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Title: Oxidative stress, inflammation and risk of neurodegeneration in a population sample

Running Title: Inflammation, MCI & Brain

Authors:
Nicolas Cherbuin PhD[1,*]
Erin Walsh PhD[1]
Bernhard T. Baune PhD, MD, MPH, FRANZCP[2,3]
Kaarin J. Anstey PhD[1,4]

Affiliations:
Centre for Research on Ageing, Health and Wellbeing, Australian National University, Canberra, Australia[1]
Department of Psychiatry, University of Münster, Germany[2]
Department of Psychiatry/Florey Institute, University of Melbourne, Australia[3]
School of Psychology/NeuRA, University of New South Wales, Sydney, Australia[4]

***Correspondence:**
Nicolas Cherbuin, Centre for Research on Ageing, Health and Wellbeing, 54 Mills Road, Australian National University, Canberra, ACT 2601, Australia.
Tel: (612) 61253858; Fax: (612) 61251558; Email: nicolas.cherbuin@anu.edu.au

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Abstract

Background: Inflammation and oxidative stress (OS) have been clearly linked to neurodegeneration. However, studies investigating the associations between peripheral markers of inflammation and cognitive decline have produced mixed results. This is possibly due to the fact that markers are typically tested individually despite the fact that biologically they function interactively. Thus, the aim of this study was to investigate the association between a combination of OS/inflammation markers and outcomes including MCI diagnosis, cognitive decline, and hippocampal atrophy.

Methods: OS/inflammation status was assessed in 380 older community-living individuals. Thirteen blood markers were assayed. Principal component analysis (PCA) of all markers was conducted to identify the more salient inflammatory components. Associations between significant principal components, MCI diagnosis, previous change in MMSE score and hippocampal atrophy were investigated through logistic and linear multiple regression.

Results: Two factors (PC1&PC2) reflecting predominantly broad pro-inflammatory activity and two factors (PC3&PC4) reflecting predominantly OS activity were identified by PCA analysis. PC3 and PC4 were predictive of MCI. PC3 was also predictive of prior MMSE change. PC1, PC2, and PC3 were significantly associated with hippocampal atrophy.

Conclusions: Combined analysis of complex and interacting biomarkers revealed a protective association between anti-oxidant activity and MCI that is consistent with lifestyle factors shown to reduce risk of cognitive decline. Oxidative stress and broad systemic inflammation were also found to be associated with hippocampal atrophy further highlighting the benefits of the PCA methodology applied in this study.

Text

Introduction

In order to develop strategies to slow down or prevent neurodegenerative processes which occur increasingly with ageing, it is necessary to better understand the subtle biological mechanisms involved. Two broad and inter-related mechanisms, oxidative stress and inflammation, have been shown to be strongly implicated in neurodegeneration[1-3]. Oxidative stress (OS) is a bi-product of energy metabolism in the human body. It refers to the elevated cellular production of reactive oxygen species (ROS) that are not buffered by anti-oxidant activity. Seminal research conducted by Denham Harman originally suggested that OS was the principal cause of DNA and cellular damage which accumulates with ageing and leads to senescence and thus underpinned the free-radical theory of ageing[4]. However, research conducted over the past 30 years revealed that ROS also contributes to important cellular communication functions in close proximity to the producing source (mostly the mitochondrion), and that excess ROS can also contribute to DNA and cellular damage more distally if their levels are not sufficiently buffered by anti-oxidants either produced in the cell or absorbed from exogenous sources (e.g. diet)[5].

Similarly, inflammatory processes are essential to tissue repair and to fight infections, are involved in physiological cell signalling pathways, and when excessively or chronically activated lead to over-production of ROS, increased tissue damage and impaired cellular function[6,7]. Moreover, inflammation is not a uniform biological mechanism that is simply up or down-regulated in the presence of injury or infection. Instead, it is a complex system of feedback loops in which signalling proteins (e.g. cytokines) may not only have pro- or anti-inflammatory actions, but at least for some, have anti-inflammatory actions at low levels and pro-inflammatory actions at higher levels and vice versa.

A large number of studies have focused on single markers, and while many identified significant associations between OS/inflammation markers and a variety of outcome measures, there is a great deal of heterogeneity in the direction and magnitude of findings and how different markers interact together is not well understood. For example, Lai and colleagues[1] conducted a meta-analysis of the difference in single peripheral inflammatory markers between individuals with Alzheimer's disease and those with normal cognition. Of the 19 markers that could be analysed only one led to findings that showed low heterogeneity between studies. For all others very large heterogeneity between studies was identified. As an illustration, for IL6, while the meta-analysis identified a significant difference, the majority of studies reported either non-significant or negative findings.

Few studies have investigated inflammatory markers in Mild Cognitive Impairment (MCI) but where markers overlap between studies, findings have been mixed or contradictory[8]. For example, in a recent review the marker most investigated was TNFα with 5 reports (three indicating no associations and two reporting elevated levels). For IL8, two studies reported no associations while one reported decreased levels. For other markers, either a single study was available or where two studies were available they often report contradictory findings. The reason for heterogeneous findings is not clear but given the complex regulating mechanisms and feedback loops between different inflammatory markers it is likely that the combined actions of several cytokines are associated with clinical outcomes (e.g. MCI), rather than variation in a single marker. In addition, from a methodological perspective an approach which tests a long list of single markers is also problematic as it heightens the possibility of erroneous findings due to false positive or, if corrections for multiple comparisons are implemented, it

becomes very difficult to detect other than very large effects unless unusually large sample sizes are available.

To address these issues in the present study we aimed to assess the influence of ROS and inflammation on cognitive decline by identifying salient combinations of several biomarkers (**Figure 1)**. We used principal component analysis (PCA) to summarise the contribution of 13 biomarkers to components representing different states of OS and inflammation across a large sample of community-living individuals. Elevated OS/inflammation levels have already been demonstrated in clinical dementia[1], but there is a pressing need for early markers of disease progression. Because of this, we tested associations between different OS/inflammatory components with MCI diagnosis and prior change in cognitive function (decrease in MMSE scores). In addition, we also conducted further analyses to determine whether those factors associated with cognitive decline are also associated with prior change in one of the structures most affected by early neurodegenerative processes, the hippocampus[9].

Methods and Materials

Study population

Participants from the PATH Through Life (PATH) project[10] with detailed blood inflammatory markers available (n=422) and who were free of neurological disorders (n=380) were included in this study. All participants provided written informed consent and the study was approved by the Australian National University Ethics Committee.

Inflammatory cytokines and OS markers

Available measures considered as markers of inflammation were Tumor Necrosis Factor alpha and TNF receptors (TNFα, TNF-R1, TNF-R2), and interleukins (IL1β, IL4, IL6, IL8, IL10).

Measure considered as OS markers included nitric oxide (NO), neopterin (NEO), total anti-oxidant capacity (TAC), as well as related markers of DNA damage strongly associated with OS including malondialdehyde (MDA) and 8-hydroxy-2-deoxyguanosine (GUA). All markers were assessed in duplicates as recommended by assay manufacturers (see supplementary material for details).

Diagnosis and cognitive change

MCI and dementia diagnoses were established based on a neuropsychological assessment and consensus diagnosis if clinical criteria (DSM-IV/Petersen/Winblad[11,12], see supplementary material) were met. Cognitive change was operationalised as a change in MMSE score between wave 1 and wave 4.

MRI scan acquisition and image analysis

Neuroimaging data was only available for a sub-sample of participants (n=226) who were imaged with a fast-field echo sequence for T1-weighted 3-D structural scan acquired on the same scanner for all participants. All images were processed using FreeSurfer (v. 5.3)[13]. The only regions of interest considered in this study were the left and right hippocampi (HC) because it is particularly sensitive to inflammatory processes and known to be implicated in cognitive decline and Alzheimer's disease[14,15].

Statistical analysis

Statistical analyses were computed in the R statistical package (version 3.2). Missing values (<1% of all variables analysed) were imputed by chained equations with the package "mice" using the "pmm" algorithm[16]. Descriptive analyses were conducted using Chi-square tests for categorical data and t-tests to compare groups on continuous variables. The principal

components of the observed cytokine response across all inflammatory and oxidative stress markers were extracted through a principal component analysis (PCA) using the R package "prcomp"[17]. Principal components were selected based on an eigen value >1. Sensitivity analyses were conducted to determine whether including a greater number of factors, or analysing inflammatory and OS markers in separate PCAs led to substantially different results. Associations between principal components and outcome measures were investigated by logistic regression for MCI and by linear regression analyses for continuous measures (change in MMSE and in HC volume) while controlling for age, sex, education and (for HC analyses only) intra-cranial volume. Additional information is provided in the supplementary material

Results

The sample's demographic characteristics and OS/inflammation markers' levels are presented in **Table 1**.

Principal component analysis

Pearson bivariate correlations are presented in **Figure 2** and indicate that none of the markers were exclusively positively or negatively associated with other measures. TNF-R1 and TNF-R2 were the most highly correlated measures. Notably, the shared variance between pairs of markers was on average relatively low and ranged from 0.04% to 23% (with one exception TNF-R1-TNF-R2 at 86%). NO and TAC shared least variance with other markers while IL4 and IL8 shared most.

Results of the PCA analysis are presented in supplementary material (**Table E1** and **Figure E1**). Using an eigen-value cut-off of 1, four main principal components (PC1-PC4) which explained 54.86% of the variance in pro-inflammatory and OS markers were identified. The relative contribution of the different markers to each PC is presented in **Figure 3**. PC1 can be

interpreted as consisting of relatively unspecific heightened pro-inflammatory response particularly involving TNFα and TNF receptors, in the context of low OS activity. PC2 suggests a lower pro-inflammatory response in the context of a somewhat increased anti-oxidant activity. PC3 is indicative of decreased antioxidant activity (TAC) and increased DNA damage (MDA, GUA) in the context of a low inflammatory response. And PC4, is also suggestive of lower anti-oxidant activity involving increased GUA and low inflammation, but unlike PC3, in the context of decreased MDA.

Associations between principal components, MCI, and cognitive decline

Analyses testing associations between the main PCs and MCI diagnosis at wave 4 or change in MMSE score between wave 1 and wave 4 are presented in **Table 2** see also sensitivity analyses in the supplementary material). They show that PC3 (OR: 1.76, 95%CI 1.36-2.31, p=0.00003) and PC4 (OR: 1.45, 95%CI 1.11-191, p=0.007) (lower anti-oxidant activity) were associated with a 76% and 45% increased risk of MCI. In addition, PC3 was associated with less prior decline in MMSE in the whole sample and in the MCI sub-sample, but not in the normal subsample. Moreover, PC2 (pro-inflammatory response) was associated with larger prior decline in MMSE but only in the whole sample. And finally, PC1 (pro-inflammatory response) was associated with a greater prior decline in MMSE score but only in cognitively normal participants. However, after correction for multiple comparisons the associations between PC3/PC4 and MCI/MMSE change were the only ones to remain significant.

Association between principal components and hippocampal atrophy

Analyses testing associations between the main PCs and change in hippocampal volume between wave 1 and wave 4 are presented in **Table 2**. They show that PC1 (pro-inflammatory response) was consistently associated with a greater prior hippocampal atrophy. However, this

effect was only significant after correction for multiple comparisons for the right HC in the whole sample and the normal sub-sample. The only other significant principal component after correction for multiple comparison was PC3 for the left (trend) and right HC in the MCI sub-sample.

Main contributors to the effects of principal components

Additional analyses investigating the markers contributing to significant principal component (PC1 & PC3; see supplementary material and **Table 2&3**) showed that higher TAC levels were associated with decreased risk of MCI and a lower decrease in MMSE, while TAC was associated with greater left HC atrophy. TNFα was associated with greater HC atrophy in the whole sample and the "normal" sub-sample NEO was associated with greater HC.

Discussion

This study's main findings were that the pattern of OS/inflammation observed in the population surveyed was associated with having MCI, prior cognitive decline and hippocampal atrophy. Importantly, lower anti-oxidant activity was found to be the strongest predictor of hippocampal atrophy and cognitive decline.

The PCA analysis revealed four significant principal components which together explained over 54% of the variability in all markers. Of the four principal components identified one component (PC3, high anti-oxidant and low OS damage), was significantly associated with a decreased risk of MCI after stringent correction for multiple comparisons. Follow up analyses indicated that this effect was mostly attributable to total anti-oxidant levels with every one pg

increase in TAC being associated with an 8% decreased risk of MCI. Notably, consistent associations were also detected for MMSE score change, not only in the whole sample, but also within the normal and MCI sub-samples. This is important because it suggests a potent and continuous effect of TAC across the whole cognitive range. Further, attesting to the robustness of these findings, is the fact PC3 and TAC were also significantly associated with past hippocampal atrophy although only in MCI participants. The reason for the lack of association in the whole sample or normal sub-sample is not clear but we speculate that OS damage may only start to be detectable in the pre-clinical stages of dementia-related neurodegeneration and may not yet be detectable in those without MCI. Another component (PC4) reflecting high OS activity through lipid peroxidation (MDA) in the context of higher anti-oxidant activity but lower DNA damage (GUA) was also associated with hippocampal atrophy but not MCI or MMSE. It is not clear why PC4, which like PC3 reflected TAC activity, was not associated as consistently with outcome measures. One possible explanation is that TAC contribution to PC3 is greater than to PC4 and therefore may afford a greater protective effect to this factor. Alternatively, this difference may reflect NO activity which is decreased in the presence of OS and importantly, whose down-regulation plays a significant role in the development of hypertension. Thus, PC4 may be less strongly associated with cardio-vascular disease which is a potent risk factor for neurodegeneration and cognitive decline.

These are important results as they show that more subtle analyses can reveal how pro-inflammatory states rather than individual markers are associated with neurodegenerative processes. It is also particularly notable that of all the markers investigated total anti-oxidant capacity is the only one being consistently associated with neurodegeneration and cognitive decline in the population investigated. These findings may have important clinical and population health implication as it suggests that targeting anti-oxidant levels may be more

effective than lowering specific pro-inflammatory cytokines. This also seems consistent with current evidence indicating that administration (as opposed to use which is conflated with confounders in population studies) of anti-inflammatory treatments is not effective at decreasing cognitive decline or dementia risk[18,19], while interventions that boost anti-oxidant levels including diet, omega 3 fatty acids, and exercise have been found to be on average very effective[20-23].

In conclusion, this study showed that systemic OS/inflammatory states are associated with cognitive decline and neurodegeneration and that higher anti-oxidant activity appears to be particularly protective. Future investigations are needed to identify the main determinants of anti-oxidant and pro-inflammatory states. Moreover, this study has also made an important methodological contribution by showing that using PCA to assess how combinations of markers, rather than individual ones, might lead to the detection of more meaningful associations between complex pathological mechanisms and health and functional outcomes.

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Table/Figure Legends

Table 1. Participants' demographic characteristics.

Table 2. Associations between principal components of oxidative stress and inflammation and their principal contributing markers, and MCI and cognitive decline (MMSE change in previous 12 years) in the whole sample and MCI or normal sub-samples.

Table 3. Associations between principal components of oxidative stress and inflammation and their principal contributing markers, and hippocampal atrophy in the whole sample and MCI or normal sub-samples.

Figure 1. Path depicting the spectrum of inflammation and oxidative stress spanning normal cell signalling function to pathological chronic inflammation and their link to neurodegeneration and cognitive decline.

Figure 2. Bivariate correlation matrix between oxidative stress and OS/inflammation markers.

Figure 3. Main oxidative stress and inflammatory markers' contribution to each of the four significant principal components (PC1-PC4) identified in the PCA analysis. Red line represents the significance threshold.

Table 1. Participants' demographic characteristics.

Measures	Whole Sample (n=380)	Males (n=211)	Females (n=169)	T/chi-sq Test (P value)	Normal (n=299)	MCI (n=81)	T/chi-sq Test (P value)
Age, years (SD)	75.35 (1.41)	75.37 (1.41)	75.32 (1.40)	0.29 (0.774)	75.31 (1.37)	75.48 (1.54)	-0.86 (0.392)
Education, years (SD)	14.22 (2.67)	14.76 (2.63)	13.56 (2.58)	4.46 (0.000)	14.43 (2.53)	13.46 (3.04)	2.64 (0.009)
MMSE, score (SD)	28.77 (1.49)	28.53 (1.66)	29.06 (1.19)	-3.61 (0.000)	29.08 (1.14)	27.60 (2.00)	6.35 (0.000)
BMI, kg/m2 (SD)	26.46 (4.79)	26.76 (4.43)	26.07 (5.20)	1.38 (0.169)	26.32 (4.54)	26.96 (5.62)	-0.95 (0.345)
Hypertension, n (%)	284 (74.74%)	155 (73.46%)	129 (76.33%)	0.27 (0.602)	230 (76.92%)	54 (66.67%)	3.03 (0.082)
Diabetes, n (%)	62 (16.32%)	38 (18.01%)	24 (14.20%)	0.74 (0.391)	42 (14.05%)	20 (24.69%)	4.54 (0.033)
APO*E4 genotype, n (%)	103 (27.11%)	56 (26.54%)	47 (27.81%)	0.03 (0.872)	72 (24.08%)	31 (38.27%)	5.80 (0.016)
IL1β, pg/ml (SD)	0.04 (0.04)	0.04 (0.04)	0.04 (0.05)	-1.09 (0.277)	0.04 (0.04)	0.03 (0.04)	0.86 (0.389)
IL4, pg/ml (SD)	0.21 (0.18)	0.21 (0.18)	0.21 (0.17)	0.07 (0.943)	0.21 (0.18)	0.21 (0.15)	-0.00 (0.999)
IL6, pg/ml (SD)	0.66 (0.79)	0.69 (0.76)	0.62 (0.84)	0.81 (0.417)	0.61 (0.68)	0.66 (0.61)	-0.67 (0.504)
IL8, pg/ml (SD)	2.61 (1.89)	2.57 (2.00)	2.66 (1.76)	-0.48 (0.631)	2.49 (1.73)	2.82 (2.10)	-1.26 (0.209)
IL10, pg/ml (SD)	0.56 (0.48)	0.55 (0.34)	0.58 (0.61)	-0.59 (0.558)	0.57 (0.50)	0.53 (0.25)	1.02 (0.309)
NO, pg/ml (SD)	22.26 (13.13)	22.16 (12.42)	22.39 (14.00)	-0.16 (0.872)	22.12 (12.35)	22.80 (15.75)	-0.36 (0.720)
TAC, mmol/l (SD)	64.24 (10.48)	65.24 (10.76)	62.99 (10.01)	2.11 (0.036)	65.52 (10.87)	59.52 (7.20)	5.90 (0.000)
NEO, pg/ml (SD)	3.01 (2.72)	3.19 (2.85)	2.79 (2.53)	1.42 (0.157)	2.97 (2.68)	3.17 (2.86)	-0.58 (0.561)
MDA, pg/ml (SD)	85.69 (36.51)	84.74 (31.59)	86.88 (41.92)	-0.55 (0.581)	84.26 (36.39)	90.99 (36.67)	-1.47 (0.144)
GUA, ng/ml (SD)	12.89 (4.49)	13.42 (4.52)	12.22 (4.38)	2.63 (0.009)	12.63 (4.44)	13.84 (4.61)	-2.11 (0.037)
TNFα, pg/ml (SD)	2.41 (1.34)	2.41 (1.14)	2.40 (1.55)	0.06 (0.955)	2.42 (1.33)	2.36 (1.38)	0.34 (0.735)
TNF-R1, ng/ml (SD)	1.31 (0.49)	1.32 (0.45)	1.29 (0.54)	0.58 (0.559)	1.30 (0.49)	1.31 (0.47)	-0.15 (0.881)
TNF-R2, ng/ml (SD)	1.43 (0.54)	1.45 (0.49)	1.41 (0.59)	0.79 (0.432)	1.42 (0.54)	1.47 (0.52)	-0.65 (0.515)

Significance: $p<0.05$

Table 2. Associations between principal components of oxidative stress and inflammation and their principal contributing markers, and MCI and cognitive decline (MMSE change in previous 12 years) in the whole sample and MCI or normal sub-samples.

	PCA Components					Significant Contributing Markers			
	MCI	MMSE Change				MCI	MMSE Change		
		Whole	MCI	Normal			Whole	MCI	Normal
PC1	0.03	0.026	-0.145	0.076**	TAC	-0.079***	0.023***	0.053**	0.010*
	p = 0.694	p = 0.464	p = 0.144	p = 0.026		p = 0.00002	p = 0.0002	p = 0.038	p = 0.068
PC2	0.121	-0.120**	-0.296*	-0.057	MDA	0.004	-0.002	-0.009*	0.001
	p = 0.275	p = 0.016	p = 0.052	p = 0.219		p = 0.294	p = 0.175	p = 0.056	p = 0.595
PC3	0.567***	-0.171***	-0.432**	-0.027					
	p = 0.00003	p = 0.004	p = 0.024	p = 0.616					
PC4	0.371***	-0.038	0.011	-0.052					
	p = 0.008	p = 0.539	p = 0.953	p = 0.393					
Constant	-0.124	16.725***	15.526*	19.569***	Constant	6.284	15.101***	15.976*	18.214***
	p = 0.987	p = 0.00001	p = 0.096	p = 0.00000		p = 0.400	p = 0.0001	p = 0.092	p = 0.00001
Observations	380	380	81	299	Observations	380	380	81	299
Log Likelihood	-176.37	-611.226	146.309	-427.195	Log Likelihood	-175.786	-610.498	-147.626	-429.353
Akaike Inf. Crit.	368.74	1,240.45	310.619	872.391	Akaike Inf. Crit.	363.572	1,235.00	309.251	872.706
Note:	p<0.1; p<0.05; p<0.01								

Table 3. Associations between principal components of oxidative stress and inflammation and their principal contributing markers, and hippocampal atrophy in the whole sample and MCI or normal sub-samples.

	Hippocampal Volume Change					
	Whole		MCI		Normal	
	L	R	L	R	L	R
Principal Components						
PC1	26.149**	36.936***	61.634**	87.797**	21.354*	36.865***
	p = 0.029	p = 0.007	p = 0.039	p = 0.029	p = 0.100	p = 0.010
PC2	-15.395	30.578*	196.403**	119.576	0.09	-7.299
	p = 0.311	p = 0.074	p = 0.020	p = 0.143	p = 0.996	p = 0.680
PC3	-11.817	-1.47	179.416**	274.933***	-24.216	-19.147
	p = 0.556	p = 0.948	p = 0.017	p = 0.003	p = 0.244	p = 0.396
PC4	9.323	-4.246	-23.939	86.09	9.899	-15.669
	p = 0.639	p = 0.848	p = 0.698	p = 0.260	p = 0.640	p = 0.493
Constant	2,629.676**	2,051.83	8,918.137*	2,047.93	2,775.109**	2,287.667*
	p = 0.027	p = 0.125	p = 0.052	p = 0.699	p = 0.028	p = 0.097
Observations	226	226	25	25	201	201
Log Likelihood	-1,599.30	1,624.85	-164.09	167.193	-1,423.12	-1,440.08
Akaike Inf. Crit.	3,218.60	3,269.69	348.18	354.385	2,866.23	2,900.16
Significant Contributing Markers						
TAC	1.921	1.563	-21.601***	-11.131	2.495	2.027
	p = 0.294	p = 0.449	p = 0.004	p = 0.305	p = 0.196	p = 0.334
NEO_(log)	-89.806*	-132.189**	-310.593***	236.561	-85.165	-174.297**
	p = 0.085	p = 0.025	p = 0.003	p = 0.130	p = 0.177	p = 0.012
Constant	2,469.540**	1,813.16	-6,241.718*	8,610.19	2,618.812**	2,187.38
	p = 0.040	p = 0.182	p = 0.098	p = 0.198	p = 0.042	p = 0.118
Observations	226	226	25	25	201	201
Log Likelihood	-1,600.59	-1,627.41	-166.979	-180.27	-1,423.77	-1,440.67
Akaike Inf. Crit.	3,217.17	3,270.82	349.958	376.53	2,863.53	2,897.33
Note:	p<0.1; p<0.05; p<0.01					

Figure 1.

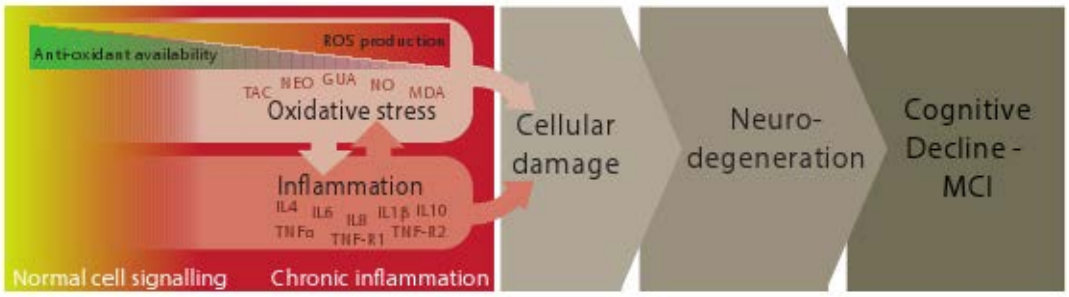

Anti-oxidant availability
ROS production
TAC NEO GUA NO MDA
Oxidative stress
Inflammation
IL4 IL6 IL8 IL1β IL10
TNFα TNF-R1 TNF-R2
Normal cell signalling
Chronic inflammation
Cellular damage
Neuro-degeneration
Cognitive Decline - MCI

Figure 2.

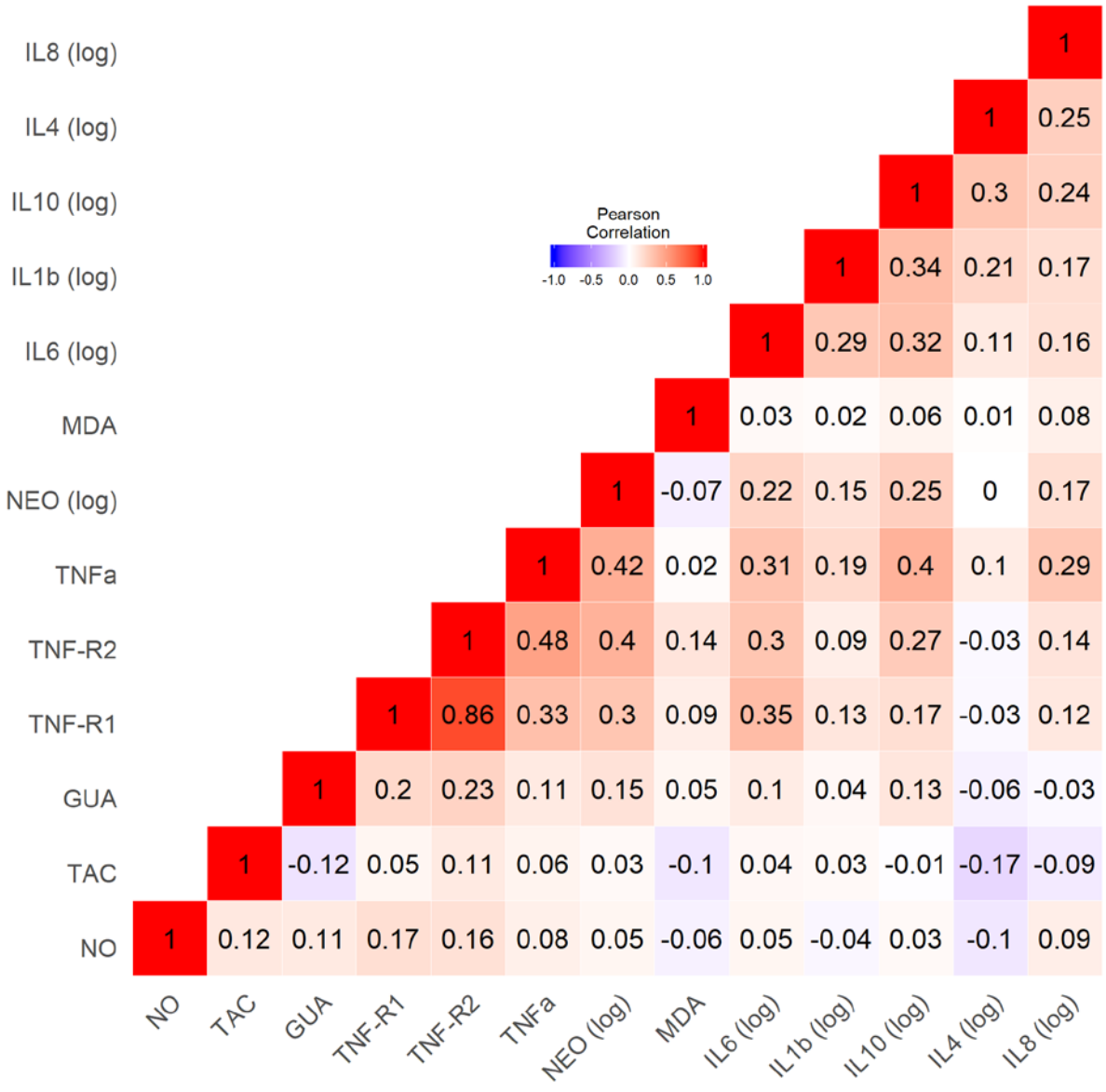
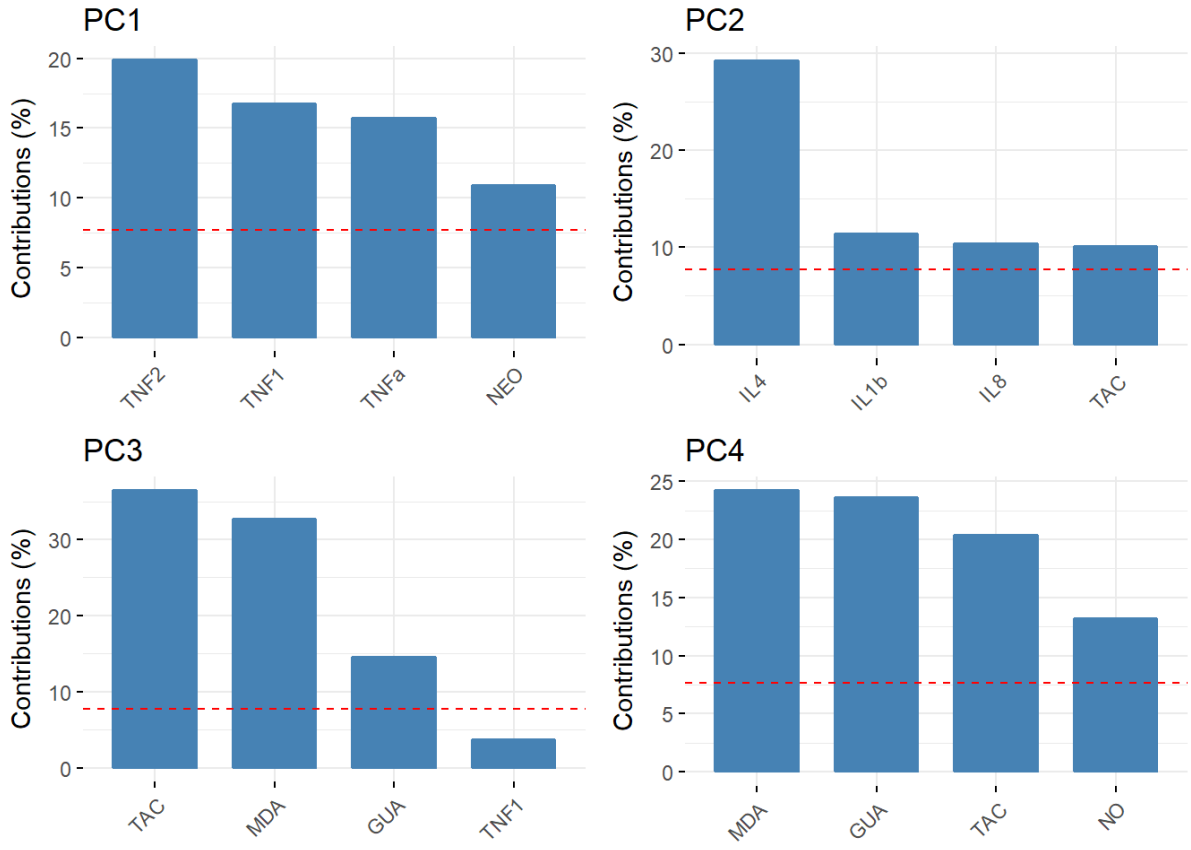

Pearson Correlation
-1.0 -0.5 0.0 0.5 1.0
IL8 (log)
IL4 (log)
IL10 (log)
IL1b (log)
IL6 (log)
MDA
NEO (log)
TNFa
TNF-R2
TNF-R1
GUA
TAC
NO
1 0.12 0.11 0.17 0.16 0.08 0.05 -0.06 0.05 -0.04 0.03 -0.1 0.09
1 -0.12 0.05 0.11 0.06 0.03 -0.1 0.04 0.03 -0.01 -0.17 -0.09
1 0.2 0.23 0.11 0.15 0.05 0.1 0.04 0.13 -0.06 -0.03
1 0.86 0.33 0.3 0.09 0.35 0.13 0.17 -0.03 0.12
1 0.48 0.4 0.14 0.3 0.09 0.27 -0.03 0.14
1 0.42 0.02 0.31 0.19 0.4 0.1 0.29
1 -0.07 0.22 0.15 0.25 0 0.17
1 0.03 0.02 0.06 0.01 0.08
1 0.29 0.32 0.11 0.16
1 0.34 0.21 0.17
1 0.3 0.24
1 0.25
1
NO TAC GUA TNF-R1 TNF-R2 TNFa NEO (log) MDA IL6 (log) IL1b (log) IL10 (log) IL4 (log) IL8 (log)

Figure 3.


PC1
Contributions (%)
20
15
10
5
0
TNF2
TNF1
TNFa
NEO
PC2
Contributions (%)
30
20
10
0
IL4
IL1b
IL8
TAC
PC3
Contributions (%)
30
20
10
0
TAC
MDA
GUA
TNF1
PC4
Contributions (%)
25
20
15
10
5
0
MDA
GUA
TAC
NO

Methods and Materials

Inflammatory cytokines and OS markers

Available measures considered as markers of inflammation were Tumor Necrosis Factor alpha and TNF receptors (TNFα, TNF-R1, TNF-R2), and interleukins (IL1β, IL4, IL6, IL8, IL10). Measure considered as OS markers included nitric oxide (NO), neopterin (NEO), total anti-oxidant capacity (TAC), as well as related markers of DNA damage strongly associated with OS including malondialdehyde (MDA) and 8-hydroxy-2-deoxyguanosine (GUA). All markers were assessed in duplicates as recommended by assay manufacturers. Serum/plasma samples were collected after a fast of at least 8 hours, stored at -80C, and were all processed with the same pipeline at the same time at the ANU Phenomics Facility. TNFα, TNF-R1, TNF-R2, IL1β, IL4, IL6, IL8, IL10 were processed on the Mesoscale platform with the human high sensitivity cytokine and pro-inflammatory panel 1 assays (Rockville, MD, USA) using plasma samples. Other measures were assessed on ELISA assays as follows: NO (ELISA OxiSelect™, Jomar Bioscience, STA-802), Neo (Human BioAssay, Jomar Biosciences, #3410), TAC (Abcam, #Ab65329), MDA (ELISA OxiSelect™, Jomar Bioscience, STA-832), GUA (Oxidative Damage EIA kit, Sapphire Biosciences, #190-58932) using serum sample. The mean coefficient of variation across all assays was 23.7% (range 0.89%-60.8%). IL1α was also assayed but did not pass quality control and therefore was not included in analyses.

Diagnosis and cognitive change

At wave 1 Mild Cognitive Impairment (MCI) and dementia diagnoses were assessed in a two-step process. Participants were first assessed on a number of cognitive measures (MMSE, symbol-digit modalities test, simple and complex reaction time, digits backwards, immediate and delayed recall, purdue pegboard) as part of the main survey. If they performed below specific thresholds they were administered a full neuropsychological assessment during a face-to-face interview on which a consensus diagnosis was established based on published criteria (see [11,12] for detailed methodology). At the fourth wave, because of the higher prevalence of MCI and dementia in this age group, all participants were administered a neuropsychological assessment and given a diagnosis if they met clinical criteria. In addition to formal MCI diagnosis based on Petersen/Winblad criteria[13,14], cognitive change was operationalised in the present study as a change in MMSE score between wave 1 and wave 4.

MRI scan acquisition and image analysis

Neuroimaging data was only available for a sub-sample of participants (n=226) who were imaged with a fast-field echo sequence at wave 1 with a 1.5 Tesla Philips Gyroscan ACS-NT scanner (Philips Medical Systems, Best, The Netherlands) for T1-weighted 3-D structural scan (TR/TE/FA=28.05/2.64 ms/30º, matrix size = 256×256; in plane resolution of 1mm x 1 mm) and at wave 4 on a Siemens 1.5 T Espree scanner (TR/TE/FA=1160 ms/4.24 ms /15°, in plane resolution of 0.5 × 0.5 mm). All images were pre-processed using the MINC imaging toolbox (MINC; http://en.wikibooks.org/wiki/MINC) including image intensity normalisation and B_0

inhomogeneity correction[13]. Further image analysis was carried out using FreeSurfer (v. 5.3)[14]. The only regions of interest considered in this study were the left and right hippocampi (HC). This choice was made because the HC has been shown to be particularly sensitive to inflammatory processes and to be implicated in cognitive decline and Alzheimer's disease. In addition, focusing on few well-justified regions of interest decreases the risk of false positive (type 1 error) being detected.

Results

Principal Component Analysis (PCA)

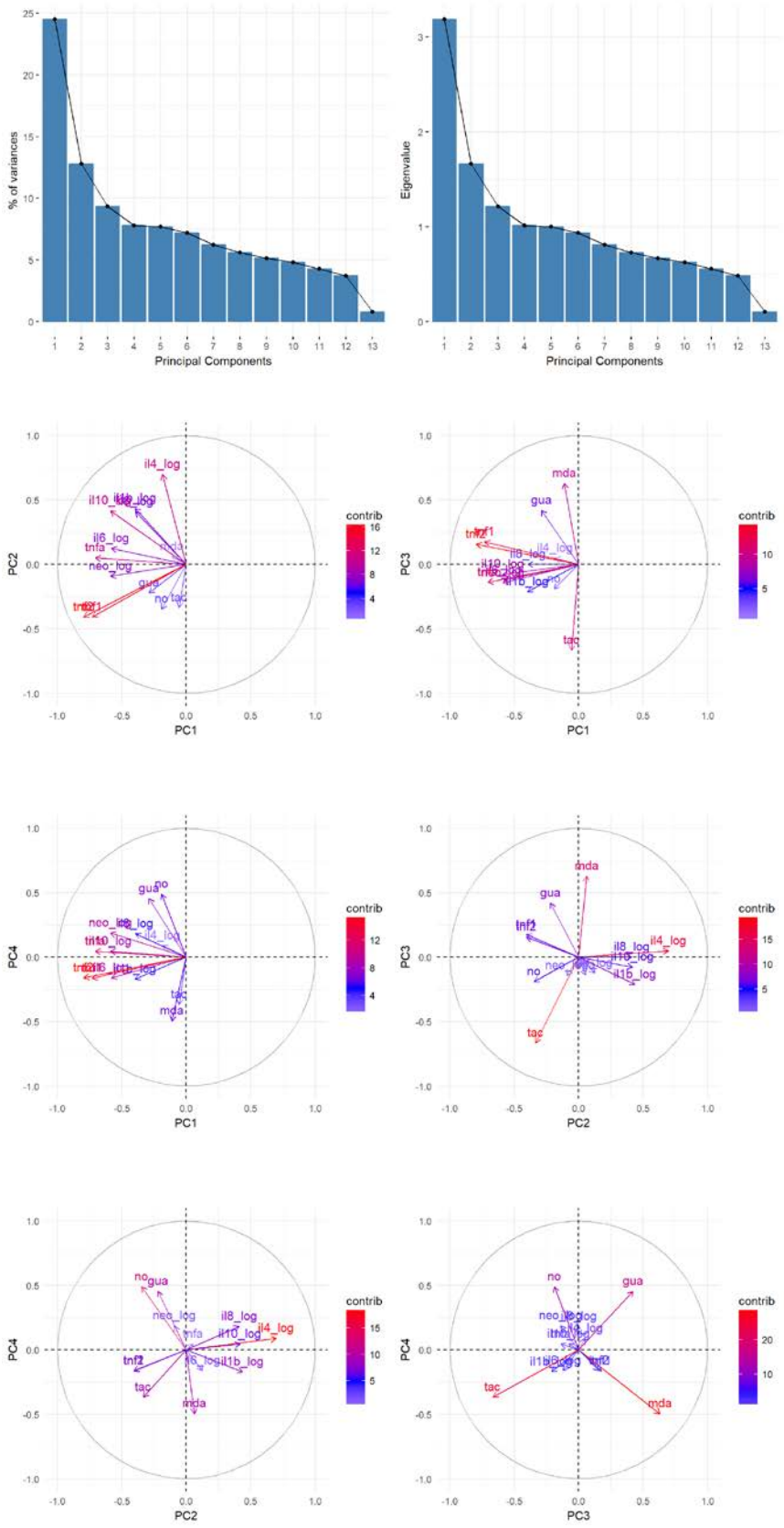
Table E1. Factor loadings or OS/inflammatory markers on each principal component. Note that only PC1-PC4 were found to be significant.

```
##                PC1        PC2         PC3        PC4        PC5
## il1b_log -0.22237408  0.33840595 -0.1918047526 -0.17006321  0.31293666
## il4_log  -0.10360431  0.54095569  0.0441812211  0.08819677 -0.11170763
## il6_log  -0.32569568  0.09756884 -0.1106483744 -0.15982149  0.18772286
## il8_log  -0.22129678  0.31835323  0.0003030546  0.18112437 -0.56675598
## il10_log -0.33037873  0.32270933 -0.0694459750  0.04532924  0.12368004
## no       -0.10726339 -0.26839630 -0.1720219851  0.48602838 -0.44574763
## tac      -0.03130027 -0.26002605 -0.6038220950 -0.36356803 -0.05836458
## neo_log  -0.32983785 -0.07151318 -0.1283663730  0.18576855  0.15486235
## mda      -0.06226482  0.04818823  0.5713826131 -0.49207066 -0.27322103
## gua      -0.16314608 -0.17137049  0.3814251352  0.45171296  0.44811529
## tnfa     -0.39634227  0.03978990 -0.1259997327  0.04566925 -0.07477140
## tnfr1    -0.40949698 -0.31640211  0.1592836526 -0.16287354 -0.06381216
## tnfr2    -0.44617173 -0.31573857  0.1425202025 -0.15871136 -0.08150807
##                PC6         PC7         PC8        PC9         PC10
## il1b_log -0.35861360  0.042905726 -0.308237681 -0.59608889  0.2214152112
## il4_log  -0.02357512 -0.370146578  0.530768456 -0.07054489 -0.0989875811
## il6_log  -0.25241514 -0.272734851 -0.437699506  0.61798213 -0.2065020962
## il8_log   0.10613762  0.117140493 -0.333318702 -0.19764168 -0.4987647099
## il10_log -0.15069666  0.231873066  0.366625147  0.25866029  0.2466879512
## no       -0.53560792  0.008203675 -0.023070456  0.04215581  0.3588910390
## tac      -0.20379123  0.266688902  0.323039913 -0.08792368 -0.4177366499
## neo_log   0.47650887  0.299973300 -0.152421045 -0.10906983  0.2029411942
## mda      -0.23886915  0.445389249 -0.004088298  0.05990805  0.1088405267
## gua      -0.27820460  0.234273054  0.130265792 -0.09399719 -0.4779143390
```

```
## tnfa      0.27071370  0.274042292  0.094708821  0.23328851  0.0577317893
## tnfr1      0.01558136 -0.435573010  0.035363026 -0.20341213  0.0004358371
## tnfr2      0.09848886 -0.200066423  0.175649547 -0.14900932  0.0289676759
##                PC11        PC12         PC13
## il1b_log -0.09740801 -0.17450137 -0.051482802
## il4_log   0.42098130 -0.25116214 -0.007849243
## il6_log   0.22273706 -0.03022824 -0.081410574
## il8_log  -0.13643730  0.22969907 -0.019151202
## il10_log -0.28055069  0.58487939  0.071365707
```

```
## no        0.15616142 -0.09694174 -0.010607842
## tac       0.16772561  0.02235838  0.057815319
## neo_log   0.62697868  0.14669601  0.056057363
## mda       0.26080887 -0.08839994  0.061820834
## gua       0.01454932 -0.08077259  0.021310489
## tnfa     -0.37200235 -0.66870522  0.130818358
## tnfr1     -0.07132442  0.11889415  0.651470661
## tnfr2     -0.09499683  0.06040670 -0.729931102
```

Figure E1. Proportion of variance in OS/inflammatory markers explained by principal components (top left) and their eigen values (top right). Spatial depiction in bivariate space of the markers contributing to different principal components (lower figure).



Sensitivity analyses

In addition to the analyses presented above and in the main article, a number of sensitivity analyses were conducted to test the robustness of these results. First, the same PCA analyses were conducted with a different number of principal components ranging from 5 to 7 to determine whether the choice of the number of principal component might have influenced the results. None of these analyses produced substantially different results but they involved increased complexity (i.e. due to more components across which the variance in biomarkers was spread) and decreased ease of interpretation. Consequently, the 4 PC model was retained.

Secondly, to further confirm the robustness of the PCA results, the main analyses were repeated using only cases with complete data (n=225). As shown in Figure E2 the principal components structure remained generally unchanged although contributions from significant markers were marginally different.

Figure E2. Main oxidative stress and inflammatory markers' contribution to each of the four significant principal components (PC1-PC4) identified in the PCA analysis using only complete cases (left), and imputed data (right). Red line represents the significance threshold.

Associations between principal components and outcome measures were also consistent between complete cases and imputed data analyses as shown in Table E2. Note that given the sample size was about halved when incomplete cases were excluded, it is remarkable that associations remained so consistent between the analyses.

Table E2. Associations between principal components of oxidative stress and inflammation, and MCI and cognitive decline (MMSE change in previous 12 years) in the whole sample and MCI or normal sub-samples using only complete cases (left), and imputed data (right).

	PCA Components Complete Cases					PCA Components Imputed				
	MCI	MMSE Change				MCI	MMSE Change			
		Whole	MCI	Normal			Whole	MCI	Normal	
PC1	0.206	0.048	-0.232	0.133**	PC1	0.03	0.026	-0.145	0.076*	
	p = 0.084	p = 0.326	p = 0.120	p = 0.006		p = 0.694	p = 0.464	p = 0.144	p = 0.026	
PC2	0.131	-0.073	-0.239	-0.017	PC2	0.121	-0.120*	-0.296	-0.057	
	p = 0.378	p = 0.265	p = 0.204	p = 0.783		p = 0.275	p = 0.016	p = 0.052	p = 0.219	
PC3	1.048**	-0.175*	-0.730*	0.030	PC3	0.567**	-0.171**	-0.432*	-0.027	
	p = 0.00001	p = 0.029	p = 0.025	p = 0.703		p = 0.00003	p = 0.004	p = 0.024	p = 0.616	
PC4	-0.534**	0.030	0.131	0.069	PC4	0.371**	-0.038	0.011	-0.052	
	p = 0.008	p = 0.719	p = 0.637	p = 0.395		p = 0.008	p = 0.539	p = 0.953	p = 0.393	
Constant	-2.217	10.976*	5.492	14.003**	Constant	-0.124	16.725**	15.526	19.569**	
	p = 0.819	p = 0.033	p = 0.667	p = 0.009		p = 0.987	p = 0.00001	p = 0.096	p = 0.00000	
Observations	225	225	46	179	Observations	380	380	81	299	
Log Likelihood	-89.948	-375.638	-84.916	-268.083	Log Likelihood	-176.37	-611.226	-146.309	-427.195	
Akaike Inf. Crit.	195.897	769.276	187.832	554.167	Akaike Inf. Crit.	368.74	1,240.45	310.619	872.391	
Note:	p<0.05; p<0.01									

Finally, to further confirm the stability of results a different approach to data reduction was tested. This involved categorising participants based on whether they were high or low on measures of inflammation and oxidative stress. All markers were z-scored, and reversed where warranted to reflect consistent effects (higher inflammation or higher OS). An average of these measures was computed separately for the inflammatory and OS markers and partitioned into high and low inflammation and high and low OS. Each participant was then categorised into one of four groups: low inflammation-low OS (LILO, reference), low inflammation-high OS (LIHO), high inflammation-low OS (HILO), or high inflammation and high OS (HIHO). Similar analyses as those conducted above were repeated to test associations between groups, cognitive decline and HC atrophy. They revealed that while most associations were weaker and did not reach significance, they supported a similar pattern of association. Moreover, the HIHO group was found to have a significantly greater decrease in MMSE and a higher risk of MCI (trend) than the reference group (LILO; results not shown).

Limitations

This study had a number of limitations but also several strengths. As this study tested associations it cannot demonstrate causal links. While it investigated a large number of OS/inflammatory markers several others were not investigated. Moreover, systemic blood markers were investigated and the extent to which their levels apply to the central nervous system is hotly debated. However, past research has shown that systemic markers can cross the blood-brain barrier and impact on cerebral health[22]. Particular strengths were the large sample investigated, the investigation of brain and cognitive outcome measures, and the robust statistical methods used.