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 $\epsilon 4$ (*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.

There is evidence of iron misregulation^{1–3} (reviewed in Thompson *et al.*⁴ and Ke and Qian⁵) and of oxidative stress (reviewed in Christen⁶, Perry *et al.*⁷ and Praticò⁸), 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 $\epsilon 4$ (*APOE4*), 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 CERAD²⁷ 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 described²⁹ 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 described³⁰ 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

Genotypes	AD		MCI		Controls	
	%	(n)	%	(n)	%	(n)
<i>TF</i> C2-negative	57.6	(110)	71.0	(49)	66.5	(179)
<i>TF</i> C2 heterozygotes	34.0	(65)	24.6	(17)	25.7	(69)
<i>TF</i> C2 homozygotes	8.4	(16)	4.3	(3)	7.8	(21)
<i>HFE</i> C282Y-negative	84.3	(161)	75.4	(52)	88.1	(237)
<i>HFE</i> C282Y heterozygotes	15.7	(30)	23.2	(16)	11.5	(31)
<i>HFE</i> C282Y homozygotes	0	(0)	1.4	(1)	0.4	(1)
<i>HFE</i> H63D-negative	72.2	(138)	75.4	(52)	72.1	(194)
<i>HFE</i> H63D heterozygotes	26.2	(50)	24.6	(17)	24.9	(67)
<i>HFE</i> 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
<i>TF</i> C2-positive	81	90	3.2	(1.2–8.3)	0.016
<i>TF</i> 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
<i>HFE</i> C282Y-positive	30	32	4.1	(1.6–10.5)	0.004
<i>HFE</i> 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 *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.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:iron⁴⁹ and transferrin:iron ratios² 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 Christen⁶, Perry *et al*⁷ and Praticò⁸), particularly to lipids⁵²⁻⁵⁴ 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, F₂-isoprostanes⁶⁴ (reviewed in Praticò⁸). 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 enzymes⁵⁵⁻⁵⁶ 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 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.²⁵ The antioxidant activity of apoE has been supported by studies in mice⁷²⁻⁷³ and the association of *APOE4* 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 *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.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 *APOE4* in that causality. They may help to explain why *APOE4* 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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REFERENCES

- 1 Connor JR, Menzies SL, St Martin SM, Mufson EJ. A histochemical study of iron, transferrin, and ferritin in Alzheimer's diseased brains. *J Neurosci Res* 1992;**31**(1):75-83.
- 2 Loeffler DA, Connor JR, Juneau PL, Snyder BS, Kanaley L, DeMaggio AJ, Nguyen H, Brickman CM, LeWitt PA. Transferrin and iron in normal, Alzheimer's disease, and Parkinson's disease brain regions. *J Neurochem* 1995;**65**(2):710-24.
- 3 Smith MA, Harris PL, Sayre LM, Perry G. Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. *Proc Natl Acad Sci U S A* 1997;**94**(18):9866-8.
- 4 Thompson KJ, Shoham S, Connor JR. Iron and neurodegenerative disorders. *Brain Res Bull* 2001;**55**(2):155-64.
- 5 Ke Y, Qian ZM. Iron misregulation in the brain: a primary cause of neurodegenerative disorders. *Lancet Neurol* 2003;**2**:246-53.
- 6 Christen Y. Oxidative stress and Alzheimer disease. *Am J Clin Nutr* 2000;**71**(2):621S-9S.
- 7 Perry G, Nunomura A, Hirai K, Zhu X, Perez M, Avila J, Castellani RJ, Atwood CS, Aliev G, Sayre LM, Takeda A, Smith MA. Is oxidative damage the fundamental pathogenic mechanism of Alzheimer's and other neurodegenerative diseases? *Free Radic Biol Med* 2002;**33**(11):1475-9.
- 8 Praticò D. Alzheimer's disease and oxygen radicals: new insights. *Biochem Pharmacol* 2002;**63**(4):563-7.
- 9 Perry G, Taddeo MA, Petersen RB, Castellani RJ, Harris PL, Siedlak SL, Cash AD, Liu Q, Nunomura A, Atwood CS, Smith MA. Adventitiously-bound redox active iron and copper are at the center of oxidative damage in Alzheimer disease. *Biomaterials* 2003;**16**(1):77-81.
- 10 Feder JN, Penny DN, Irrinki A, Lee VK, Lebrón JA, Watson N, Tsuchihashi Z, Sigal E, Bjorkman PJ, Schatzman RC. The hemochromatosis gene product complexes with the transferrin receptor and lowers its affinity for ligand binding. *Proc Natl Acad Sci U S A* 1998;**95**:1472-7.
- 11 van Rensburg SJ, Carstens ME, Potocnik FC, Aucamp AK, Taljaard JJ. Increased frequency of the transferrin C2 subtype in Alzheimer's disease. *Neuroreport* 1993;**4**(11):1269-71.
- 12 Namekata K, Imagawa M, Terashi A, Ohta S, Oyama F, Ihara Y. Association of transferrin C2 allele with late-onset Alzheimer's disease. *Hum Genet* 1997;**101**(2):126-9.
- 13 Van Landeghem GF, Sikström C, Beckman LE, Adolfsson R, Beckman L. Transferrin C2, metal binding and Alzheimer's disease. *Neuroreport* 1998;**9**(2):177-9.
- 14 Hussain RI, Ballard CG, Edvardson JA, Morris CM. Transferrin gene polymorphism in Alzheimer's disease and dementia with Lewy bodies in humans. *Neurosci Lett* 2002;**317**(1):13-6.
- 15 Lleó A, Blesa R, Angelopoulos C, Pastor-Rubio P, Villa M, Oliva R, Bufill E. Transferrin C2 allele, hemochromatosis gene mutations, and risk for Alzheimer's disease. *J Neural Neurosurg Psychiatry* 2002;**72**(6):820-1.
- 16 Moalem S, Percy ME, Andrews DF, Kruck TP, Wong S, Dalton AJ, Mehta P, Fedor B, Warren AC. Are hereditary hemochromatosis mutations involved in Alzheimer disease? *Am J Med Genet* 2000;**93**(1):58-66.

- 17 Sampietro M, Caputo L, Casatta A, Meregalli M, Pellagatti A, Tagliabue J, Annoni G, Vergani C. The hemochromatosis gene affects the age of onset of sporadic Alzheimer's disease. *Neurobiol Aging* 2001;**22**(4):563-8.
- 18 Candore G, Licastro F, Chiappelli M, Franceschi C, Lia D, Rita Balistreri C, Piazza G, Colonna-Romano G, Grimaldi LM, Caruso C. Association between the HFE mutations and unsuccessful ageing: a study in Alzheimer's disease patients from Northern Italy. *Mech Ageing Dev* 2003;**124**(4):525-8.
- 19 Combarros O, García-Román M, Fontalba A, Fernández-Luna JL, Llorca J, Infante J, Berciano J. Interaction of the H63D mutation in the hemochromatosis gene with the apolipoprotein E epsilon 4 allele modulates age of onset of Alzheimer's disease. *Dement Geriatr Cogn Disord* 2003;**15**(3):151-4.
- 20 Pulliam JF, Jennings CD, Kryscio RJ, Davis DG, Wilson D, Montine TJ, Schmitt FA, Markesbery WR. Association of HFE mutations with neurodegeneration and oxidative stress in Alzheimer's disease and correlation with APOE. *Am J Med Genet* 2003;**119B**(1):48-53.
- 21 Lehmann DJ, Williams J, McBroom J, Smith AD. Using meta-analysis to explain the diversity of results in genetic studies of late-onset Alzheimer's disease and to identify high-risk subgroups. *Neuroscience* 2001;**108**(4):541-54.
- 22 Petersen RC, Smith GE, Waring SC, Ivnik RJ, Tangalos EG, Kokmen E. Mild cognitive impairment: clinical characterization and outcome. *Arch Neurol* 1999;**56**(3):303-8.
- 23 Saunders AM, Strittmatter WJ, Schmechel D, St George-Hyslop PH, Pericak-Vance MA, Joo SH, Rosi BA, Gusella JF, Crapper-MacLachlan DR, Alberts MJ, Hulette C, Crain B, Goldgaber D, Roses AD. Association of apolipoprotein E allele epsilon 4 with late-onset familial and sporadic Alzheimer's disease. *Neurology* 1993;**43**(8):1467-72.
- 24 Farrer LA, Cupples LA, Haines JL, Hyman B, Kukull WA, Mayeux R, Myers RH, Pericak-Vance MA, Risch N, van Duijn CM. Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium. *JAMA* 1997;**278**(16):1349-56.
- 25 Miyata M, Smith JD. Apolipoprotein E allele-specific antioxidant activity and effects on cytotoxicity by oxidative insults and beta-amyloid peptides. *Nat Genet* 1996;**14**(1):55-61.
- 26 Roth M, Huppert FA, Tym E, Mountjoy CQ. *CAMDEX: The Cambridge examination for mental disorders of the elderly*. Cambridge: Cambridge University Press, 1988.
- 27 Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, Vogel FS, Hughes JP, van Belle G, Berg L. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurology* 1991;**41**(4):479-86.
- 28 McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. 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* 1984;**34**(7):939-44.
- 29 Namekata K, Oyama F, Imagawa M, Ihara Y. Human transferrin (TF): a single mutation at codon 570 determines Tf C1 or Tf C2 variant. *Hum Genet* 1997;**100**(3-4):457-8.
- 30 Merryweather-Clarke AT, Poinon JJ, Shearman JD, Robson KJH. Global prevalence of putative haemochromatosis mutations. *J Med Genet* 1997;**34**:275-8.
- 31 Merryweather-Clarke AT, Poinon JJ, Shearman JD, Robson KJH, Jouanolle AM, Mosser A, David V, Le Gall J-Y, Halsall DJ, Elsey TS, Kelly A, Cox TM, Clare M, Bomford A, Vandewalle JL, Rochette J, Borot N, Coppin H, Roth M-P, Ryan E, Crowe J, Totaro A, Gasparini P, Roetto A, Camaschella C, Darke C, Wallace DF, Saeb-Parsy K, Dooley JS, Worwood M, Walker AP. Polymorphism in intron 4 of HFE does not compromise haemochromatosis mutation results. *Nat Genet* 1999;**23**:271.
- 32 Wenham PR, Price WH, Blandell G. Apolipoprotein E genotyping by one-stage PCR. *Lancet* 1991;**337**(8750):1158-9.
- 33 Ihaka R, Gentleman R. R: a language for data analysis and graphics. *J Comput Graph Stat* 1996;**5**:299-314.
- 34 Buss S, Muller-Thomsen T, Hock C, Alberici A, Binetti G, Nitsch RM, Gal A, Finckh U. No association between DCP1 genotype and late-onset Alzheimer disease. *Am J Med Genet* 2002;**114**(4):440-5.
- 35 Lehmann D, Johnston C, Cortina Borja M, Smith A. Age influences the synergy between butyrylcholinesterase K variant and apolipoprotein E epsilon 4 in late-onset Alzheimer's disease. In: Doctor B, Quinn D, Rotundo R, eds. *Structure and function of cholinesterases and related proteins*. New York: Plenum, 1998:159-66.
- 36 Dautry-Varsat A, Ciechanover A, Lodish HF. pH and the recycling of transferrin during receptor-mediated endocytosis. *Proc Natl Acad Sci U S A* 1983;**80**(8):2258-62.
- 37 Burt MJ, George PM, Upton JD, Collett JA, Frampton CM, Chapman TM, Walmsley TA, Chapman BA. The significance of haemochromatosis gene mutations in the general population: implications for screening. *Gut* 1998;**43**(6):830-6.
- 38 Merryweather-Clarke AT, Worwood M, Parkinson L, Mattock C, Poinon JJ, Shearman JD, Robson KJH. The effect of HFE mutations on serum ferritin and transferrin saturation in the Jersey population. *Br J Haematol* 1998;**101**:369-73.
- 39 Phatak PD, Ryan DH, Cappuccio J, Oakes D, Braggins C, Provenzano K, Eberly S, Sham RL. Prevalence and penetrance of HFE mutations in 4865 unselected primary care patients. *Blood Cells Mol Dis* 2002;**29**(1):41-7.
- 40 Njajou OT, Houwing-Duistermaat JJ, Osborne RH, Vaessen N, Verger J, Heeringa J, Pols HA, Hofman A, van Duijn CM. A population-based study of the effect of the HFE C282Y and H63D mutations on iron metabolism. *Eur J Hum Genet* 2003;**11**(3):225-31.
- 41 Beckman L, Beckman G. Transferrin C2 as an enhancer of cyto- and genotoxic damage. *Prog Clin Biol Res* 1986;**209B**:221-4.

- 42 **Wong CT**, Saha N. Effects of transferrin genetic phenotypes on total iron-binding capacity. *Acta Haematol* 1986;**75**(4):215-8.
- 43 **Morris CM**, Kerwin JM, Edwardson JA. Non-haem iron histochemistry of the normal and Alzheimer's disease hippocampus. *Neurodegeneration* 1994;**3**(4):267-75.
- 44 **Connor JR**, Menzies SL. Cellular management of iron in the brain. *J Neurol Sci* 1995;**134**(suppl):33-44.
- 45 **Connor JR**, Snyder BS, Arosio P, Loeffler DA, LeWitt P. A quantitative analysis of isoferitins in select regions of aged, parkinsonian, and Alzheimer's diseased brains. *J Neurochem* 1995;**65**(2):717-24.
- 46 **Kawamata T**, Tooyama I, Yamada T, Walker DG, McGeer PL. Lactotransferrin immunocytochemistry in Alzheimer and normal human brain. *Am J Pathol* 1993;**142**(5):1574-85.
- 47 **Schipper HM**, Cisse S, Stopa EG. Expression of heme oxygenase-1 in the senescent and Alzheimer-diseased brain. *Ann Neurol* 1995;**37**(6):758-68.
- 48 **Smith MA**, Wehr K, Harris PL, Siedlak SL, Connor JR, Perry G. Abnormal localization of iron regulatory protein in Alzheimer's disease. *Brain Res* 1998;**788**(1-2):232-6.
- 49 **Fleming J**, Joshi JG. Ferritin: isolation of aluminum-ferritin complex from brain. *Proc Natl Acad Sci U S A* 1987;**84**(22):7866-70.
- 50 **Piñero DJ**, Hu J, Connor JR. Alterations in the interaction between iron regulatory proteins and their iron responsive element in normal and Alzheimer's diseased brains. *Cell Mol Biol (Noisy-le-grand)* 2000;**46**(4):761-76.
- 51 **Rogers JT**, Randall JD, Cahill CM, Eder PS, Huang X, Gunshin H, Leiter L, McPhee J, Sarang SS, Utsuki T, Greig NH, Lahiri DK, Tanzi RE, Bush AI, Giordano T, Gullans SR. An iron-responsive element type II in the 5'-untranslated region of the Alzheimer's amyloid precursor protein transcript. *J Biol Chem* 2002;**277**(47):45518-28.
- 52 **Palmer AM**, Burns MA. Selective increase in lipid peroxidation in the inferior temporal cortex in Alzheimer's disease. *Brain Res* 1994;**645**(1-2):338-42.
- 53 **Lovell MA**, Ehmman WD, Butler SM, Markesbery WR. Elevated thiobarbituric acid-reactive substances and antioxidant enzyme activity in the brain in Alzheimer's disease. *Neurology* 1995;**45**(8):1594-1601.
- 54 **Sayre LM**, Zelasko DA, Harris PL, Perry G, Salomon RG, Smith MA. 4-Hydroxynonenal-derived advanced lipid peroxidation end products are increased in Alzheimer's disease. *J Neurochem* 1997;**68**(5):2092-7.
- 55 **Smith CD**, Carney JM, Starke-Reed PE, Oliver CN, Stadtman ER, Floyd RA, Markesbery WR. Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer disease. *Proc Natl Acad Sci U S A* 1991;**88**(23):10540-3.
- 56 **Hensley K**, Hall N, Subramaniam R, Cole P, Harris M, Aksenov M, Aksenova M, Gabbita SP, Wu JF, Carney JM, Lovell M, Markesbery WR, Butterfield A. Brain regional correspondence between Alzheimer's disease histopathology and biomarkers of protein oxidation. *J Neurochem* 1995;**65**(5):2146-56.
- 57 **Good PF**, Werner P, Hsu A, Olanow CW, Perl DP. Evidence of neuronal oxidative damage in Alzheimer's disease. *Am J Pathol* 1996;**149**(1):21-8.
- 58 **Lyras L**, Cairns NJ, Jenner A, Jenner P, Halliwell B. An assessment of oxidative damage to proteins, lipids, and DNA in brain from patients with Alzheimer's disease. *J Neurochem* 1997;**68**(5):2061-9.
- 59 **Nunomura A**, Perry G, Hirai K, Aliev G, Takeda A, Chiba S, Smith MA. Neuronal RNA oxidation in Alzheimer's disease and Down's syndrome. *Ann N Y Acad Sci* 1999;**893**:362-4.
- 60 **Shan X**, Tashiro H, Lin CL. The identification and characterization of oxidized RNAs in Alzheimer's disease. *J Neurosci* 2003;**23**(12):4913-21.
- 61 **Gabbita SP**, Lovell MA, Markesbery WR. Increased nuclear DNA oxidation in the brain in Alzheimer's disease. *J Neurochem* 1998;**71**(5):2034-40.
- 62 **Mecocci P**, MacGarvey U, Beal MF. Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. *Ann Neurol* 1994;**36**(5):747-51.
- 63 **Hayn M**, Kremser K, Singewald N, Cairns N, Nemethova M, Lubec B, Lubec G. Evidence against the involvement of reactive oxygen species in the pathogenesis of neuronal death in Down's syndrome and Alzheimer's disease. *Life Sci* 1996;**59**(7):537-44.
- 64 **Montine KS**, Kim PJ, Olson SJ, Markesbery WR, Montine TJ. 4-Hydroxy-2-nonenal pyrolytic adducts in human neurodegenerative disease. *J Neuropathol Exp Neurol* 1997;**56**(8):866-71.
- 65 **Cash AD**, Perry G, Ogawa O, Raina AK, Zhu X, Smith MA. Is Alzheimer's disease a mitochondrial disorder? *Neuroscientist* 2002;**8**(5):489-96.
- 66 **Hensley K**, Carney JM, Mattson MP, Aksenova M, Harris M, Wu JF, Floyd RA, Butterfield DA. A model for beta-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer disease. *Proc Natl Acad Sci U S A* 1994;**91**(8):3270-4.
- 67 **Kim HC**, Yamada K, Nitta A, Olariu A, Tran MH, Mizuno M, Nakajima A, Nagai T, Kamei H, Jhoo WK, Im DH, Shin EJ, Hjelte OP, Ottersen OP, Park SC, Kato K, Mirault ME, Nabeshima T. Immunocytochemical evidence that amyloid beta (1-42) impairs endogenous antioxidant systems in vivo. *Neuroscience* 2003;**119**(2):399-419.
- 68 **Braak H**, Del Tredici K, Schultz C, Braak E. Vulnerability of select neuronal types to Alzheimer's disease. *Ann N Y Acad Sci* 2000;**924**:53-61.
- 69 **LeVine SM**, Macklin WB. Iron-enriched oligodendrocytes: a reexamination of their spatial distribution. *J Neurosci Res* 1990;**26**(4):508-12.
- 70 **Connor JR**, Menzies SL. Relationship of iron to oligodendrocytes and myelination. *Glia* 1996;**17**(2):83-93.
- 71 **Nunomura A**, Perry G, Aliev G, Hirai K, Takeda A, Balraj EK, Jones PK, Ghanbari H, Wataya T, Shimohama S, Chiba S, Atwood CS, Petersen RB, Smith MA. Oxidative damage is the earliest event in Alzheimer disease. *J Neuropathol Exp Neurol* 2001;**60**(8):759-67.
- 72 **Ramassamy C**, Averill D, Belfert U, Theroux L, Lussier-Cacan S, Cohn JS, Christen Y, Schoofs A, Davignon J, Poirier J. Oxidative insults are associated with apolipoprotein E genotype in Alzheimer's disease brain. *Neurobiol Dis* 2000;**7**(1):23-37.
- 73 **Shea TB**, Rogers E, Ashline D, Ortiz D, Sheu MS. Apolipoprotein E deficiency promotes increased oxidative stress and compensatory increases in antioxidants in brain tissue. *Free Radic Biol Med* 2002;**33**(8):1115-20.
- 74 **Montine KS**, Olson SJ, Amarnath V, Whetsell WO Jr, Graham DG, Montine TJ. Immunohistochemical detection of 4-hydroxy-2-nonenal adducts in Alzheimer's disease is associated with inheritance of APOE4. *Am J Pathol* 1997;**150**(2):437-43.
- 75 **Ramassamy C**, Averill D, Belfert U, Bastianetto S, Theroux L, Lussier-Cacan S, Cohn JS, Christen Y, Davignon J, Quirion R, Poirier J. Oxidative damage and protection by antioxidants in the frontal cortex of Alzheimer's disease is related to the apolipoprotein E genotype. *Free Radic Biol Med* 1999;**27**(5-6):544-53.
- 76 **Tamaoka A**, Miyatake F, Matsuno S, Ishii K, Nagase S, Sahara N, Ono S, Mori H, Wakabayashi K, Tsuji S, Takahashi H, Shoji S. Apolipoprotein E allele-dependent antioxidant activity in brains with Alzheimer's disease. *Neurology* 2000;**54**(12):2319-21.
- 77 **Lehmann DJ**, Wiebusch H, Marshall SE, Johnston C, Warden DR, Morgan K, Schappert K, Poirier J, Xuereb J, Kalsheker N, Welsh KI, Smith AD. HLA class I, II and III genes in confirmed late-onset Alzheimer's disease. *Neurobiol Aging* 2001;**22**(1):71-7.
- 78 **Feder JN**, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, Dormishian F, Domingo Jr R, Ellis MC, Fullan A, Hinton LM, Jones NL, Kimmel BE, Kronmal GS, Lauer P, Lee VK, Loeb DB, Mapa FA, McClelland E, Meyer NC, Mintier GA, Moeller N, Moore T, Morikang E, Prass CE, Quintana L, Starnes SM, Schatzman RC, Brunke KJ, Drayna DT, Risch NJ, Bacon BR, Wolff RK. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nat Genet* 1996;**13**:399-408.
- 79 **Ruddy DA**, Kronmal GS, Lee VK, Mintier GA, Quintana L, Domingo Jr R, Meyer NC, Irrinki A, McClelland EE, Fullan A, Mapa FA, Moore T, Thomas W, Loeb DB, Harmon C, Tsuchihashi Z, Wolff RK, Schatzman RC, Feder JN. A 1.1-Mb transcript map of the hereditary hemochromatosis locus. *Genome Res* 1997;**7**:441-56.
- 80 **Merryweather-Clarke AT**, Pointon JJ, Jouanolle AM, Rochette J, Robson KJH. Geography of HFE C282Y and H63D mutations. *Genet Test* 2000;**4**:183-98.
- 81 **Growdon JH**, Locascio JJ, Corkin S, Gomez-Isla T, Hyman BT. Apolipoprotein E genotype does not influence rates of cognitive decline in Alzheimer's disease. *Neurology* 1996;**47**(2):444-8.
- 82 **Slooter AJ**, Houwing-Duistermaat JJ, van Harskamp F, Cruys M, Van Broeckhoven C, Breteler MM, Hofman A, Stijnen T, van Duijn CM. Apolipoprotein E genotype and progression of Alzheimer's disease: the Rotterdam Study. *J Neurol* 1999;**246**(4):304-8.