Running head: OxStress in AD

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Markers of oxidative damage to lipids, nucleic acids and proteins and antioxidant enzymes activities in Alzheimer's disease brain: a meta-analysis in human pathological specimens

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Abstract
Oxidative stress and decreased cellular responsiveness to oxidative stress are thought to influence brain aging and Alzheimer's disease, but the specific patterns of oxidative damage and the underlying mechanism leading to this damage are not definitively known. The objective of this study was to define the pattern of changes in oxidative-stress related markers by brain region in human Alzheimer's disease and mild cognitive impairment brain tissue. Observational case-control studies were identified from systematic queries of PubMed, ISI Web of Science and Scopus databases and studies were evaluated with appropriate quality measures. The data was used to construct a region-by-region meta-analysis of malondialdehyde, 4-hydroxynonenal, protein carbonylation, 8-hydroxyguanine levels and superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase activities. We also evaluated ascorbic acid, tocopherol, uric acid and glutathione levels. The analysis was complicated in several cases by publication bias and/or outlier data. We found that malondialdehyde levels were slightly increased in the temporal and occipital lobes and hippocampus, but this analysis was significantly impacted by publication bias. 4-hydroxynonenal levels were unchanged in every brain region. There was no change in 8-hydroxyguanine level in any brain region and protein carbonylation levels were unchanged except for a slight increase in the occipital lobe. Superoxide dismutase, glutathione peroxidase and reductase and catalase activities were not decreased in any brain region. There was limited data reporting non-enzymatic antioxidant levels in Alzheimer's disease brain, although glutathione and tocopherol levels appear to be unchanged. Minimal quantitative data is available from brain tissue from patients with mild cognitive impairment. While there is modest evidence supporting minor regional changes in markers of oxidative damage, this analysis fails to identify a consistent pattern of pro-oxidative changes and accumulation of oxidative damage in bulk tissue analysis in the setting of Alzheimer's disease, as has been widely reported.

Keywords:
Mild cognitive impairment, malondialdehyde, hydroxynonenal, carbonylation, hydroxyguanine, superoxide dismutase, glutathione, catalase, tocopherol, ascorbic acid.

Introduction

One major proposed mechanism to explain changes associated with cellular and organismal aging is that oxidative damage to lipid, protein and nucleic acids accumulates as cells become less capable of coping with oxidative stress.[1] This potentially leads to membrane instability, accumulation of damaged proteins and acquired mutation, all of which may contribute to cellular dysfunctions associated with aging and aging related diseases. The role of oxidative stress as it contributes to neuronal dysfunction in the context of aging and Alzheimer's disease (AD) is of particular interest and has generated an enormous observational and experimental literature. Despite this, large scale clinical trials of antioxidative therapy have not yet produced a compelling therapy for AD.[2,3] The large volume of literature and heterogeneity of results makes a comprehensive understanding of the changes occurring in human brain in Alzheimer's disease elusive. The purpose of this analysis to comprehensively assemble high-quality observational studies which have quantitatively measured markers of oxidative stress or activities of anti-oxidative molecules in the brains of subjects with Alzheimer's disease.

Methods

Literature search

A systematic review of the literature was performed by querying PubMed, Scopus and ISI Web of Science databases for combinations of relevant keywords: "human" AND "Alzheimer's" or "Mild cognitive impairment" AND "[name of marker of oxidative stress or enzyme]". Observational case-control studies reporting quantitative measurements of malondialdehyde, hydroxynonenal, protein carbonylation, 8-hydroxyguanosine, superoxide dismutase activity, glutathione peroxidase activity, glutathione reductase activity or catalase activity, tocopherol, ascorbic acid, uric acid and glutathione levels in human brain tissue were accepted for quality assessment and inclusion. To maximize the completeness of data collection, this systematic review was supplemented by less-structured techniques including reviewing the references from the identified studies and from appropriate review articles and by searching GoogleScholar. Qualitative studies and studies of fractionated tissues looking at markers in subcellular organelles were excluded; only bulk tissue analysis was included in this analysis. The search was completed by February 2017.

Quality measures

Studies which were identified as relevant to the current analysis were evaluated for quality. The diagnosis of Alzheimer's disease was required to be made from pathological examination of brain tissue by CERAD criteria or Braak and Braak staging; a clinical diagnosis alone was not acceptable. A clinical diagnosis was acceptable for studies of CSF or of mild cognitive impairment. Control tissue was required to be age-matched to the Alzheimer's disease subjects, or a minimum of 60 years of age. Adequate description of the analytical methodology was required and complete regional reporting of the data was required.

Statistical analysis

Summary statistics were extracted from each article and converted where necessary to mean and standard deviation. Meta-analysis was performed separately for each marker/enzyme in each brain region. For those data points represented by 0, 1 or 2 studies only, the data was presented in table 1, but no formal meta-analysis was performed (in the event of 2 studies, the data was presented as a weighted mean). Data was analyzed with MIX 1.7 software and presented as effect size (using Hedge's G) in the form of forest plots. The meta-analysis was weighted by the inverse of variance in the random effect model. Heterogeneity was assessed with the Q and I^2 statistics. The presence of publication bias was assessed with a funnel plot analyzed by Egger's test and when there was evidence of publication bias, it was corrected by the trim and fill method. When publication bias was detected, the corrected effect size and confidence interval were reported in Table 1. Outliers were not excluded from the forest plots or table 1, but they were discussed and the result of excluding outliers was presented in the text and/or in the supplement.

Results

Oxidative damage to lipids, proteins and nucleic acids

Lipid (per)oxidation in the brain was most-frequently evaluated by measuring the markers malondialdehyde, hydroxynonenal and acrolein. Malondialdehyde may be measured directly or by the thiobarbituric acid reactive substances assay (TBARS) -- while malondialdehyde is not the only source of TBARS in the brain, it is the major source

Table 1: Oxidative changes in Alzheimer's disease and mild cognitive impairment

Tissue of origin	Comparison group	Oxidative markers	% Change (vs controls)	Effect size Hedges g (95% CI)	Total N (AD/control)	Number studies
Frontal lobe	Alzheimer's disease	Malondialdehyde	**↑ 13.0%†**	0.37 (0.08 – 0.65) †	180 / 151	13 studies
		HNE	No change	0.41 (-0.15 – 2.00)	41 / 37	3 studies
		Protein carbonylation	No change	0.37 (-0.15 -0.90)	36 / 25	3 studies
		8-hydroxyguanine	No change	0.64 (-0.50 – 1.78)	55 / 52	6 studies
		Superoxide dismutase	No change	0.17 (-0.27 – 0.61)	98 / 87	9 studies
		Glutathione	No change	-0.09 (-0.58 – 0.39)	53 / 47	6 studies
		Glutathione peroxidase	No change	0.23 (-0.07 – 0.52)	106 / 87	9 studies
		Glutathione reductase	No change	0.42 (-0.13 – 0.96)	30 / 24	3 studies
		Catalase	No change	0.09 (-0.36 – 0.65)	94 / 61	7 studies
	MCI	No data				
Parietal lobe	Established AD	Malondialdehyde	No change	0.04 (-0.29 – 0.37) †	103 / 91	9 studies
		HNE	No change	0.33 (-0.24 – 0.89)	27 / 22	2 studies
		Protein carbonylation	No change	1.09 (-0.35 – 2.53)	34 / 28	3 studies
		8-hydroxyguanine	No change	0.50 (-0.11 – 1.11)	62 / 62	6 studies
		Superoxide dismutase	**↑ 12.0%**	0.39 (0.00 – 0.77)	61 / 50	7 studies
		Glutathione peroxidase	No change	0.15 (-0.36 – 0.65)	33 / 29	4 studies
		Glutathione reductase	No change	0.46 (-0.05 - -0.98)	32 / 28	3 studies
		Catalase	No change	-0.02 (-1.10 - 1.06)	37 / 29	4 studies
	MCI	Protein carbonylation	↑ 55%	1.09 (0.03 – 2.14)	6 / 12	1 study
Occipital lobe	Established AD	Malondialdehyde	**↑ 21.2%**	0.59 (0.05 – 1.13)	50 / 44	6 studies
		Protein carbonylation	**↑ 12.0%**	0.55 (0.03 – 1.08)	36 / 26	3 studies
		8-hydroxyguanine	No change	0.04 (-1.55 - 1.78)	28 / 27	3 studies
		Superoxide dismutase	No change	0.35 (-0.52 – 1.23)	45 / 35	5 studies
		Glutathione	No change	-0.26 (-1.55 – 1.03)	22 / 19	3 studies
		Glutathione peroxidase	No change	-0.62 (-1.46 – 0.23)	13 / 10	1 study
		Glutathione reductase	No change	0.31 (-0.52 – 1.14)	13 / 10	1 study
		Catalase	No change	-0.09 (-0.65 – 0.46)	39 / 29	4 studies
	MCI	No data				
Temporal lobe	Established AD	Malondialdehyde	**↑ 11.4%†**	0.21 (0.00 – 0.41) †	177 / 156	14 studies
		HNE	No change	0.41 (-0.73 – 1.54)	41 / 42	4 studies
		Protein carbonylation	No change	0.27 (-0.25 – 0.79)	30 / 28	3 studies
		8-hydroxyguanine	No change	0.08 (-0.67 – 0.84)	64 / 65	7 studies
		Superoxide dismutase	No change	0.44 (-0.14 – 1.02)	92 / 62	8 studies
		Glutathione peroxidase	No change	0.07 (-0.31 – 0.46)	59 / 51	6 studies
		Glutathione reductase	No change	0.11 (-0.42 – 0.63)	30 / 28	3 studies
		Catalase	No change	0.18 (-0.72 – 1.09)	57 / 40	5 studies
	MCI	Malondialdehyde	↑ >100%	2.71 (1.39 – 4.04)	9 / 10	1 study
		HNE	↑ >100%	2.29 (0.84 – 3.73)	7 / 7	1 study
Hippocampus	Established AD	Malondialdehyde	**↑ 18.5%**	0.59 (0.13 – 1.04)	55 / 33	4 studies
		HNE	No change	0.91 (-0.15 – 1.96)	32 / 32	3 studies ‡
		Protein carbonylation	No change	0.70 (-0.12 – 1.52)	29 / 28	3 studies
		8-hydroxyguanine	No change	0.41 (-0.61 – 1.44)	7 / 8	1 study
		Superoxide dismutase	No change	0.31 (-0.43 – 1.05)	54 / 43	5 studies
		Glutathione	No change	0.47 (-0.76 – 1.70)	40 / 29	4 studies
		Glutathione peroxidase	No change	0.10 (-0.46 – 0.67)	28 / 24	3 studies
		Glutathione reductase	No change	0.51 (-0.18 – 1.20)	19 / 23	2 studies
		Catalase	No change	0.82 (-0.26 – 1.91)	40 / 27	4 studies
	MCI	HNE	↑ 46%	1.43 (0.21 – 2.65)	7 / 6	1 study
Amygdala	Established AD	Malondialdehyde	No change	0.24 (-0.31 – 0.78)	32 / 23	3 studies
		Superoxide dismutase	Inconsistent	2.86 (-0.28 – 6.00)	32 / 22	3 studies
		Glutathione peroxidase	No change	0.04 (-0.78 – 0.87)	13 / 10	1 studies
		Catalase	No change	-0.38 (-1.01 – 0.25)	32 / 22	3 studies
	MCI	No data				
Cerebellum	Established AD	Malondialdehyde	No change	0.20 (-0.20 – 0.61)	53 / 44	6 studies
		HNE	No change	0.44 (-0.02 – 0.92)	38 / 35	4 studies
		Protein carbonylation	No change	0.29 (-0.80 – 1.37)	33 / 30	3 studies
		8-hydroxyguanine	No change	-0.40 (-0.80 – 1.60)	50 / 53	5 studies
		Superoxide dismutase	No change	-0.28 (-1.26 – 0.70)	53 / 47	5 studies
		Glutathione peroxidase	No change	-0.06 (-0.49 – 0.36)	43 / 42	4 studies
		Glutathione reductase	No change	0.41 (-0.20 – 1.02)	24 / 20	2 studies
		Catalase	No change	0.09 (-0.36 – 0.54)	42 /37	4 studies
	MCI	Malondialdehyde	No change	-0.37 (-1.28 – 0.54)	9 / 10	1 study
		HNE	No change	1.14 (-0.02 – 2.30)	7 / 7	1 study

Effect size based on comparison to aged neurological control brain tissue. Results in bold = p<0.05, *= p<0.01, AD = Alzheimer's disease, MCI = mild cognitive impairment, HNE = 4-hydroxy-2-*trans*-nonenal, n.s. = not significant. † = corrected for publication bias, ‡ = all studies originated from a single research group.

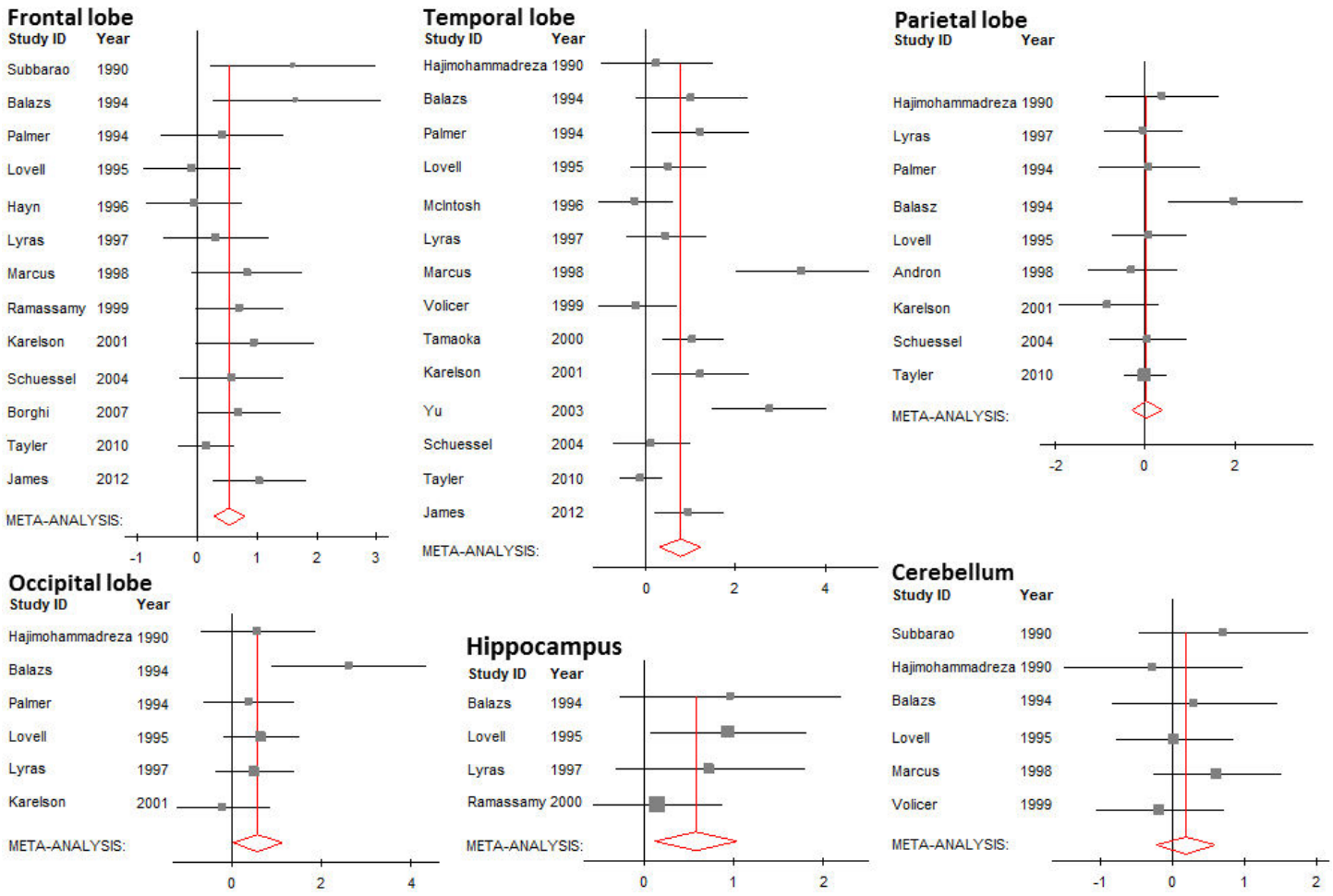

Figure 1: Malondialdehyde levels in Alzheimer's disease brain

In the raw analysis, malondialdehyde levels appear to be significantly increased in the frontal, temporal and occipital lobes and in the hippocampus. There is significant evidence of publication bias in the analyses for the frontal, temporal and parietal lobes – correction for this bias reduces the observed increase in the frontal and temporal lobes, although both remain significantly increased. The analysis of the occipital lobe is impacted by an outlier study; were the analysis repeated without Balazs 1994, it would no longer suggest a significant increase. There is no statistical heterogeneity in the analyses, except in the temporal lobe which is highly heterogeneous ($p<0.0001$). The increase in malondialdehyde level in the hippocampus is not impacted by publication bias, heterogeneity or outlier effects.

and we have considered the TBARS assay and other more selective assays for malondialdehyde to be essentially equivalent. Malondiadehyde appears to be increased the frontal lobe, temporal and hippocampus and is slightly increased in the occipital lobe in AD subjects compared to age matched controls (Table 1, Figure 1). However, this analysis is complicated by strong evidence for publication bias, outlier data and heterogeneity. For the frontal lobe, trim and fill analysis predicted the presence of four unpublished studies in the negative portion of the distribution; correcting for this reduces the effect size from 0.54 (95%CI 0.28 – 0.80), a 19.5% increase to an effect size 0.36 (95%CI 0.14 to 0.56), a 13.0% increase compared to controls. For the temporal lobe, trim and fill analysis predicted the presence of 5 studies in the negative portion of the distribution of studies and would reduce the estimated effect size from HG=0.78 (95%CI 0.32 – 1.24), a 42% increase ($p<0.0001$) to HG=0.21 (95%CI 0.00 – 0.41), an 11.4% increase over controls ($p=0.05$). The parietal lobe analysis also had some evidence of publication bias, with two imputed studies, however this did not substantially alter the conclusion; there was no evidence of any change in malondialdehyde level in the parietal lobe with or without correction for publication bias. There was a trend toward an increase in malondialdehyde level in the occipital lobe in Alzheimer's disease (HG= 0.50, 95% CI 0.03 – 0.97, $p=0.04$); however, there was one outlier study and analysis without this study would reduce this to non-significant levels.[4] There was no change in malondialdehyde level in the amygdala or cerebellum. The analysis of the temporal lobe also had a very high degree of heterogeneity ($Q=46.9$, $I^2=72.2\%$, $p<0.0001$) – there was no statistical heterogeneity in the analysis for any other brain region.

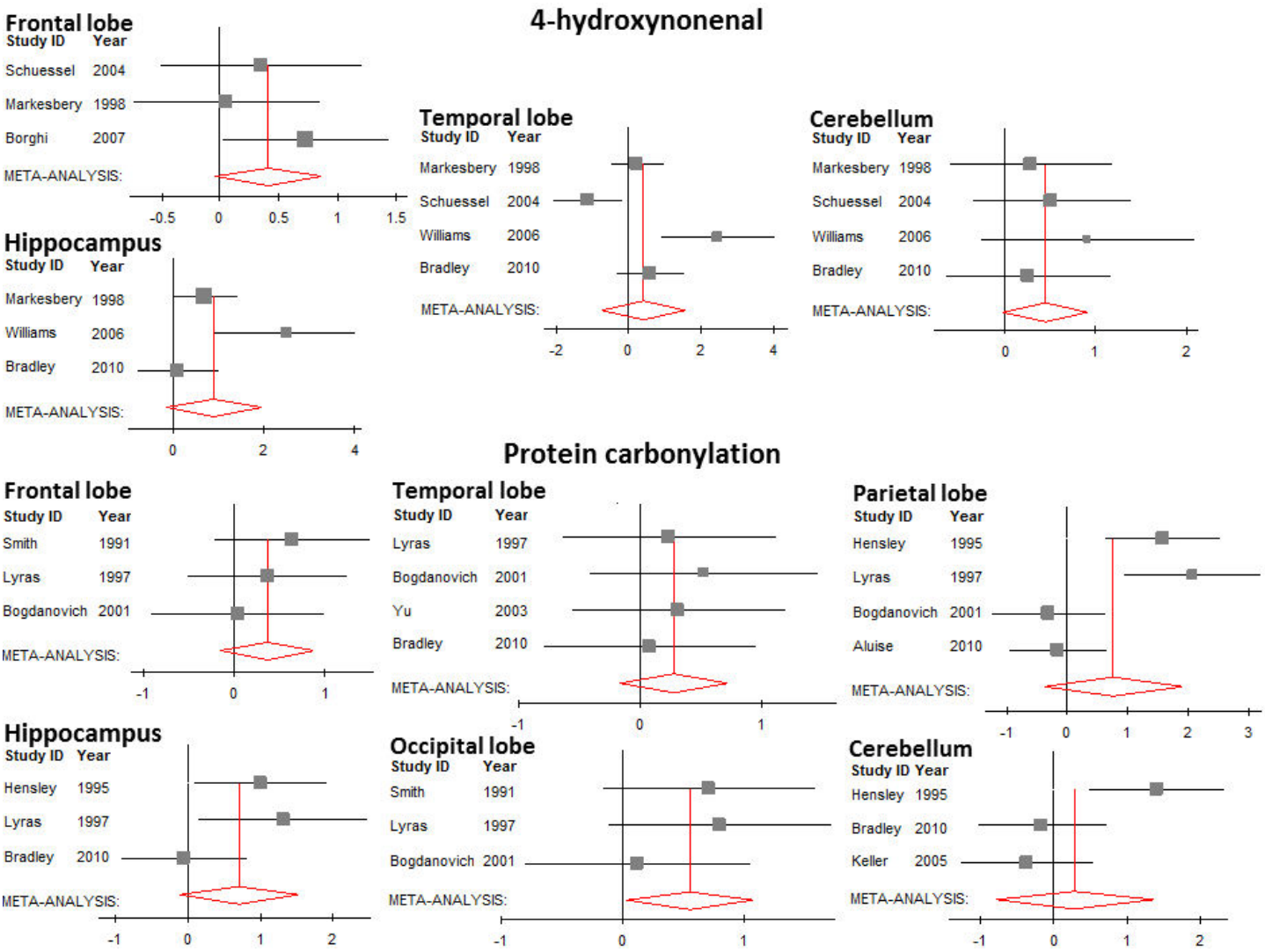

Figure 2: Hydroxynonenal and protein carbonylation levels in Alzheimer's disease brain

There is no significant increase in 4-hydroxynonenal in any studied brain region, although there is significant heterogeneity in the analysis. Protein carbonylation levels are similarly unchanged in most studied brain regions with the exception of the occipital lobe which has a 12% increase in protein carbonylation, p=0.04.

Two other relevant markers of oxidative lipid damage are 4-hydroxy-2-nonenal and acrolein. In one study, hydroxynonenal levels were reported to be unchanged in multiple brain regions, but the data were not shown; we approached the authors of this study to obtain the original data which was included in this analysis.[5] In meta-analysis, hydroxynonenal was not increased in any brain region (Figure 2). For acrolein, only three quantitative studies were available all of which originated from a single research group. Data from this group frequently behaved as outliers, so this data needs to be independently confirmed. For this reason we did not feel we could accurately estimate an effect size and acrolein was omitted from Table 1; however, the analysis is included in the supplement. Various isoprostane and neuroprostane measurements have been reported, but none have been reported in at least three separate studies to justify meta-analysis.

Comparatively little data is available describing the levels of lipid damage in subjects who have died with MCI. The available data shows increased temporal lobe accumulation of TBARS, acrolein and HNE, but the reproducibility of this data has not been demonstrated. This small amount of data is difficult to effectively evaluate in meta-analysis.
The presence of oxidative damage to proteins may be broadly assessed in tissue by measurement of carbonyl residues. Protein carbonylation was significantly increased in the occiput and in the hippocampus in Alzheimer's disease, while there were no significant changes noted in other brain regions. This was a small analysis with 2 to 4 studies per brain region, so evaluation of publication bias was limited.

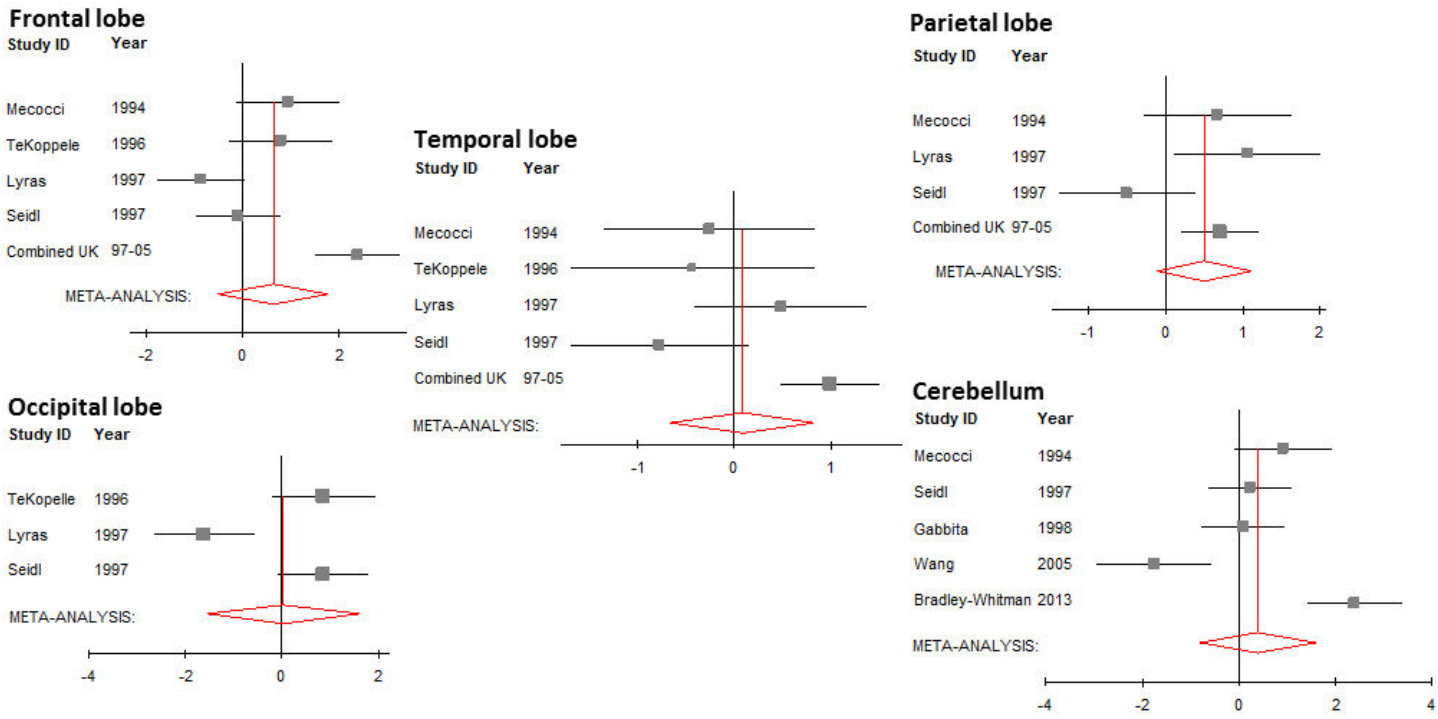

Figure 3: Nuclear 8-hydroxyguanine levels in Alzheimer's disease brain
8-hydroxyguanine levels are unchanged in AD. Three studies (Gabbita 1998, Wang 2005 and Bradley-Whitman 2013) were contributed by the University of Kentucky (UK); these were aggregated prior to inclusion in this analysis except in the cerebellum where the results of the three studies were highly discordant.

Numerous DNA-damage markers have been evaluated in AD tissue; for this review nuclear 8-hydroxyguanine was chosen as a representative marker as it is probably the most-studied marker. There was no significant change in 8-hydroxyguanine level in any brain region (Figure 3). Three studies in this analysis originated from one laboratory and these three suggested a general trend toward increased 8-hydroxyguanine levels in multiple brain regions; however, this result was not confirmed by any of the five other research groups. Detailed analysis for this marker is included in the supplementary material. Because one group contributing multiple studies can distort the weighting paradigm in random effects analysis, the data in Figure 3 was presented with the three studies aggregated into a single a result (except in the case of the cerebellum where the results of these three studies were discordant). A conventional meta-analysis with each study included separately is included in the supplement; this analysis did not significantly alter the conclusion.

There is again very little data on protein carbonylation levels or nuclear DNA damage in brain in mild cognitive impairment.

Antioxidant enzyme activity
Metabolic control of oxidation and reduction reactions within cells is maintained partly due to the action of key enzymes including superoxide dismutase, catalase and glutathione peroxidase and reductase. Superoxide dismutase activity was not changed in any AD brain region except for a modest increase in the parietal lobe (effect size 0.39, 95% CI 0.00-0.77, p=0.05; Figure 4). One study described superoxide dismutase activity in mild cognitive impairment and found no changes except for a modest decrease in activity in the hippocampus.[6] The glutathione system in AD appears to be broadly unaffected – there is no significant change in either glutathione peroxidase or glutathione reductase in any of the brain regions studied (Figure 5). There is no significant change in catalase activity in AD in any brain region (Figure 6). There was no evidence of publication bias in any of these analyses (although it is difficult to reliably detect publication bias in analyses this small) and the level of heterogeneity was low; this is described in detail in the supplement.

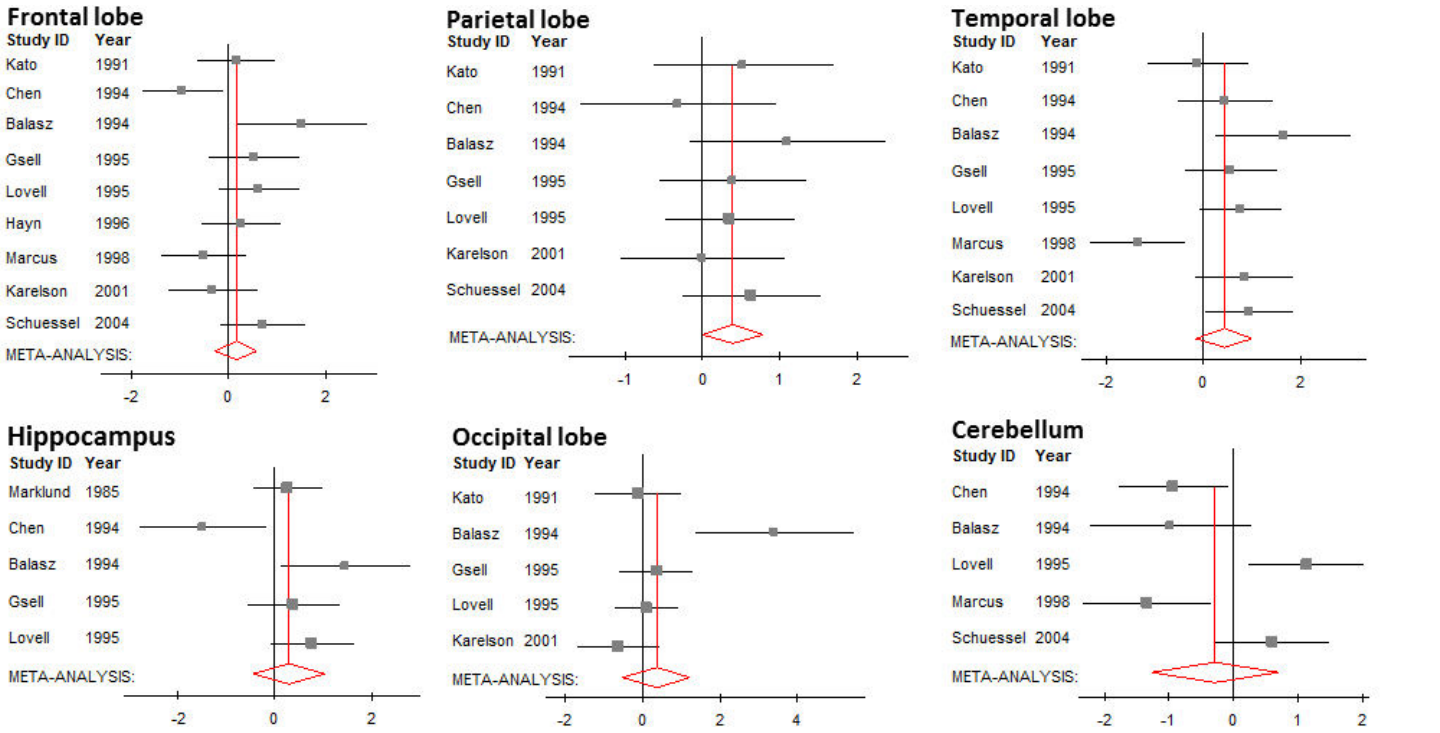

Figure 4: Superoxide dismutase activity in Alzheimer's disease brain

Superoxide dismutase activity is significantly increased only in the parietal lobe. One study has been excluded because the assay used measured total protein abundance rather than enzymatic activity.[89] Additionally, Richardson 1993 (abstract only) and Ramassamy 1999 (data not reported, but described as unchanged) were excluded

Non-enzymatic antioxidants

There is very little data available on the levels of non-enzymatic antioxidants in the brain. The best-characterized molecule is glutathione which has been described in seven different studies. There does not appear to be an alteration in glutathione levels in the frontal or occipital lobes or in the hippocampus. Tocopherol levels have been reported in Alzheimer's disease brain in three studies, but there is not enough data to analyze individual brain regions separately. We analyzed all of the reported data together in Figure 7, and there was no evidence for an alteration in tocopherol levels. To supplement this analysis, we collected studies of CSF levels of alpha-tocopherol which also appeared to be unchanged in the setting of Alzheimer's disease. Finally, there was not one single study which reported ascorbic acid levels in the brain in Alzheimer's disease. There was compelling evidence for depletion of ascorbic acid in CSF (five studies with no intra-study heterogeneity, effect size -0.69, 95%CI -1.02 to -0.35; Figure 7). There was insufficient data to analyze uric acid levels in the brain, but the limited available data did not suggest a significant change.[7]

Discussion

The field of oxidative stress as it relates to AD is large, with primary data coming from many different systems and supplemented by a large and rapidly growing narrative review literature. While this volume of data indicates intense interest in this topic, its utility is diminished by obfuscating or masking the complete picture of the oxidative changes in the AD brain. The purpose of this analysis was to quantitatively address this problem specifically for oxidative-stress related changes in the human AD brain. The pattern of oxidative changes identified in this analysis suggests that the antioxidant enzyme system in the brain is largely intact in AD and the global accumulation of oxidative damage is less substantial than has generally been reported. A deficiency in the classical antioxidative enzymes is not present in any brain region. Additionally, the availability of data describing the levels of non-enzymatic antioxidant molecules like ascorbic acid, tocopherols and uric acid is extremely limited (and in the case of ascorbic acid, completely absent). The availability of data for oxidative damage in mild cognitive impairment is also limited, both in terms of quality and quantity. These were surprising limitations given the volume of research in this field. Overall, our findings suggest that redox balance is well-regulated in the brain.

Comparing this analysis to our previous report on circulating markers of oxidative stress in AD demonstrates the extent of oxidation in peripheral blood is much greater than the changes in the brain for most markers.[8] This pattern is

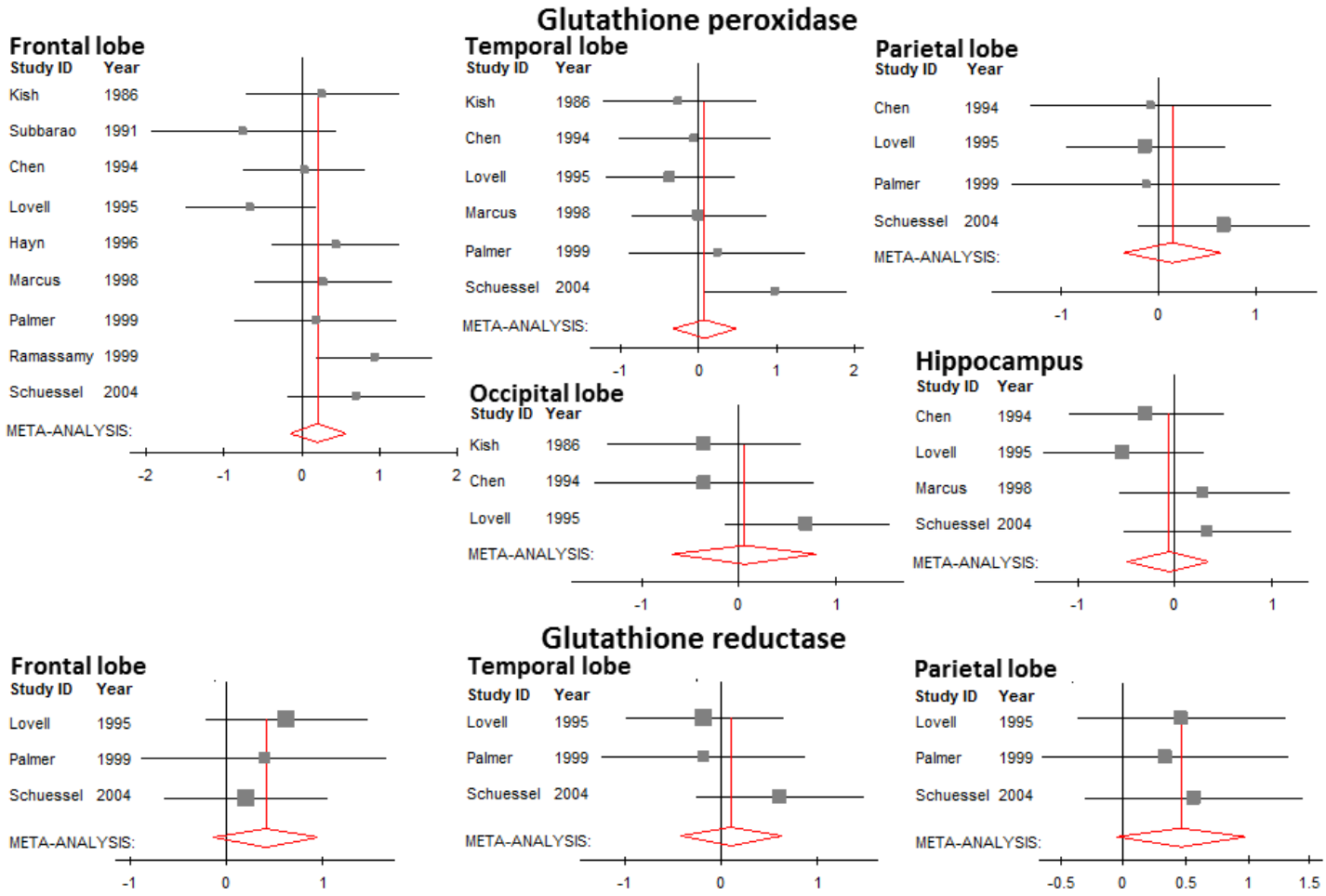

Figure 5: Glutathione peroxidase and reductase activities in Alzheimer's disease brain

There is no evidence of significant change in glutathione peroxidase activity in any brain region. There was no evidence of heterogeneity of publication bias in this analysis. For glutathione reductase, adequate data to perform meta-analysis was only available for the brain regions shown. There was no evidence of a significant change in glutathione reductase activity in any brain region in Alzheimer's disease.

unchanged if we look at the magnitude of the effect size or at the absolute change in level for markers of lipid and protein oxidation. We previously reported a significant increase in DNA oxidation in circulating lymphocytes in AD, but in this analysis we found no evidence of increased nuclear DNA oxidation (indicated by 8-hydroxyguanine levels) in any brain region in AD. Both studies found little evidence to support a derangement in the major antioxidative enzymes superoxide dismutase, glutathione peroxidase and reductase and catalase activity – it is unlikely that modulation of these systems is a major factor in the development of AD. In our study of peripheral derangements in pathways related to oxidative balance, the most compelling changes were in the levels of non-enzymatic antioxidants, particularly ascorbic acid, tocopherols, carotenoids and uric acid, all of which were profoundly depleted in blood from subjects with AD. Remarkably, very little data is available on the levels of these antioxidants in AD brain. Based on the limited data available we found that tocopherol levels and glutathione levels were unchanged in AD brain, but there was sparse data on uric acid levels and none on ascorbic acid or carotenoid levels in AD brains.

Lower levels of ascorbic acid occur naturally as we age, but the strongest data supporting the role of ascorbic acid in neuronal health is that Alzheimer's populations experience ascorbic acid levels in blood lower than that observed inother older populations [8,9,10]. Ascorbic acid, often in conjunction with glutathione, is considered a 'first line of defense" against oxidative stress, which has been proposed to be a driving force behind development of pathological factors associated with the AD [11]. Once these pathologies emerge, they may contribute the oxidative environment, further depleting antioxidant reserves [12]. Recent research in animal models highlights alternate roles for ascorbic acid in delaying the progression of disease outside of its general antioxidant capacity. These include mitochondrial function [13], DNA methylation, and enzymatic co-factor [14,15]. Avoiding ascorbic acid deficiency in early stages of disease

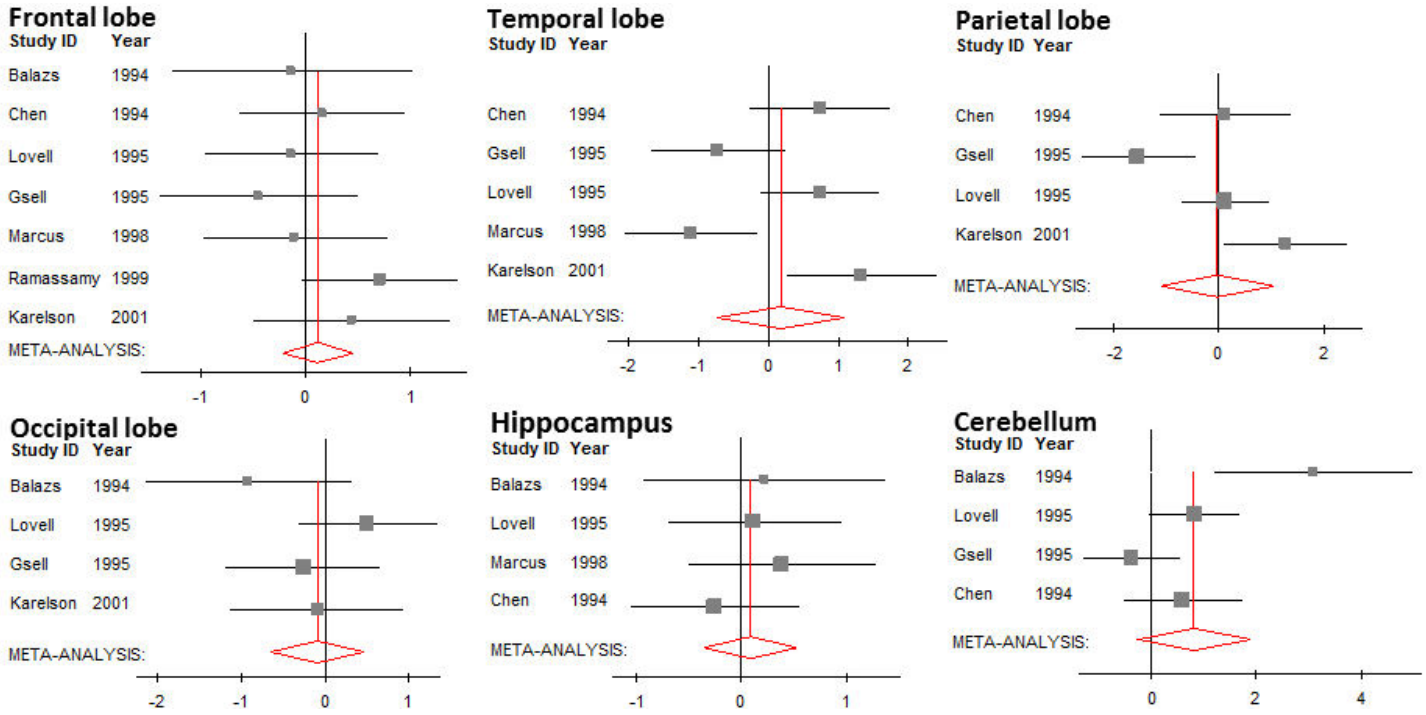

Figure 6: Catalase activity in Alzheimer's disease brain

There is no evidence of significant change in catalase activity in any brain region in Alzheimer's disease. There is significant heterogeneity in the data in the cerebellum and temporal and parietal lobes. It is difficult to evaluate to publication bias given the small number of studies, but for the frontal lobe which has the largest number of studies, there is no evidence of publication bias.

pathology may play a role in protecting neuronal health, and also protect against escalating oxidative damage. We did note that ascorbic acid levels were consistently depleted in studies of AD CSF, although this is not a perfect surrogate of brain levels. The absence of this data is particularly striking in view of the number of clinical trials of supplementation of ascorbic acid and other non-enzymatic antioxidants for patients with AD (none of which has demonstrated a convincing improvement in cognition).[16,17]

It is worth noting that two *in vivo* studies from a single lab have claimed that glutathione was decreased in brain in AD patients compared to healthy controls using proton magnetic resonance spectroscopy [18,19]. This is in striking contrast to the postmortem data reported in this meta-analysis where there appears to be no difference in glutathione levels across multiple brain regions. Most of the *in vivo* data suggesting a negative correlation between glutathione levels in brain and AD disease state are in animal models [20,21]. To date, only twenty-one AD and twenty-two MCI subjects have had levels of glutathione recorded using proton MRS, where the authors found a significant reduction in glutathione in AD and MCI patients in the frontal cortex and hippocampus compared to controls [18,19]. Both studies are difficult to interpret in our view due to poor case selection and inadequate age and education matching – neither study met criteria to be included in this analysis. While *in vivo* measures of levels of antioxidants in human brain would be valuable, these studies do not alter our conclusions that glutathione levels appear to be unchanged in AD.

Publication bias suggests that studies which do not detect a statistically significant result are less likely to be published and when present, this bias can distort the result of a meta-analysis in favor of a "positive" result. Evidence for publication bias was particularly strong for the analysis of brain malondialdehyde levels in AD. While this is not the most specific or robust marker of lipid peroxidation, it is the most commonly studied marker of lipid peroxidation in AD brain and the presence of publication bias undermines the strength of this dataset. It is important to note that publication bias is inferred due to an asymmetric distribution of imprecise (small) studies around the effect size reported in the most precise (largest) studies. It is conceptually possible to have such a distribution which does not represent publication bias, but the typical alternative causes of this are unlikely here.[22] The methodologies for evaluating malondialdehyde levels are well-established and not difficult to apply to large populations and the criteria for case selection were comparable across the studies. In a few cases, publication bias could be directly observed when authors stated that they performed malondialdehyde measurements or measurements of other relevant variables, but did not present the data

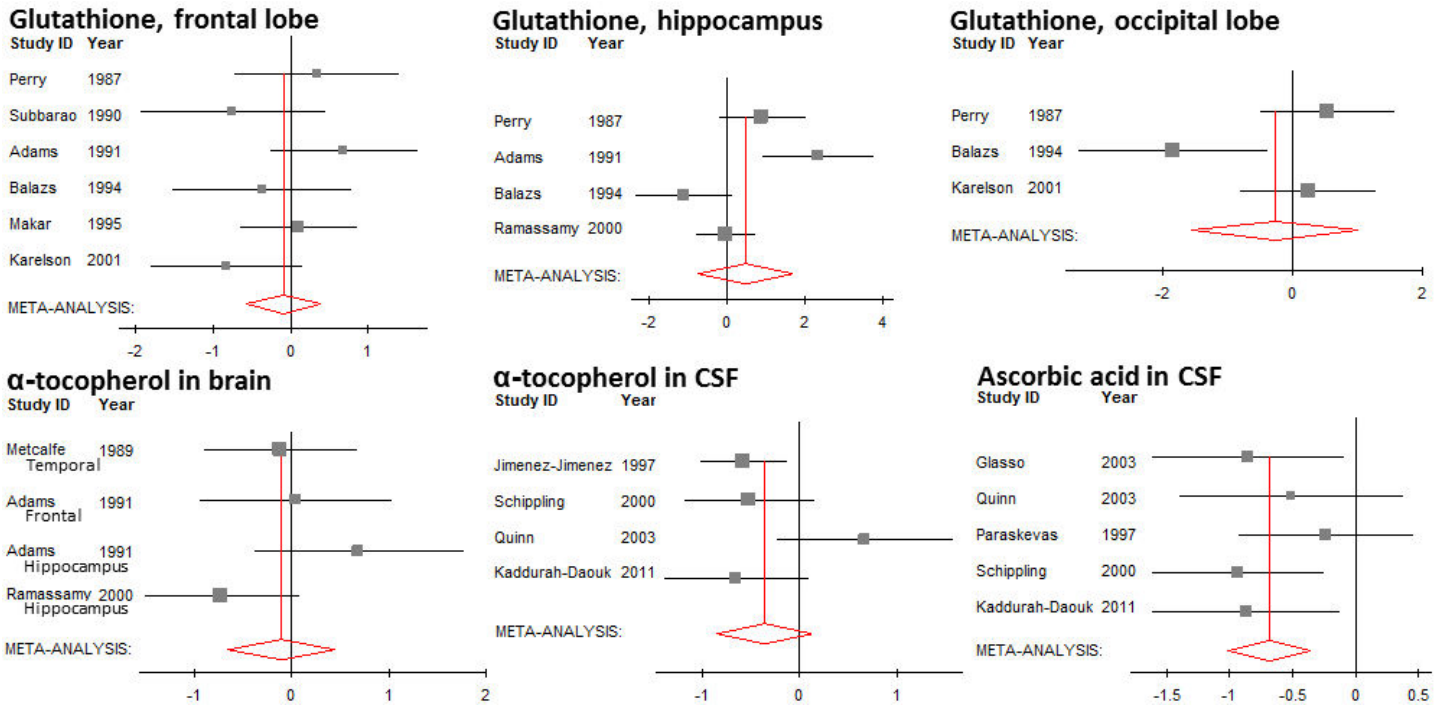

Figure 7: Non-enzymatic antioxidant levels in Alzheimer's disease brain

The major finding of this portion of the analysis is that there is not much data available describing the levels of non-enzymatic antioxidants in the brain. There is enough data for glutathione to make an analysis in three regions (the frontal lobe, occipital lobe and hippocampus) and there is no change in glutathione level in any of these regions. Three studies have measured alpha-tocopherol levels in the brain. There is not enough data to perform a region-by-region analysis, but we have lumped all the regions together to produce a general estimate of alpha-tocopherol levels in Alzheimer's disease brain – there does not appear to be a significant change. Additionally, alpha-tocopherol level in CSF was unchanged. Finally, we were unable to find a single study reporting ascorbic acid levels in the brain in Alzheimer's disease, but five studies have evaluated CSF and the ascorbic acid level in CSF in Alzheimer's disease appears significantly depleted.

because they did not show a significant difference.[5,23] (In one of these cases, we were able to obtain the original data from the authors to try to mitigate the impact of this publication bias on our analysis). In the case of malondialdehyde levels in AD brain, we believe that there is a true publication bias which led to an exaggeration of the estimated effect size. Because of the relatively small number of studies available for the other markers, it is hard to estimate the degree to which publication bias impacted the effect sizes for the remainder of the analysis. In the analysis of oxidized nucleic acids, one group found a much larger effect size than any other group. Because this group published three different studies on this topic, there was the appearance that increased oxidized guanine was a reliable finding in AD brain despite the failure of four other groups to replicate this finding -- 8-hydroxyguanine levels are convincingly unchanged in AD brain[24-26]. Random effects analysis is particularly vulnerable to this type of outlier effect when multiple outlier studies are contributed by a single research group, so in our analysis the studies from this group were aggregated into a single result prior to analysis. Finally, we discovered one case of frank redundant publication where neither study mentioned the duplicated dataset.[27,28] All of these issues created artifacts in the literature which may have led a casual reader to assume the finding of increased markers of oxidative stress in AD brain is stronger than it actually is. For this reason, a comprehensive critical analysis of the topic is both helpful and necessary.

This analysis is limited to bulk tissue analysis and may therefore miss important changes occurring in particular cell types or sub-cellular compartments. Some qualitative literature suggests that certain subgroups of neurons are particularly vulnerable to oxidative damage at an early stage of AD pathology.[29] There is also an argument that oxidative damage is present selectively in mitochondria. Increased mitochondrial DNA oxidation in AD has been reported in the absence of a significant alteration in nuclear DNA oxidation.[30] The distinction between bulk tissue analysis and local findings is important, however, because it may lead to different hypotheses. For instance, it is entirely possible that selective accumulation of oxidative damage in particular neurons and/or particular sub-cellular fractions may have little to do with global or focal increased oxidative stress, but rather represents failure of other cellular homeostatic systems. Neuronal axons adjacent to β-amyloid aggregates accumulate massive numbers of ectopic and dysfunctional

lysosomes.[31] This may lead to failed clearance of basal levels of oxidative damage in affected neurons as this lysosome pool is not available to participate in autophagic processes.[32] This could conceptually explain why the brain antioxidant systems appear to be broadly intact in AD, yet there are focal accumulations of markers of oxidative damage. Moreover, focal accumulation of oxidative damage or transient oxidative events in regionally specific areas may remain relevant to AD pathology. There are numerous reports that detail various redox-sensitive second messenger pathways affecting cellular survival, gene expression, and discrete signaling.[33-36] This includes the redox-sensitive second messenger phosphatidylinositol 5-phosphate, which integrates oxidative stress and the complexities of lipid signaling as a whole.[34-37] Should oxidative stress be relevant for AD pathology, it might be more relevant to investigate these redox-sensitive second messengers and their downstream effectors for increased context relevance and signal amplitude following transient oxidative events. It is also possible that oxidative changes are more prominent in specific patient subgroups, particularly related to *APOEε4* carrier status, and this would not be captured by the current analysis.[38,39] Additionally, vascular amyloid deposition is prominent in a sub-group of patients with AD in the form of cerebral amyloid angiopathy and may be a mechanism for focal or widespread induction of oxidative stress pathways through microhemorrhage, ischemia and perivascular inflammation.[40,41] It is also possible that inconsistencies in post-mortem interval may impact the findings; however, the included studies had comparable post-mortem intervals in the control and AD groups as a requirement of inclusion. There is limited systematic data available for the impact of post-mortem interval on most of these markers, but for those where data is available the post-mortem interval appears to be a minor variable in the cortex, particularly within the first 24-48 hours of death.[42-45]

Finally, the pattern of larger oxidative change in blood than in the brain certainly raises the possibility that systemic derangements leading to a pro-oxidative environment may be upstream of brain injury in AD. This hypothesis would also be supported by the observation of increased risk of dementia in patients who have pro-inflammatory/pro-oxidative diseases like diabetes, obesity and hypercholesterolemia.[46,47] We previously found that a treatment which lowered peripheral levels of oxidative stress improved AD-like neuropathology in an experimental animal model of AD without altering brain levels of oxidative changes.[48] The failure to translate this result in human clinical trials of anti-oxidative therapies is noteworthy, but none of the available trials has documented that the intervention(s) employed resolved antioxidant deficiencies and actually improved antioxidant capacity or markers of oxidative stress, so the hypothesis has not yet been thoroughly tested in clinical trial.[2,3] As we previously argued, it may be necessary to tailor antioxidant interventions to address individual patients' deficiencies to obtain an optimal clinical result.[8]

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