1-1-2014

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10.2174/1567205011666140618103827
Francois M., Leifert W., Martins R., Thomas P., & Fenech M. (2014). Biomarkers of Alzheimer's disease risk in peripheral tissues; focus on buccal cells. Current Alzheimer Research, 11(6), 519-531. The published manuscript is Available at EurekaSelect here

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Running Title:

Peripheral Biomarkers of Alzheimer's Disease

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Abstract

Alzheimer's disease (AD) is a progressive degenerative disorder of the brain and is the most common form of dementia. To-date no simple, inexpensive and minimally invasive procedure is available to confirm with certainty the early diagnosis of AD prior to the manifestations of symptoms characteristic of the disease. Therefore, if population screening of individuals is to be performed, more suitable, easily accessible tissues would need to be used for a diagnostic test that would identify those who exhibit cellular pathology indicative of mild cognitive impairment (MCI) and AD risk so that they can be prioritized for primary prevention. This need for minimally invasive tests could be achieved by targeting surrogate tissues, since it is now well recognized that AD is not only a disorder restricted to pathology and biomarkers within the brain. Human buccal cells for instance are accessible in a minimally invasive manner, and exhibit cytological and nuclear morphologies that may be indicative of accelerated ageing or neurodegenerative disorders such as AD. However, to our knowledge there is no review available in the literature covering the biology of buccal cells and their applications in AD biomarker research. Therefore, the aim of this review is to summarize some of the main findings of biomarkers reported for AD in peripheral tissues, with a further focus on the rationale for the use of the buccal mucosa (BM) for biomarkers of AD and the evidence to date of changes exhibited in buccal cells with AD.

Keywords

Alzheimer's disease, peripheral biomarkers, buccal mucosa, mild cognitive impairment, diagnosis.

1. Need for predictive biomarkers of AD

Alzheimer's disease (AD) is the sixth leading cause of death in the United States [1] and the most common form of dementia. AD patients have been reported with cognitive impairment characterized by impaired ability to register new information, reasoning, visuospatial abilities and language functions. AD patients also exhibit behavioural symptoms such as for instance, mood fluctuations, apathy, compulsive or obsessive behaviours and loss of interest, often correlated with loss of cognitive functions [2-5]. Previously, clinical diagnosis of AD were based upon criteria outlined by the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA), published in 1984 including memory impairments, visuospatial and language impairment (aphasia) as measured by the Mini-Mental State Examination (MMSE) [6]. These criteria were recently revised by the NINCDS-ADRDA to incorporate biomarkers of brain amyloid-beta (cerebrospinal fluid (CSF) Amyloid-β 1-42, positive positron emission tomography (PET) amyloid imaging) and downstream neuronal degeneration (CSF Tau, magnetic resonance imaging of brain atrophy, PET imaging of fluorodeoxyglucose uptake) in the diagnosis of AD [5]. Although NINCDS-ADRDA does not encourage the use of such biomarkers within tests for routine diagnostic purposes, they can and should be used to increase certainty of diagnostic in research and clinical trials. However, the current suite of tests used in clinical diagnosis can only provide a possible or probable diagnostic of AD in living subjects and the definitive diagnostic can only be made during post-mortem. This is achieved by the observation of the extracellular senile plaques and intracellular neurofibrillary tangles in the specific areas of the brain such as the entorhinal cortex and hippocampus [7,8]. The number of new AD cases is dramatically increasing with an estimated 81.1 million people worldwide being affected by dementia by 2040 [9] and since the pathogenic processes of AD are likely to begin years before clinical symptoms are observed, the need of predictive biomarkers has become urgent. Moreover

AD does not only alter the quality of life, health and wellbeing of those affected but also leads to a significant social financial burden [10,11].

2. Peripheral tissue as source for AD biomarkers

A biomarker, as defined by the National Institutes of Health Biomarkers Definitions Working Group, is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [12]. A potential biomarker should be useful for detecting early stages of a disease and exhibit high levels of sensitivity and specificity. The scientific community has been actively investigating potential early biomarkers of AD. Currently, the majority of investigators have used blood, CSF or brain imaging. In terms of direct brain imaging, Pittsburgh B (PiB) compound was used and shown to be able to readily detect amyloid-β (Aβ) protein aggregation forming senile plaques in specific regions of the brain, however it has been shown in some case reports that the accumulation of large plaques are necessary for PiB imaging to be useful [13,14]. Additionally, CSF has been used to identify changes in $A\beta_{42}$ and Tau protein levels [15,16]. However, these methods of investigations are not ideal for screening populations since they are either too invasive and/or expensive [15,17,18]. Therefore, if screening of populations of individuals for the early detection of AD is to be performed, more suitable, easily accessible tissues need to be utilized introducing diagnostic tests at much lower costs together with high specificity and sensitivity. This need for minimally invasive tests could be achieved by targeting surrogate tissues reflecting systemic susceptibility as recent evidence indicates that AD is a disorder that is not completely restricted to pathology and biomarkers within the brain, but significant biological changes also appear in non-neural tissues such as fibroblasts, blood and buccal cells [19-23] and is summarized in Table 1.

2.1. Fibroblasts

The plausibility that AD risk is reflected in cellular biomarkers in peripheral tissue has been investigated by studying well-known markers of genomic instability that have been reported to increase with age, and therefore suggest that the capacity for repair of DNA damage may also be altered in AD [24-26]. Micronuclei (MN) are a well validated and robust biomarker of whole chromosome loss and/or breakage that originate from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division and have been shown to be predictive of increased cancer risk, cardiovascular mortality and have been found to be elevated in neurodegenerative disorders [27-30]. In fibroblasts for example, MN frequency has been shown to be increased with advancing age [31] as well as in AD [32]. Down's syndrome is also considered a premature ageing syndrome with a high rate of conversion to dementia and is associated with abnormally high levels of DNA damage [33,34]. Furthermore, Down's syndrome (trisomy 21) patients express brain changes by their 4^{th} decade of life that are histopathologically indistinguishable from AD [35]. As the amyloid-β protein precursor (AβPP) gene is encoded on chromosome 21 [36], it has been suggested that one of the underlying mechanisms of AD could be the altered gene dosage and subsequent expression of AβPP, leading to accumulation of the aggregating form of Aβ peptide following proteolysis. Peripheral tissue such as skin fibroblasts from familial and sporadic AD has been shown to exhibit a 2-fold increase in the number of trisomy 21 cells when compared to controls [35]. Moreover, an increase in immunostaining of amyloid peptides ($A\beta_{40}$, $A\beta_{42}$) as well as an imbalance between free cholesterol and cholesterol ester pools has been observed in fibroblasts of AD [37]. The capacity of fibroblasts to spread in culture was also observed to be altered in AD with a decrease of cytosolic free calcium ($p<0.001$) [38]. Furthermore an increase of total bound calcium in fibroblasts was observed when compared to age-matched controls [39].

2.2. Olfactory epithelium

Anosmia or olfactory dysfunction resulting in loss of smell is common in neurodegenerative diseases such as Parkinson's or AD and may appear as one of the early symptoms. Furthermore, olfactory dysfunction has been found to be commonly associated with memory deficiency in transgenic mouse models of AD [40,41]. In humans, the olfactory epithelium was shown to be a peripheral tissue that exhibited increased oxidative damage in AD. HNE-pyrrole (a product of lipid oxidation) and heme oxygenase-1 (a catalytic enzyme involved in degradation of heme) levels were found to be increased in neurons and epithelial cells from olfactory biopsy sections in AD compared to healthy controls ($p<0.002$ and $p<0.0001$, respectively), thus confirming the presence of oxidative damage at a peripheral level in AD [42]. Increased levels of Aβ and hyperphosphorylated Tau were also observed in the olfactory epithelium in AD [21]. Detection was performed by immunohistochemistry and a significant increase in frequency of both Aβ ($p<0.001$) and hyperphosphorylated Tau ($p<0.05$) was observed when compared to controls [21]. Post-mortem neuropathological examination of participants' brains were also undertaken and a significant correlation ($r = 0.37$, $p<0.001$) was found between Aβ plaque frequency in olfactory epithelium and averaged Aβ frequency in multiple cortical regions (i.e. hippocampus, entorhinal cortex, amygdala, superior/middle temporal gyri, angular gyrus, mid-frontal gyrus, and anterior cingulate cortex) [21]. Additionally, there was a significant correlation found between hyperphosphorylated Tau in olfactory epithelium and hyperphosphorylated Tau in brains ($p<0.05$) [21]. Therefore, the presence of Aβ and Tau immunostaining could also be investigated in peripheral tissue such as olfactory epithelium for potential early AD biomarkers.

2.3. Whole blood

Since blood can be sampled easily and may reflect pathological changes in AD, it is not surprising that this tissue has been commonly investigated as a source for AD biomarkers [43-45]. For instance, following completion of a genome-wide association study (Alzheimer's Disease Neuroimaging Initiative) [46], TOMM40 (translocase of outer mitochondrial membrane 40) was found to be a potential gene associated with AD (TOMM40 risk alleles were two times more frequent than in controls) and therefore an additional risk for developing AD [46]. The expression of this gene has been found to be significantly down-regulated in blood from AD compared to controls [44]. Another study, the Australian, Imaging, Biomarkers and Lifestyle study (AIBL) observed lower levels of red blood cell folate in AD patients compared to healthy controls ($p=0.004$), albeit serum folate did not show significant differences [47]. A recent study conducted by Leidinger et al. identified 140 differentially expressed microRNAs (mi-RNAs), non coding RNAs that play key roles in the regulation of gene expression, in blood of Alzheimer's patients when compared to controls and further validated a 12-miRNAs signature of AD [48]. Using this newly developed signature, AD patients were separated from the control group with 95.1% specificity and 91.5% sensitivity. Additionally, this signature presented a separation of MCI versus control with 81.1% specificity and 87.7% sensitivity [48]. Although these studies on whole blood samples have shown interesting results, studies on blood components (i.e. white blood cells, platelets and plasma) have also brought to light several promising findings as discussed below.

2.4. White blood cells

Tau protein, one of the main proteins known to be associated with AD interacts with microtubules, actin filaments and intermediate filaments to play a key role in regulating the organisation and integrity of the cytoskeleton [49]. An increase in the phosphorylation levels of Tau was reported to occur due to the compromised function of protein phosphatase 2A in

AD brains [50,51]. Tau protein was shown to be elevated in CSF of AD patients and is an accepted biological marker of AD [15,16]. In lymphocytes, both phosphorylated and non phosphorylated forms of Tau were detected by Western blot and shown to be significantly increased in AD compared to controls (approximately 2-fold increase), with a direct correlation between phosphorylated Tau and disease duration [52]. Another protein, chitotriosidase (chitinase) a chitinolytic enzyme secreted by activated mononucleated cells that has previously been shown to exhibit a higher activity in CSF in AD [53,54], also showed a significantly increased level of expression (19-fold) in macrophages [55]. Evidence of the nuclear accumulation of γH2AX, a protein that becomes phosphorylated following induction of DNA double strand breaks, has been observed in astrocytes of AD brains [56]. Peripheral DNA damage, including single and double strand breaks, has been shown to increase in leukocytes of MCI and AD when compared to controls ($p<0.001$) [57]. Individuals with MCI have also been used to study biomarkers of AD since this group shows an approximate 50% of conversion into AD over 4 years [58] and it is interesting to note that the level of primary DNA damage is lower, although not significant, in AD compared with MCI [57]. This is suggestive that this type of DNA damage decreases as the disease progresses further. Oxidative stress which results in the accumulation of oxidized DNA base adduct 8-hydroxy-2deoxyguanosine (8-OHdG) is also believed to be involved in a number of neurodegenerative diseases [59-61] and has been shown to occur prior to the pathology hallmarks of AD [62]. An approximate 5-fold increase in 8-OHdG was observed in CSF of AD compared with controls ($p<0.001$) and may partly explain the DNA damage that has been observed in AD cases [63]. The comet assay, which can be used to assess both single and double strand breaks in DNA, has also been utilized after enzyme treatment to demonstrate that peripheral leukocytes exhibit a significant increase in oxidative DNA damage markers i.e. oxidized DNA pyrimidines and purines in MCI and AD with respect to controls ($p<0.002$ and $p<0.001$, respectively) [57]. More evidence has come from genomic instability markers

such as MN which were shown to increase in frequency in lymphocytes with age [64] and AD when compared to healthy controls [22,65,66].

Another marker of genetic instability, telomere length, is known to change with ageing and in some cell types involves progressive telomere shortening. Telomeres are highly conserved DNA sequence repeats (of TTAGGG) involved in the maintenance of genome stability. Telomere length can be assessed by a variety of methods including southern blot, flow cytometry, quantitative fluorescence *in situ* hybridisation (FISH) or by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) [67-70]. Shortened telomeres in blood have been shown to be associated with an increased risk of cardiovascular disease and degenerative disease such as cancers [71-73]. Telomere length has also been investigated in white blood cells of confirmed AD cases and found to be significantly shorter in those of AD patients compared with young and old controls ($p<0.0001$) [19]. Studies have shown a decrease in telomere length in lymphocytes isolated from AD that was correlated ($r = -0.77$) with a decrease in the MMSE scores indicating a possible link between telomere length and cognitive decline in AD [74].

Lymphocytes from AD cases or first degree relatives also show substantial differences relative to controls with respect to intracellular lipid pods [75]. Oil Red O (ORO) staining (indicative of accumulation of neutral lipids) has been used to demonstrate higher levels of neutral lipids in peripheral blood mononuclear cells of probable AD patients [75]. The study by Pani et al. 2009 demonstrated that approximately 85% of isolated lymphocytes from AD had high neutral lipids levels (mainly cholesterol ester) as well as an increased content of the Acetyl-Coenzyme A acetyltransferase-1 protein (the enzyme that catalyses the formation of cholesterol esters in cells) compared with cognitively normal age-matched controls. These

240 data suggest that intracellular cholesterol ester levels are systemically increased in AD
241 patients and support the hypothesis of altered lipid metabolism in AD.
242
243 AD pathology has also been linked to proteins that are involved in maintaining the cell-cycle.
244 For example hyperphosphorylated Tau is linked to the activity of cyclin-dependent protein
245 kinases [76,77]; AβPP metabolism is monitored by cell-cycle dependent changes and is also
246 up-regulated by mitogenic stimulation [78-80]; and finally Aβ (a product of AβPP processing)
247 has been identified as mitogenic in *in vitro* studies [81,82]. A recent study using lymphocytes
248 from AD patients demonstrated the potential of G1/S checkpoint proteins as biomarkers of
249 AD. In that study, increased expression of Cyclin E, Rb, CDK2 and E2F-1 was observed and
250 gave specificity/sensitivity scores of 84/81%, 74/89%, 80/78% and 85/85%, respectively [83].
251 These studies suggest that altered cell-cycle mechanisms may be indirectly involved in the
252 process of AD onset and development.
253
254 *2.5. Platelets*
255 Platelets have also been investigated in AD and found to express changes with the disease
256 state. For instance the ratio of two isoform products of AβPP processing (130kDa/110kDa)
257 that occurs in platelets was studied as a potential biomarker and found to be decreased in
258 platelet membranes in AD and MCI compared with their respective controls [84,85]. The
259 presence of phosphorylated and non phosphorylated Tau protein was detected by
260 immunofluorescence as well as different variant forms of Tau using Western blot
261 techniques. The different immunoreactive fractions of Tau separated by Western were
262 combined to obtain a ratio of high (>80 kDa) and low (<80 kDa) molecular weight bands and
263 when quantified by imaging was found to be significantly increased in AD compared to
264 healthy controls ($p=0.0001$) [23]. The results from this study confirmed that peripheral
265 markers such as platelet Tau isoforms could serve as potential biological markers of AD.

2.6. Plasma

Plasma is obtained with relative ease and has been used widely to identify potential biomarkers of AD. Plasma sampled from AD individuals has previously shown an approximate 4.8-fold increase in chitotriosidase levels when compared to healthy controls ($p<0.001$) [86]. YKL-40, a homolog to chitotriosidase was recently described in early stages of AD with significantly higher protein levels found in CSF ($p<0.0001$) as well as in plasma ($p=0.014$) compared to controls [87,88], and more importantly, presented a strong ability to predict onset and progression of dementia [87]. For instance, it was found that a high YKL-40/$A\beta_{42}$ ratio in CSF demonstrated strong predictive values of a faster cognitive decline, and that levels of YKL-40 significantly correlated ($r = 0.5948$, $p<0.0001$) with levels of phosphorylated Tau in CSF [87]. Analysis of plasma has some advantages as an approach to population-based screening of AD as it is well accepted and less invasive than CSF sampling, for example. A review of longitudinal studies that examined plasma levels of Aβ indicates that higher baseline levels of $A\beta_{40}$ might predict higher risk of conversion towards AD [89] and that higher levels of $A\beta_{42}$ were also associated with a 3-fold increase of AD risk [20]. Importantly, higher level of baseline plasma amyloid in people free of dementia appears to be a predictive marker of a faster cognitive decline in those individuals who converts to AD [90]. An intensive study investigating biomarkers for diagnosis of AD in the Australian Imaging, Biomarkers and Lifestyle study of ageing (AIBL) cohort identified a list of 21 plasma-based biomarkers that showed a significant fold change between AD and healthy controls. The top 10 biomarkers with the most differences ($p<0.0001$) were as follows; insulin like growth factor binding protein 2, pancreatic polypeptide, cortisol, vascular cell adhesion molecule 1, superoxide dismutase, interleukins 10 and 17, albumin, calcium and Zinc (isotope 66) [43]. More recently a study from Mapstone et al. [91] discovered and validated a list of 10 phospholipid fatty acids that were depleted in healthy controls who would

convert to MCI or AD within a 2-3 year timeframe This panel of metabolites was still depleted after conversion and allowed separation of converters from controls that remained cognitively normal with more than 90% accuracy. Importantly, the ROC curve generated in their study showed an area under the curve (AUC) of 0.96 and a specificity and sensitivity of both 90% [91]. The evidence discussed above suggests that AD is a systemic disorder involving a change in a myriad of biological parameters that can be reflected in peripheral tissues.

3. Focus on buccal cells as a peripheral tissue

Buccal mucosa (BM), like the brain and skin epithelium cells, are derived from differentiated ectodermal tissue during embryogenesis and therefore would be a potential surrogate non-neural tissue that may have the potential to reflect the underlying pathological changes observed in AD. Buccal cells have been used as a source of tissue in a variety of biochemical and molecular biology studies using an assortment of different techniques to collect the cells including; cotton swabs [92], cytobrushes [92-94], a “swish and spit” method [95-97], a modified Guthrie card [98] and a method of rubbing cheeks against teeth to exfoliate cells [94]. The results from those studies demonstrated that high quantities of buccal cells (more than a million per sampling) could be obtained and then subsequently used in a variety of assays; such as DNA analysis using PCR or other genotype tests [95,96,99-102], for isolation of mRNA for gene expression profiling, Western blots for detection of proteins and immunocytochemistry [103-105], high-performance liquid chromatography (HPLC) [106] and ion transporter assays [107]. Ideally invasive procedures should be avoided in AD patients due to age and presenting medical issues, therefore buccal cells could offer an appropriate alternative as a relatively non-invasive and easily accessible source of tissue for analysis. Furthermore, buccal cells have been shown to be osmotically stable in hypotonic solutions including water [108] making them more easily processed with less risk of losing intracellular

318 contents during investigation procedures. Additionally, it has been found that buccal cells
319 can be readily preserved during transportation for cytology and immunocytochemistry
320 studies by isolation directly into buccal cell buffer [109]. Therefore it would be possible to
321 isolate buccal cells from patients in remote regions and facilitate storage of samples in
322 laboratories.
323
324 *3.1. Morphological changes in buccal cells*
325 For the BM to be a valuable tissue to study for biomarkers of AD, the BM would need to
326 exhibit changes within the cells that correlate well with the disease state. Structurally, the
327 BM is a stratified squamous epithelium consisting of four distinct layers [110-112] as shown
328 in Figure 1. First the stratum corneum lines the oral cavity. Below this layer, is located the
329 *stratum granulosum*, and the *stratum spinosum* containing populations of differentiated,
330 apoptotic and necrotic cells. The next layer contains the *rete pegs* or *stratum germinativum*
331 composed of basal cells, which, by cell division and DNA replication regenerate and maintain
332 the profile, structure and integrity of the BM [113]. The basal cells are believed to
333 differentiate and migrate to the keratinized surface layer in 7 to 21 days. With normal ageing
334 the efficiency of cell regeneration decreases [112,114] resulting in a thinner epidermis and
335 underlying cell layers [115]. The protective function of the *stratum corneum* is not altered
336 [116] but the *rete pegs* adopts a more flattened appearance [117,118].
337
338 Since buccal cells and the nervous system are derived from the same germ cell layer, the
339 ectoderm, the regenerative potential of BM might be affected in parallel with the
340 regenerative potential of the brain, which is found to be altered in AD [119]. One study
341 investigated the BM's different cell types and its composition in AD compared with age-
342 matched controls by the use of the buccal cytome assay [120]. Frequencies of the various
343 cell types were scored and an alteration of the BM composition was shown to occur in AD. A

significant decrease in the frequency of basal cells, karyorrhectic and condensed chromatin cells ($p<0.0001$) were found in the AD cohort [120] as shown in Figure 2. The odds ratio of being diagnosed with AD for a combined karyorrhectic and basal cell frequency of <41 per 1000 cells was shown to be 140 with a specificity of 96.8% and a sensitivity of 82.4% [120]. This segregation of cell types has also been shown in an automated manner using imaging analysis by laser scanning cytometry (LSC) [121], making this cytome assay more feasible for scoring on a larger study scale. Another study [122], aimed at assessing morphologic and cytometric aspects of cells of the oral mucosa of AD patients using the Papanicolaou staining method [123]. A visual assessment of cell types was made by microscopy and cytological parameters were measured using the Image J analysis software. The results of that study demonstrated a significant reduction in the number of intermediate cells ($p<0.05$) as well as in the nuclear:cytoplasmic area ratio ($p<0.0001$) in the AD group compared to the controls [122]. Both studies suggest that changes occur in the BM of those diagnosed with AD in terms of cytological features and cell type composition which may indicate a decrease in the regenerative capacity of the BM in AD.

3.2. Cytokeratins – Biochemical cell type segregation

The frequency of basal buccal cells as discussed in the previous section was found to be lower in AD, using the buccal cytome assay, which scores cells on morphological features. Therefore, an epithelial cell differentiation marker may allow a more definite and precise identification of basal cells, as compared with visual assessment by the buccal cytome assay. Indeed, buccal cells contain groups of structural proteins called cytokeratins (CK) [124], that are found to be expressed in a tissue specific manner [125,126]. Buccal cells normally express CK 4, 5, 13, 14 and possibly 19 depending on their cell types [125,127]; CK5 and CK14 are predominantly expressed in the basal layer but after a period of differentiation and migration, buccal cells begin expressing CK4 and CK13 accompanied with a progressively

370 reduced expression of CK5 and CK14 [128]. Furthermore, in other epithelial tissues such as
371 the olfactory epithelium, basal cells were shown to express keratin 8 [129]. An example of
372 the differences in cytokeratin immunostaining of buccal cells observed by our group is
373 shown in Figure 3, where some cells were found to be positive for CK5 or CK13, others were
374 both CK5 and CK13 positive, whilst yet another population of buccal cells were negative for
375 CK5 and CK13 (Figure 3). Another study also showed that CK10 and CK8 were detected in
376 low amounts in buccal cells using immunocytochemistry techniques [128]. Interestingly,
377 differential expression of CK proteins, such as CK5, has been observed in carcinomas of the
378 BM [127,130]. For instance, in mucoepidermoid carcinoma there was a strong correlation of
379 high levels of CK5 expression (in oral mucosa) with poorer survival times ($p<0.001$).
380 Specifically, at the completion of that study, 12 (of 13) patients with high levels of CK5
381 expression were deceased, compared with 6 patients out of the 18 patients with the lowest
382 values of CK5 expression [130]. In another study investigating dementia, levels of keratin
383 autoantibodies when quantified by enzyme-linked immunosorbent assay (ELISA) in serum
384 from patients with dementia, including 68% of patients diagnosed with AD, were found to be
385 significantly increased compared to healthy controls ($p<0.05$) [131]. It was speculated that
386 the increase in presentation of the keratin antigen to the immune-competent cells may
387 result from the degenerative process of the brain. Since CK expression has been widely
388 shown to differ in the BM with cell types [125,127], developmental stage [132,133], tissue
389 differentiation [126,134-138] and pathological conditions [139-145], CK proteins could
390 provide information on the proliferation and differentiation status which may be dependent
391 on the disease state. Furthermore CK staining of BM may offer a convenient
392 immunocytochemical manner of identifying cell types which could be scored in a
393 quantitative and automated manner in AD patients using cellular imaging techniques such as
394 laser scanning cytometry.
395

3.3. Buccal cells and Tau

Accumulation of Tau forming neurofibrillary tangles (NFTs) in the brain is one of the main hallmarks of AD and has a major role in neuronal death. Hattori et al. [103] demonstrated the presence of putative multiple isoforms of Tau on Western blots that were the non-phosphorylated form of Tau protein in buccal cells with the prominent appearance of two bands at approximately 65 kDa and 110 kDa, using the monoclonal BT-2 antibody. Using ELISA techniques, total Tau protein was shown to be significantly elevated within buccal cells of AD compared with age-matched controls ($p<0.01$). Furthermore, the increase in Tau of oral epithelium was shown to be significantly correlated with the Tau level in CSF ($r = 0.43$, $p=0.011$) and was also higher in AD subjects when diagnosed at a younger age of onset than with patients at later age of onset [103]. Therefore it is feasible that oral epithelium Tau may be a measurable and useful predictive biomarker of AD in buccal cells; however this unique observation has not been verified yet in other studies and awaits replication.

3.4. Buccal cells and Amyloid

Aβ is the main component of senile plaques appearing in the brains of AD and is generated by the processing of its precursor AβPP. Since AβPP is ubiquitously expressed, it may be involved in stimulation and proliferation of keratinocytes where they are mostly expressed in the basal layer [146]. It is feasible that differences of AβPP expression in the BM could therefore also reveal information regarding the regeneration potential of the BM in AD. The expression of AβPP was shown to be present in the buccal pouch of hamsters and AβPP is believed to promote the development of oral carcinogenesis [147]. The biopsy of oral tissues for instance has been advocated as an alternate method of detecting amyloid deposition in amyloidosis [148] confirming that amyloid can accumulate to detectable levels in peripheral tissue such as the liver in systemic amyloidosis [149]. AβPP has previously been investigated in young adult Wistar rats and localized by immunohistochemistry in several peripheral

422 tissues, i.e. liver, kidney, spleen, pancreas, salivary gland, testis and ovary [150]. Since AβPP
423 is a protein ubiquitously expressed in humans, it is likely that Aβ protein which is processed
424 from AβPP and its' variants (e.g. monomers, dimers, oligomers, etc…) may be a plausible
425 target to be investigated in the BM of AD patients [151]. It is plausible that a genetic or
426 acquired predisposition for amyloidogenic processing of AβPP could be evident not only in
427 the brain but also in epithelial tissues.
428
429 *3.5. Buccal cells and DNA damage*
430 Genomic DNA damage has been shown to be associated with AD as discussed earlier [152].
431 Genomic instability has been reported to increase with age and therefore the capacity for
432 DNA damage repair may also be altered [24-26]. In buccal cells a buccal micronucleus
433 cytome assay was developed by Thomas et al. to score DNA damage, cell death and
434 regenerative potential [120,153]. A Down's syndrome cohort was used as a model for
435 premature ageing and presented a significantly elevated level of MN compared with both
436 the older and younger control groups ($p<0.0001$) [154]. The same buccal micronucleus
437 cytome scoring assay was performed on an Alzheimer's cohort and showed a slightly
438 elevated MN score in the AD group when compared to age-matched controls, but this
439 difference did not reach statistical significance [120]. Genomic changes such as aneuploidy
440 of both chromosomes 17 and 21, containing respectively the genes coding for Tau and AβPP
441 [155,156], has also been investigated in buccal cells. Aneuploidy levels of chromosomes 17
442 and 21 were shown to increase in buccal cells in AD and Down's syndrome compared to
443 their respective controls [157]. Additionally, DNA double strand breaks have been detected
444 in human buccal cells using an immunofluorescent antibody against γH2AX [158], therefore
445 confirming that MN and γH2AX are two important DNA damage biomarkers that can be
446 detected and may be altered in buccal cells from patients with AD. Oxidative stress has also
447 been studied in leukocytes and exfoliated BM using HPLC after DNA isolation [106] and

448 because the association between accumulated oxidative DNA damage and ageing is well
449 documented, it is possible that the BM may show changes in 8-OHdG levels from AD buccal
450 samples; however this has yet to be tested.
451
452 *3.6. Buccal cells and cytological parameters*
453 In a recent study from our group, an automated buccal cell assay was developed using laser
454 scanning cytometry (LSC) to measure buccal cell neutral lipid, nuclear DNA content and
455 nuclear shape from clinically diagnosed AD, MCI patients and age- and gender-matched
456 controls [109]. Findings showed significantly lower levels of neutral lipids in MCI and a
457 significant increase in DNA content in both MCI and AD compared to controls. The ploidy
458 distribution of nuclei was also investigated in this study and showed that the increase in
459 DNA content observed in MCI and AD cases were due to a significant decrease in the
460 proportion of 2N nuclei with a concomitant increase in the proportion of >2N nuclei.
461 Additionally, the LSC automated buccal cell assay developed by our group allowed collection
462 of "circularity" measurements providing information on the shape of buccal cell nuclei. It
463 was found that nuclei had a significantly more irregular shape in MCI and AD when
464 compared to controls [109]. These results suggest that the changes in DNA content are due
465 to hyperdiploid nuclei accumulating with the disease state. ROC curves were also used in this
466 study for each of the parameters analysed and their combination, generating AUC varying
467 from 0.763 to 0.837 [109]. It would therefore be of interest to combine this automated assay
468 with detection of other potential specific protein markers, which may increase the likelihood
469 of better predictive markers for AD.
470
471 *3.7. Buccal cells and telomere length*
472 Absolute telomere length has been investigated in buccal cells of confirmed AD cases and
473 healthy age- and gender-matched controls. A significantly shorter telomere length was

observed in buccal cells of the AD group compared to controls (p=0.01). Additionally, in the same individuals, there was a significant decrease in telomere length in white blood cells (p<0.0001) [19]. However there was no correlation between buccal cell and lymphocyte telomere length. This may be partly due to the differences in turnover rates of cell division in buccal cells compared with lymphocytes. Although the evidence is minimal to-date, buccal cells and lymphocytes appear to exhibit a reduction in telomere length in AD and therefore, suggest that other peripheral tissues inducing BM may also be used to assess reductions in telomere length in AD.

4. Future perspectives

As populations throughout the world continue to age, the prevalence of AD is expected to increase dramatically. By 2050 nearly one million new AD cases per year has been estimated, with this increasing prevalence becoming a global concern threatening to impact heavily on both social and economic levels [10,159-161]. Therefore biomarkers for an early diagnostic of the disease would tremendously benefit the community as treatment strategies would likely to be more effective in preserving brain function if administered early in the disease process prior to the development of symptoms. Evidence that pathologic changes of AD are reflected in peripheral tissues such as fibroblasts, olfactory epithelium, whole blood, platelets, white blood cells and plasma indicates that AD is a systemic disorder and that these tissues should be considered as a useful source for potential biomarkers (see Table 1). However, investigating a minimally invasive tissue such as the BM as a source of biomarkers with high specificity and sensitivity for AD is yet to be achieved. The BM is an easily accessible non neuronal tissue, which offers a simple, painless and non-expensive sampling procedure. Previous findings suggest that the regenerative potential of the buccal mucosa varies and cytological changes occur within buccal cells following the appearance of AD. However there is still little known in this area regarding buccal cell differentiation and

proliferation status. Only a few studies have investigated changes in the oral mucosa in AD investigating cytological parameters, cell type composition, qualification of Tau, MN, DNA content, lipids, telomere length as well as chromosome 17 and 21 aneuploidy (see Table 1) confirming that the BM is a potential tissue for AD diagnostic biomarkers. Therefore, further research must be undertaken in order to obtain a better understanding of the biology of buccal cells, to replicate such studies and investigate other potential markers of AD that might include lipid content, APOE gene expression, AβPP, Aβ, γH2AX, 8-OHdG as well as others. Longitudinal studies could then be undertaken to capture the variation in biomarkers with the progression of the disease and the associated cognitive decline. This review summarizes some of the knowledge gaps in buccal cells as a peripheral tissue for AD diagnostics. If combined with results from other peripheral tissues, new biomarker sets could emerge that may identify individuals who are at increased risk or are at an early stage of AD with much higher certainty. Therefore, investigations involving minimally invasive non-neural tissue for sampling biomarkers cellular origin of MCI/AD risk need to be further investigated.

5. Acknowledgements

Financial support from the CSIRO's Preventative Health Flagship is gratefully acknowledged. This project was part funded by a grant from The JO & JR Wicking Trust, which is managed by ANZ Trustees (Australia).

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1133 **Table 1:** Summary of AD biomarkers altered in peripheral tissues.

1134

Peripheral tissue investigated	Parameters measured and outcome	Reference(s)
Fibroblast	3-fold ↑ MN frequency	[32]
	2-fold ↑ Trisomy 21 levels	[35]
	1.3-fold ↑ Immunostaining of amyloid peptides ($A\beta_{40}$, $A\beta_{42}$) 1.3-fold ↓ β-Secretase 1 6-fold ↑ Rate of cholesterol esterification after 48 h 56% ↑ pool of neutral lipids	[37]
	Altered pattern of spreading in culture 70% ↓ Free calcium content	[38]
	197% ↑ Bound calcium content	[39]
Whole blood	TOMM40 alleles ↑ disease risk by 2	[46]
	10% ↓ Red blood cell folate	[47]
	AD signature of 12 mi-RNAs identified, compared with controls (95% specificity / 91.5% sensitivity)	[48]
White blood cell	31% ↓ Telomere length	[19]
Lymphocyte	↑ Neutral lipid accumulation	[75]
	2-fold ↑ Total Tau	[52]
	↑ MN frequency in chromosomes 13 and 21	[22,65,66]
	1.15-fold ↓ Telomere length correlated with ↓ MMSE scores (r = -0.77)	[74]
	↑ G1/S checkpoint proteins (Cyclin E, Rb, CDK2 and	[83]

	E2F-1)	
Leukocyte	2-fold ↑ Single and double strand breaks combined 2.6-fold ↑ DNA oxidized pyrimidines 2-fold ↑ DNA oxidized purines	[57]
Macrophage	19-fold ↑ Chitotriosidase expression level	[55]
Platelet	2.1-fold ↓ AβPP Isoforms (130kDa/110kDa) ratio in platelet membranes	[84,85]
	6.5-fold ↓ High kDa/Low kDa forms of Tau ratio	[23]
Plasma	↑ Aβ in individuals who further convert to AD	[89]
	↑ $A\beta_{42}$ predicts ↑ AD risk	[20]
	↑ Aβ predicts faster cognitive decline	[90]
	↑ Insulin growth factor binding protein 2, pancreatic polypeptide, cortisol, vascular cell adhesion molecule, superoxide dismutase, interleukin 10 ↓ Albumin, Calcium, Zinc (isotope 66), interleukin 17	[43]
	4.8-fold ↑ Chitotriosidase level	[86]
	3.7-fold ↑ YKL-40 level	[88]
	10 lipids panel predicting conversion to MCI or AD ROC curve AUC value was 0.96	[91]
Nasal cell	3.7-fold ↑ Abundance ratings for Aβ and 1.8-fold ↑ for phosphorylated Tau	[21]
	1.2-fold ↑ HNE-pyrrole and 1.5-fold ↑ Heme oxygenase-1	[42]
Buccal cell	↓ Frequencies of basal, karyorrhectic and condensed chromatin cells	[120]

	1.24-fold ↓ Nuclei/Cytoplasmic size ratio in intermediate cells 1.5-fold ↓ Intermediate cell frequency	[122]
	↑ MN frequency in Down's syndrome	[121,154]
	1.75-fold ↑ Tau correlated (r = 0.43) with ↑ Tau in CSF	[103]
	1.2-fold ↑ Aneuploidy levels of chromosome 17 1.5-fold ↑ Aneuploidy levels of chromosome 21	[157]
	2-fold ↓ Telomere length	[19]
	1.7 fold ↑ and 1.5 fold ↑ DNA content in MCI and AD, respectively 1.5 fold ↓ Neutral lipid content in MCI 1.7 fold ↓ and 1.5 fold ↓ 2N nuclei population in MCI and AD, respectively ↑ irregular nuclear shape	[109]

Abbreviations: Aβ, Amyloid-β; AD, Alzheimer's disease; AβPP, Amyloid-β protein precursor; CSF, Cerebrospinal fluid; mi-RNAs, microRNAs; MMSE, Mini-mental state examination; MN, Micronuclei.

Figure 1.

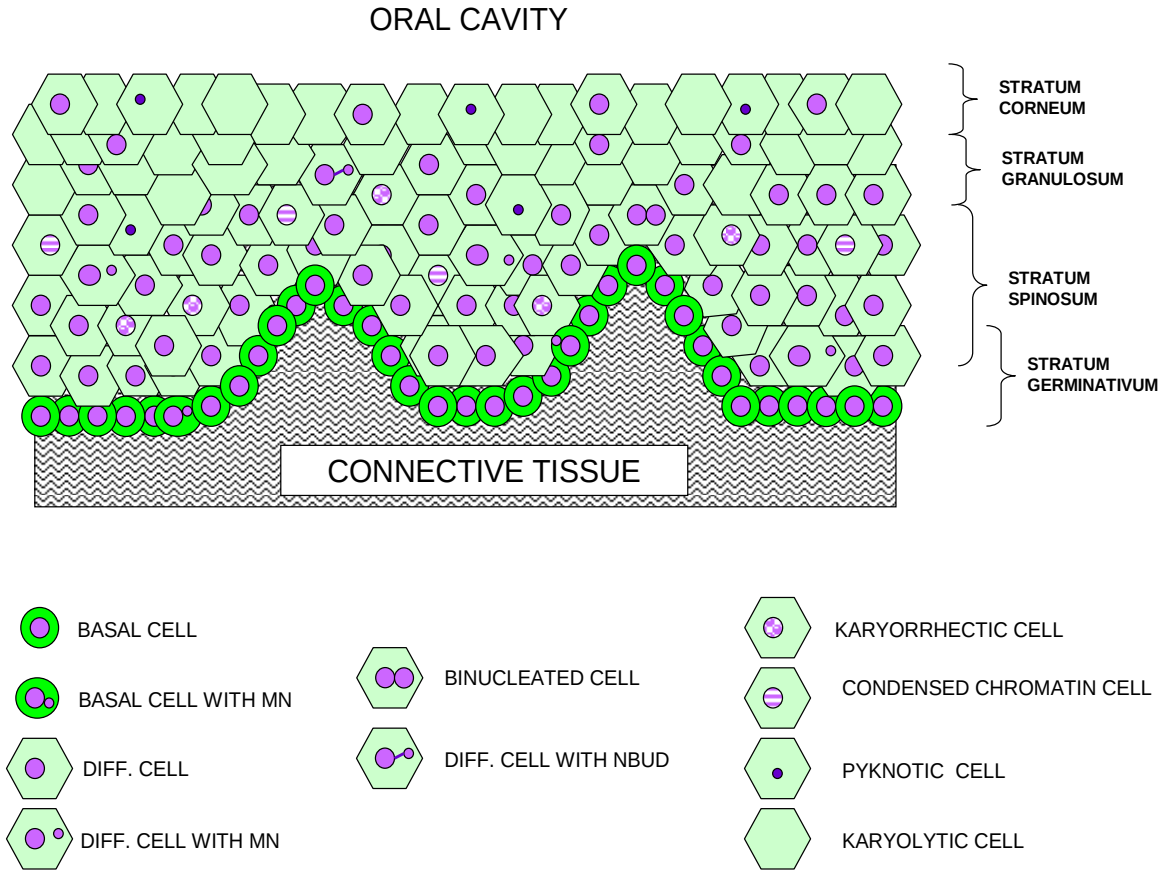
ORAL CAVITY
STRATUM CORNEUM
STRATUM GRANULOSUM
STRATUM SPINOSUM
STRATUM GERMINATIVUM
CONNECTIVE TISSUE
BASAL CELL
BASAL CELL WITH MN
DIFF. CELL
DIFF. CELL WITH MN
BINUCLEATED CELL
DIFF. CELL WITH NBUD
KARYORRHECTIC CELL
CONDENSED CHROMATIN CELL
PYKNOTIC CELL
KARYOLYTIC CELL

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Figure 2.

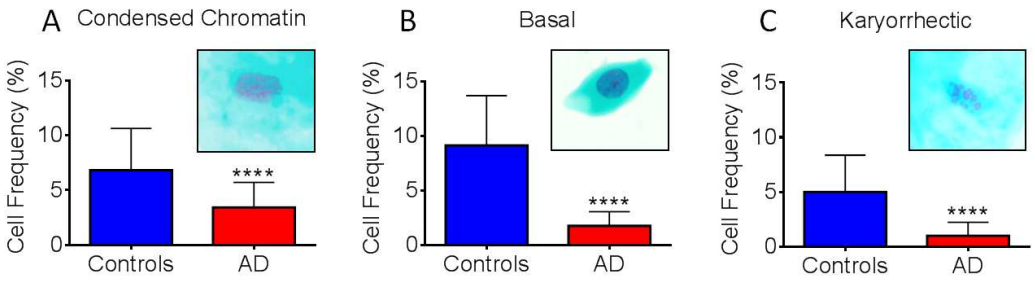

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Figure 3.

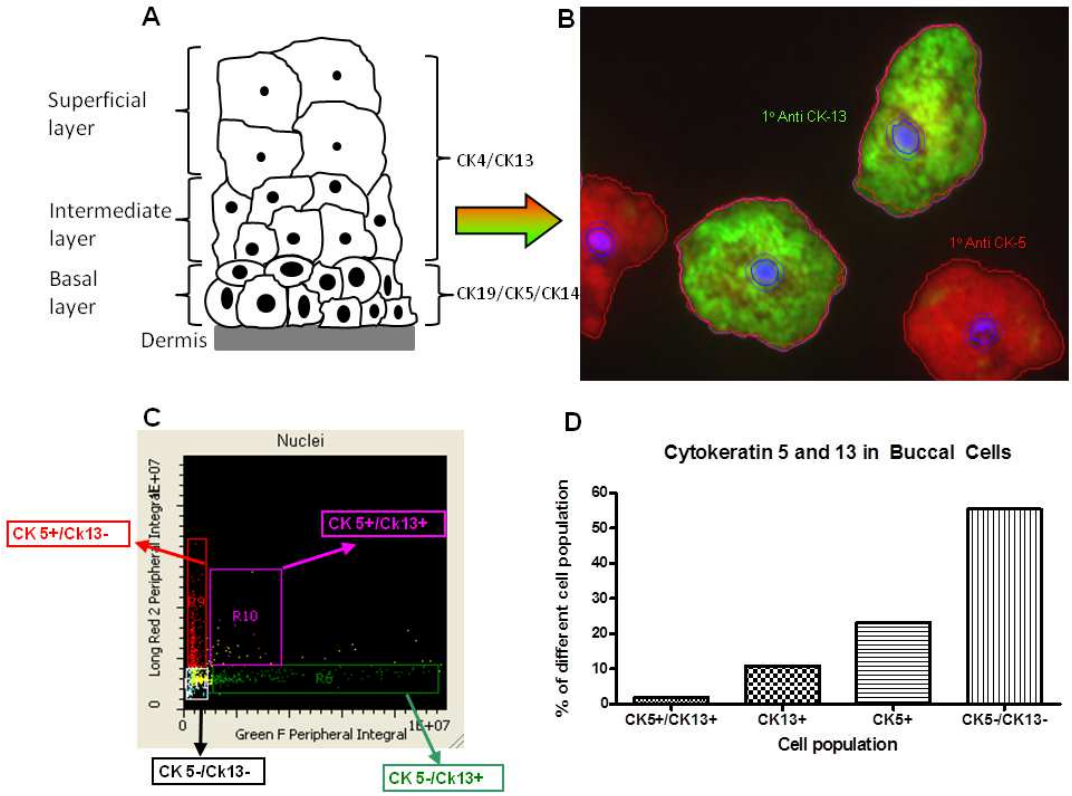

Figure legends

Figure 1: Diagrammatic representation of a cross section of normal buccal mucosa.

The schematic is illustrative of a healthy individual's buccal mucosa, highlighting the different cell layers and possible spatial relationships of the various cell types present.

Figure 2: Changes in the buccal cytome are associated with AD.

The frequency (%) of different buccal cell types scored for AD (n=31) and their age- and gender-matched controls (n=31); for (A) condensed chromatin cells, (B) basal cells and (C) karyorrhectic cells. Representative images of the buccal cell nuclei (which are one of the parameters used to define the buccal cytome in addition to the cytoplasm area and staining intensity) are shown as insets within each graph. Abbreviations: AD, Alzheimer's disease; Data are Mean +/- SD. ****p<0.0001. Adapted from Thomas et al. 2007 [120].

Figure 3: **Immunocytochemistry techniques showed a difference in expression of Cytokeratin 5 and 13 within buccal cells.**

(A) Schematic showing the differential expression of cytokeratins within the buccal cell layers. (B) Cytokeratin 5 and 13 were detected using an immunocytochemistry dual-staining technique, cells expressing cytokeratin 13 were detected with a secondary antibody 488 Alexa Fluor (Green) and cells expressing cytokeratin 5 were detected with a secondary antibody 647 Alexa Fluor (Red). (C) Using Laser Scanning Cytometry different populations of cells were scored depending on the type of cytokeratin expressed. (D) From the scattergram in (C), the percentage of buccal cell types based on cytokeratin 5/13 expression is shown.