ORIGINAL ARTICLE

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

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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 (*APOE*4) 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 *APOE*4. Since 4% of Northern Europeans carry the two iron-related variants and since iron overload is a treatable condition, these results merit replication.

There is evidence of iron misregulation[1–3] (reviewed in Thompson *et al*[4] and Ke and Qian[5]) and of oxidative stress (reviewed in Christen[6], Perry *et al*[7] and Praticò[8]), partly due to redox-active iron,[3 9] in brains of patients with Alzheimer's disease (AD). The products of the transferrin (*TF*) and haemochromatosis (*HFE*) genes interact in iron metabolism by competing for binding the transferrin receptor.[10] The combination of the *TF* C2 and *HFE* C282Y variants may result in an excess of free iron and in the generation of free radicals in neurones (see Discussion).

Both the *TF* C2 and *HFE* C282Y variants, as well as the *HFE* H63D polymorphism, have been investigated as potential risk factors for AD, with mixed results (*TF* C2,[11–15] *HFE* H63D and C282Y[15–20]). The interaction between *TF* C2 and *HFE* C282Y has not been specifically studied, to our knowledge. Sporadic AD is a complex disease. Each susceptibility gene is thus subject to interactions, which limit its overall association with disease.[21] We therefore examined the interaction between *TF* C2 and *HFE* C282Y in AD and in mild cognitive impairment (MCI)[22] in the cohort of the Oxford Project to Investigate Memory and Ageing (OPTIMA). We also looked for an interaction with apolipoprotein E ε4 (*APOE*4), as it is not only an established risk factor for AD[23 24] but is associated with oxidative stress.[25]

METHODS

All 191 cases of AD (108 women), 69 cases of MCI (30 women) as defined by Petersen *et al*[22], 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 (SPET) scans (for measuring cerebral blood flow), as well as 6-monthly cognitive assessments using CAMDEX.[26] 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 or of last examination of controls was 76.1 ± 8.9 years. Of the AD cases, 111 were neuropathologically confirmed by CERAD[27] criteria (96 "definite" and 15 "probable") and 80 were diagnosed "probable AD" by NINCDS-ADRDA criteria.[28] Possible autosomal dominant cases were excluded, based on family history. MCI cases were based on the criteria of Petersen *et al*.[22] All 269 controls were without cognitive impairment and with CAMCOG scores >80.[26]

Genotyping of *TF* C2 was performed as previously described[29] with the following modifications. The number of PCR cycles was increased from 35 to 40, *Bst*EII (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.

Abbreviations: AD, Alzheimer's disease; CERAD, The Consortium to Establish a Registry for Alzheimer's Disease; CI, confidence interval; *HFE*, the haemochromatosis gene; IRP, iron regulatory protein; MCI, mild cognitive impairment; NINCDS-ADRDA, National Institute of Neurological, Communicative Diseases and Stroke-Alzheimer's Disease and Related Diseases Association; NS, not significant; OPTIMA, the Oxford Project to Investigate Memory and Ageing; OR, odds ratio; *TF*, the transferrin gene; TfR1, transferrin receptor 1

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HFE genotyping was carried out as described[30] using the modified primer for the C282Y amplification.[31] Standard PCR methods were used for *APOE*.[32] All genotyping was undertaken blind to diagnosis. Unadjusted odds ratios (OR) were by Fisher's exact test. Adjusted odds ratios were by logistic regression analysis using ''R''.[33] Synergy factor analysis used the method of Lehmann *et al*.[21]

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, in MCI, and in 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.[34] 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.[11 13 30]

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 and MCI. The unadjusted odds ratio (OR) of AD for bi-carriers of *HFE* C282Y and *TF* C2 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.[35] 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 = 7.0, 95% CI 1.5 to 33, $p = 0.006$) and not in men (OR = 3.3, 95% CI 0.8 to 14, $p = 0.16$, NS), the interaction with gender was not significant by logistic regression analysis ($p = 0.4$). There was thus no evidence of an interaction with gender, nor with age (data not shown). Nor did carrying either variant or both affect the mean onset age of AD, which was 70.7 years for bi-carriers and 70.4 years for all others.

Table 1 *TF* C2 and *HFE* C282Y and H63D genotypes in AD, in MCI, and in controls

	AD		MCI		Controls	
Genotypes	%	(*n*)	%	(*n*)	%	(*n*)
TF C2-negative	57.6	(110)	71.0	(49)	66.5	(179)
TF C2 heterozygotes	34.0	(65)	24.6	(17)	25.7	(69)
TF C2 homozygotes	8.4	(16)	4.3	(3)	7.8	(21)
HFE C282Y-negative	84.3	(161)	75.4	(52)	88.1	(237)
HFE C282Y heterozygotes	15.7	(30)	23.2	(16)	11.5	(31)
HFE C282Y homozygotes	0	(0)	1.4	(1)	0.4	(1)
HFE H63D-negative	72.2	(138)	75.4	(52)	72.1	(194)
HFE H63D heterozygotes	26.2	(50)	24.6	(17)	24.9	(67)
HFE H63D homozygotes	1.6	(3)	0	(0)	3.0	(8)
Total number		(191)		(69)		(269)

AD, Alzheimer's disease; *TF* and *HFE*, the transferrin and haemochromatosis genes, respectively; MCI, mild cognitive impairment.

Table 2 Risk of AD associated with the *HFE* C282Y allele, by *TF* C2 carrier status

Subgroup	AD (*n*)	Controls (*n*)	OR	(95% CI)	p
TF C2-positive	81	90	3.2	(1.2–8.3)	0.016
TF C2-negative	110	179	0.8	(0.4–1.6)	0.6, NS
All	191	269	1.3	(0.8–2.2)	0.35, NS

AD, Alzheimer's disease; CI, confidence interval; NS, not significant; OR, odds ratio.

Table 3 Risk of AD associated with the *TF* C2 allele, by *HFE* C282Y carrier status

Subgroup	AD (*n*)	Controls (*n*)	OR	(95% CI)	p
HFE C282Y-positive	30	32	4.1	(1.6–10.5)	0.004
HFE C282Y-negative	161	237	1.1	(0.8–1.6)	0.5, NS
All	191	269	1.3	(0.96–1.8)	0.09, NS

AD, Alzheimer's disease; CI, confidence interval; NS, not significant; OR, odds ratio.

We also examined the interaction with *APOE*4. Tri-carriers of *HFE* C282Y, *TF* C2 and *APOE*4 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 *APOE*4 (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 *APOE*4 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.[36] The wild type HFE protein was found to bind transferrin receptor 1 and to decrease the receptor's affinity for transferrin.[10] 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.[10] The increased binding of

Table 4 Risk of AD and of MCI for bi-carriers of *HFE* C282Y and *TF* C2

	AD	MCI	Controls
Bi-carriers (*n*)	16	4	5
All others (*n*)	175	65	264
OR (95% CI)	4.8 (1.7 to 13)	3.2 (0.8 to 12)	
p	0.001	0.09, NS	

AD, Alzheimer's disease; CI, confidence interval; MCI, mild cognitive impairment; NS, not significant; OR, odds ratio.

transferrin to TfR1 raises the risk of peripheral iron overload,[37–40] 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.[41] One study found that total iron-binding capacity was lower in *TF* C2 carriers.[42] 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.[5]

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.[3 43] 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:iron[49] and transferrin:iron ratios[2] 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 mRNAs.[50] This 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*[9] 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 Christen[6], Perry *et al*[7] and Praticò[8]), particularly to lipids[52–54] but also to proteins,[55–58] RNA,[59 60] and DNA,[58 61] including mitochondrial DNA,[62] although there is also some contrary evidence.[58 63] The post mortem evidence, which has certain limitations, has recently been supported by studies in living patients using the cerebrospinal fluid markers, F_2-isoprostanes[64] (reviewed in Praticò[8]). It is thought that in AD, mitochondria supply the precursors of hydroxyl radicals, that is, redox-active iron and hydrogen peroxide.[7 9 65] The lack of a strong compensatory increase in antioxidant enzymes[55 56] may be due to the role of β-amyloid peptides in inactivating them.[66 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.[68] It may be relevant that myelination demands iron and oligodendrocytes contain high levels of iron.[69 70] Two groups have proposed, for differing reasons, that oxidative stress is an early event in AD, probably preceding β-amyloid accumulation.[8 71]

Oxidative stress and *APOE*4

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.[25] The antioxidant activity of apoE has been supported by studies in mice[72 73] and the association of *APOE*4 with oxidative stress has been shown in AD patients.[72 74–76] It has been suggested that apoE acts as an antioxidant by sequestering metals.[25] It was found that apoE bound copper, zinc, and bi- 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.[25] 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,[8 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 *APOE*4 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 *APOE*2 and *APOE*3 appear partially protected from this oxidative damage, but tri-carriers of the two iron-related variants plus *APOE*4 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 *APOE*4-negative subjects, while our current result is mainly in *APOE*4 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.[30 80] In general, the associations of susceptibility genes with complex diseases are limited by interactions with age, gender, other genes, and the environment.[21] 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 *APOE*4 in that causality. They may help to explain why *APOE*4 increases the risk of AD,[23, 24] but has not generally been found to contribute to faster progression of the disease,[81, 82] if oxidative stress is indeed an early event in AD as has been suggested.[8, 72]

Furthermore, if it is also shown that bi-carriers of these variants suffer from iron overload, we note that that is a potentially treatable condition. Approximately 4% of Northern Europeans carry both variants.

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