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# γH2AX is increased in peripheral blood lymphocytes of Alzheimer's disease patients in the South Australian Neurodegeneration, Nutrition and DNA Damage (SAND) study of aging

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ABSTRACT

An early cellular response to DNA double-strand breaks is the phosphorylation of histone H2AX to form γH2AX. Although increased levels of γH2AX have been reported in neuronal nuclei of Alzheimer's disease (AD) patients, γH2AX responses in the lymphocytes of individuals with mild cognitive impairment (MCI) and AD remain unexplored. In this study, the endogenous γH2AX level was measured, using laser scanning cytometry (LSC) and visual scoring, in lymphocyte nuclei from MCI (n = 18), or AD (n = 20) patients and healthy controls (n = 40). Levels were significantly elevated in nuclei of the AD group compared to the MCI and control groups, and there was a concomitant increase, with a significant trend, from the control group through MCI to the AD group. A significant negative correlation was seen between γH2AX and the mini mental state examination (MMSE) score, when the analysis included all subjects. Receiver Operation Characteristic curves were carried out for different γH2AX parameters; visually scored percent cells containing overlapping γH2AX foci displayed the best area under the curve value of 0.9081 with 85% sensitivity and 92% specificity for the identification of AD patients versus control. Plasma homocysteine, creatinine, and chitinase-3-like protein 1 (CHI3L1) were positively correlated with lymphocyte γH2AX signals, while glomerular filtration rate (GFR) was negatively correlated. Finally, there was a diminished γH2AX response to X-rays in lymphocytes of the MCI and AD groups compared to the control group. Our results indicate that lymphocyte γH2AX levels are a potential marker for identifying individuals at increased risk of developing AD. Prospective studies with normal healthy individuals are needed to test whether there is indeed a link between γH2AX levels and AD risk.

## 1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterised clinically by abnormal behavioural and mental effects that include loss of memory, tremors, and absent-mindedness, and is the most common cause of dementia [1–3]. The brains of patients with AD are histopathologically characterised by two hallmark lesions: deposition of amyloid-β (Aβ) plaques and the development of neurofibrillary tangles composed of hyperphosphorylated protein tau [4]. AD has reached a global prevalence of approximately 24.3 million, with 4.6 million new cases diagnosed worldwide each year [5,6]. The increase in prevalence of AD not only reduces the quality of life of those affected but also causes a significant financial burden [7].

The onset of AD involves increasingly severe cognitive deficits, progressing from mild cognitive impairment (MCI) to AD. MCI is characterised by deterioration in cognitive ability which, however, does not affect the individual's ability to carry out the activities of daily life. Individuals affected by MCI have a higher risk of developing AD with advancing age, with 14–18% of those over 70 suffering from this condition [8,9]. Our ability to detect the early stages of AD and to differentiate the stages of AD progression, to guide the choice of therapy, is limited. The Mini Mental State Examination (MMSE) is a validated research-based set of 30 questions considering memory loss, cognitive decline, visuospatial and language impairment that is currently used as a standard tool for the clinical diagnosis of AD [10,11]. However, this test lacks accuracy for the diagnosis of AD in living subjects, and

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diagnostic confirmation can only be achieved post-mortem by examination of the senile plaques and neurofibrillary tangles in brain tissue from the patient [12]. Identification of biomarkers in easily accessible tissue, to identify individuals at increased risk of AD while they are still in the early stages of illness, is a challenge for the scientific community.

Most methods for the investigation of AD are invasive, expensive, and cannot identify biomarkers [13–15]. Successful population-based screening requires readily available, minimally invasive, and inexpensive samples for a robust, low-cost diagnostic test with high specificity and sensitivity. To detect amyloid-β protein aggregation forming senile plaques in specific regions of the brain, the Pittsburgh B (PiB) compound was used and found to be able to detect these plaques readily [16,17]. Diffusion MRI and lipid biomarkers have been used as potential diagnostic and prognostic tools for AD classification [18,19]. Although formation of plaques containing Aβ peptides is a hallmark of AD [4], these have also previously been detected in non-neural tissues, such as blood, saliva, skin and other peripheral tissues [20–24], suggesting that abnormalities in Aβ processing may be exhibited in peripheral tissues other than the brain. Several studies have reported abnormalities in platelets, red blood cells, and white blood cells due to AD pathology [25–27].

Previous studies have shown loss of genome integrity due to increased DNA damage levels in neurodegenerative disease [28–32]. Furthermore, several studies have reported increased levels of DNA damage in conjunction with elevated oxidative stress and a lack of DNA repair capacity in the peripheral lymphocytes of AD individuals, compared to age-matched controls [32,33]. Phosphorylation of the C-terminal tails of the H2AX histones in the nucleosomes located in the vicinity of the break [34,35] is one of the earliest known responses to DNA double-strand break (DSB) formation in cells. Induction of DSB in live cells triggers the phosphorylation of Ser139 in the SQ motif near the carboxy-terminus of H2AX, resulting in the formation of phosphorylated H2AX, termed γH2AX [36,37]. While H2AX is distributed uniformly throughout chromatin, only H2AX molecules located close to DSBs become phosphorylated to form γH2AX [34,35,38].

The association of astrocyte degeneration and DNA damage with Alzheimer's disease has been elucidated by investigating the γH2AX signal in astrocytes from the hippocampal region (known to be the most vulnerable region of the brain in AD). γH2AX staining is stronger in the nuclei of astrocytes from AD patients compared to healthy controls, as determined by immunocytochemical techniques [39]. This suggests that DSB measured by γH2AX-positive immunostaining in the nuclei of astrocytes may be associated with impaired neuronal function and contribute to the pathogenesis of AD [39]. Additionally, a recent study reported higher γH2AX levels in hippocampal tissue of individuals with both AD pathology and clinical dementia than was seen in a normal ageing group [40].

Growing evidence shows that high blood pressure, midlife obesity, stroke, and Type 2 diabetes are associated with risk of developing AD [41–45]. Few studies have investigated endogenous γH2AX levels in normal ageing and accelerated ageing disorders. H2AX phosphorylation and the DNA damage response (DDR) have been implicated in diseases of accelerated ageing (e.g., Werner syndrome, AD, obesity, diabetes, sleep apnea, prostate cancer, cataract disease, hypertension and Hutchinson–Gilford progeria syndrome) in recent studies [39,46,47], suggesting that lack of DNA integrity due to accumulating DNA damage progressively increases with age and may contribute to, or be caused by, these accelerated ageing disorders. Overall, these studies show that accumulation of γH2AX foci is increased in individuals with greater morbidity and pathological ageing. This led to the hypothesis that individuals with MCI and AD may exhibit increased levels of γH2AX compared to healthy controls.

Identification of susceptibility to DSB of lymphocytes after exposure to ionising radiation may provide valuable information about the risk of developing diseases. A previous study reported that lymphocytes from bladder cancer patients are more susceptible to DSB (measured using the γH2AX assay) than are controls [48]. Another study, using a mouse model, reported lower induction of DNA damage responses (e.g., γH2AX foci and ATM protein levels) in old mice than in young and mature mice, suggesting inefficient DNA damage recognition or a defect in the recruitment and function of DNA repair machineries [49]. In a study of obesity in children, γH2AX induction was higher in lymphocytes of obese subjects than in overweight subjects, after treating the lymphocytes with the radiomimetic mutagen bleomycin [50]. We hypothesize that lymphocytes from MCI and AD groups exhibit higher levels of endogenous γH2AX. We also hypothesize that lymphocytes from MCI and AD groups do not respond to radiation-induced damage as efficiently as the control group.

To test these hypotheses, (i) the endogenous levels of γH2AX in lymphocytes from participants in the South Australian Neurodegeneration Nutrition and DNA Damage study (SAND) were assessed to determine whether they could be used for identifying those at risk of developing AD; and (ii) radiation-induced γH2AX levels in control, MCI, and AD groups were assessed. This is the first single-cohort study correlating H2AX phosphorylation with risk of developing AD. This was done by (i) visual scoring of γH2AX foci in lymphocytes from control, MCI, and AD patients; and (ii) developing and using an automated laser scanning cytometry (LSC) γH2AX protocol in which multiple γH2AX parameters (area, integral, MaxPixel), as well as the ploidy, were measured in thousands of lymphocytes, to identify whether increased levels of γH2AX were associated with those who were diagnosed with MCI or AD, as compared to healthy age- and gender-matched controls.

## 2. Results

### 2.1. Clinical characteristics of participants

The mean age, gender, and MMSE score of the SAND participants in the control, MCI, and AD groups are shown in Table 1. Participants were classified as control, MCI or AD on the basis of the MMSE score.

### 2.2. Scoring γH2AX signals in lymphocytes by LSC

To investigate whether the endogenous γH2AX level is significantly increased in AD compared to control, γH2AX protein was measured in lymphocytes from control, MCI and AD cases by immunofluorescence. LSC measured multiple γH2AX parameters within each nucleus, including the total γH2AX integral (a function of γH2AX intensity and size), γH2AX MaxPixel (the value of the most intense γH2AX signal/pixel within each nucleus), γH2AX area, and the number of γH2AX events (foci) per cell in all nuclei and/or in cells with different DNA content (ploidy status) and senescent cells.

#### 2.2.1. γH2AX results using all nuclear types

Fig. 2 summarises the one-way ANOVA results for the different

**Table 1**
Clinical characteristics.

| | Control | MCI | AD |
|---|---|---|---|
| Sex (M:F) | 12:28 | 6:12 | 5:15 |
| Age (years) | | | |
| | 75.75 ± 1.575 (72.57–78.93) | 74.60 ± 1.955 (78.69–70.51) | 76.85 ± 2.450 (71.72–81.98) |
| MMSE score | | | |
| | 28.60 ± 0.211 (28.17–29.03) | 26.28 ± 0.559 (25.10–27.46) | 21.00 ± 0.8645 (19.19–22.81) |

Means, standard error of the mean (SEM) are reported for each group. Abbreviations: AD, Alzheimer's disease; F, Female; M, Male; MCI, Mild cognitive impairment; MMSE, Mini Mental State Examination Score.