy factor for these two variants in AD risk was 5.1, with p = 0.015.

Although the association was significant in women (OR of AD for bi-carriers = 5.0, 95% CI 1.8 to 14, p = 0.007) and, after adjusting for age, gender, and years of education, it was 5.4 (CI 1.5 to 19.9, p = 0.01). There was a trend, not statistically significant, for an association of MCI with bi-carriers.

We also examined the interaction with APOE4. Tri-carriers of HFE C282Y, TF C2 and APOE4 appeared at still greater risk of AD: OR (versus all others) = 37.5, 95% CI 2.2 to 638, p<0.0001. Of the 14 tri-carriers of the three variants, identified in this study, 12 had AD and two had MCI.

We found no association of the HFE H63D variant with AD, or with MCI, either overall or in two-way interaction with either TF C2 or APOE4 (data not shown). But all five tri-carriers of the C282Y and H63D variants of HFE plus TF C2 either had AD (n = 4) or MCI (n = 1). The OR of AD for these tri-carriers versus all others was 12.9 (95% CI 0.7 to 242, p = 0.03).

**DISCUSSION**

Our results showed that in the Oxford population, neither TF C2 nor HFE C282Y alone was associated with AD, yet bi-carriers were at 5 times greater risk of AD. Carrying HFE H63D as well may further increase the risk. Furthermore, tri-carriers of HFE C282Y, TF C2 and APOE4 may be at still greater risk. Why should this be?

**Biology of the HFE C282Y, HFE H63D and TF C2 proteins**

Once transferrin binds to transferrin receptor 1, the complex is internalised and iron is released, as the lower pH of the endosome is reached. The wild type HFE protein was found to bind transferrin receptor 1 and to decrease the receptor’s affinity for transferrin. The C282Y variant, however, fails to bind to transferrin receptor 1 (TFR1), leaving transferrin free to bind TFR1 with high affinity. The H63D variant may also fail to reduce the affinity of transferrin for TFR1, even though this variant does bind to TFR1. The increased binding of
transferrin to TFR1 raises the risk of peripheral iron overload, although its effect on iron metabolism in the brain is largely unknown.

The TF C2 variant has been reported to be associated with various conditions related to free radicals, suggesting differences in iron metabolism of TF C2 carriers.46 One study found that total iron-binding capacity was lower in TF C2 carriers.46 This suggests that TF C2 may have a lower affinity for iron. We therefore suggest that in bi-carriers of HFE C282Y and TF C2 it is possible that more transferrin–receptor complexes are internalised by neurones (due to HFE C282Y) and iron is more readily released in endosomes (due to TF C2), leading to higher levels of free iron and the production of free radicals, which damage the membranes of vulnerable neurones. Alternatively, if the total iron-binding capacity is lower in TF C2 carriers there may be more free iron which could also result in tissue damage.

Iron and AD
Iron is essential for the activity of many enzymes and oxygen carriers, but its misregulation in the brain can lead to oxidative stress and neurodegeneration. Ferrous iron, the redox-active form, can react with oxygen to produce the superoxide radical and react with hydrogen peroxide to generate the hydroxyl radical, which can damage every category of macromolecule. There are several well-studied examples of neurodegenerative conditions due to mutations in genes of iron metabolism, which lead to oxidative stress.

There are numerous signs of iron misregulation in AD. Non-haem and redox-active iron is found in tangles and in the neurites of plaques in the AD brain.45 The expression and distribution in the brain of various proteins of iron metabolism change during ageing and in AD, for example transferrin,1 2 TFR1,44 ferritin,1 45 lactotransferrin,46 haem oxygenase-1,47 and iron regulatory protein 2 (IRP2).48 The ferritin:iron49 and transferrin:iron ratios1 both decrease in certain regions of the AD brain. The role of iron regulatory proteins (IRPs) is to maintain cellular iron homeostasis by adjusting the ratio of the expression of ferritin to that of TFR1, according to the cell’s needs for iron storage and uptake. This iron homeostasis may be lost in AD, where it has been reported that IRPs may form stable complexes with iron response elements in the 5′ or 3′ untranslated regions of mRNA that could result in increased synthesis of TFR1 and decreased expression of ferritin, leading to increased cellular uptake of iron with reduced storage. Perry et al.46 have suggested that metals bound to cytoplasmic RNA are the main sites of redox activity in neurones in AD. They highlighted the large quantity of cytoplasmic RNA in pyramidal neurones. A type II iron response element has been found in the 5′ untranslated region of the amyloid precursor protein.51 This suggests that iron is intimately involved in regulating a protein that is known to be involved in AD.

Oxidative stress in AD
There is ample evidence of oxidative damage in AD (reviewed in Christen52, Perry et al.53 and Praticò54), particularly to lipids51–54 but also to proteins,55–57 RNA55,58 and DNA.56,58 including mitochondrial DNA,52 although there is also some contrary evidence.59–61 The post mortem evidence, which has certain limitations, has recently been supported by studies in living patients using the cerebrospinal fluid markers, F2-isoprostanes62 (reviewed in Praticò54). It is thought that in AD, mitochondria supply the precursors of hydroxyl radicals, that is, redox-active iron and hydrogen peroxide.63 64 The lack of a strong compensatory increase in antioxidant enzymes55 56 may be due to the role of β-amyloid peptides in inactivating them.56 67 It has been suggested that vulnerable neurones are more sparsely myelinated and are therefore subject to higher energy turnover and are hence less resistant to oxidative stress.55 68 It may be relevant that myelination demands iron and oligodendrocytes contain high levels of iron.59 60 Two groups have proposed, for differing reasons, that oxidative stress is an early event in AD, probably preceding β-amyloid accumulation.55 71

Oxidative stress and APOE4
Apolipoprotein E (apoE) has antioxidant activity at physiological levels, in the order E2>E3>E4, as was shown in cell cultures by Miyata and Smith.69 The antioxidant activity of apoE has been supported by studies in mice70 71 and the association of APOE4 with oxidative stress has been shown in AD patients.72 73 74 It has been suggested that apoE acts as an antioxidant by sequestering metals.75 It was found that apoE bound copper, zinc, and bis- and trivalent iron, but not aluminium nor certain other metals. It is particularly relevant that, unlike copper and zinc, both ferrous and ferric forms of iron remained bound to apoE even when pH was reduced to 2.5.76 This suggests that apoE could stay attached to iron, even after the latter had dissociated from transferrin (see above), thus enabling apoE2 and E3, but not E4, to act as antioxidants if they exist in the same intracellular compartment.

Mild cognitive impairment (MCI)
If our above hypothesis is correct and if oxidative stress is an early event in AD, as suggested,71 then we should see some sign of the association in MCI. We found a tendency for bi-carriers of HFE C282Y and TF C2 to be at risk for MCI, in line with their associations with AD (table 4). Also, in both sets of tri-carriers examined (that is bi-carriers plus APOE4 and bi-carriers plus HFE H63D), there were cases of AD and MCI, but no controls whatsoever. These findings for MCI are consistent with our results for AD and with our hypothesis.

CONCLUSIONS
We suggest that bi-carriers of HFE C282Y and TF C2 may be at higher risk of AD, due to increased redox-active iron and therefore oxidative stress in the cytoplasm of vulnerable neurones. Carriers of APOE2 and APOE3 appear partially protected from this oxidative damage, but tri-carriers of the two iron-related variants plus APOE4 may be at very high risk of AD.

There have been many other reports of associations of AD with genes in the human major histocompatibility complex,77 but these have been conflicting. Although HFE C282Y is in weak linkage disequilibrium with HLA-B7 and we ourselves had found an association of HLA-B7 with AD,77 that earlier finding was solely in APOE4-negative subjects, while our current result is mainly in APOE4 carriers. Although we cannot exclude linkage disequilibrium with other nearby genes or with those on the extended haplotype of HFE C282Y,78 79 we consider it unlikely that our present results are due to linkage disequilibrium with any of the classical HLA genes.

Our results may partly explain the conflicting reports of previous association studies of AD with TF C2 and with HFE C282Y, though the rarity of the latter allele outside populations of Northern European origin will also be a factor.80 81 In general, the associations of susceptibility genes with complex diseases are limited by interactions with age, gender, other genes, and the environment.82 It is unlikely that the roles of these genes will be understood without investigating these interactions, which may differ by ethnic group.
If replicated, the findings will also contribute to our understanding of the causality of AD and of the roles of redox-active iron, of oxidative stress, and of APOE4 in that causality. They may help to explain why APOE4 increases the risk of AD, if oxidative stress has not generally been found to contribute to faster progression of the disease, if oxidative stress is indeed an early event in AD as has been suggested. Furthermore, if it is also shown that bi-carrriers of these variants suffer from iron overload, we note that this is a potentially treatable condition. Approximately 4% of Northern Europeans carry both variants.

ACKNOWLEDGEMENTS

We especially thank all patients and volunteers, members of OPTIMA, and the Department of Neuropathology, Radcliffe Infirmary. We thank Professor Sir David Weatherall for support and encouragement.

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We are most grateful to Bristol Myers Squibb, the Medical Research Council, the Tayside and Tayside Hitachi Foundations, and the Taksunawa Foundation for financial support. This work has been supported by EC contract CTQ 1999–02237.

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