Salivary Metabolomics screening of putative biomarkers during early diagnosis of Alzheimer's disease

S.Alagendran[1, 3], N.Pushpa[2], Dr. M. Rajasekaran[4] and G.Archunan[3]

Abstract: Mild cognitive impairment (MCI) imparts an increased prospect of rising Alzheimer's disease (AD) and to determine the utility of odor identification as an additional screening test in early onset of AD pathology using Sniffin' Sticks Odor Identification Test. There is high interest in the discovery of early diagnostic metabolomic biomarkers in saliva that could predict demographically MC Impairment toward AD progression. The present study is to determine metabolic variation in acetylcholine and esterase activities within saliva samples by assessment the MCI subjects and age-matched AD subjects acquire multivariate analyses the mean value of acetyl cholinesterase is 0.24mmol/L which is lower in AD type dementia patients (p value express ≤0.001% significant) and receiver operating characteristic analysis shows the sensitivity (94.8%) and specificity (96.6%) in salivary acetyl cholinesterase substantiate the potential diagnosis for Alzheimer's disease.

Key words: Saliva, acetyl choline, esterase, Alzheimer's disease (AD)

1. INTRODUCTION

The biochemical importance of Saliva is most pivotal to oral health. It lubricate oral structures, retain neutral pH by its buffering capacity, remineralizes the teeth enamel, cleanse the oral cavity, make use of antimicrobial effects, rouse wound healing, solutes tastants, serve in continuance of taste buds, take part in the digestion of the food and protects the esophageal mucosa from reiterate gastric secretion. Saliva is a mix of discharge from parotid, submandibular and sublingual glands and hundreds of minor salivary glands located just beneath the mucosal epithelium and disseminated throughout the mouth. Daily salivary output deduce be around one liter and the flow rate varies drastically over time. Since saliva collection is noninvasive and comparatively stress free, saliva is able to serve as a potential alternative and widespread diagnostic fluid (Van Nieuw Amerongen et al., 2004; Flink *et al.*, 2005).

With reference to the sensory innervation of salivary glands, definitely, nerves give you an idea about co-localization of substance P and calcitonin gene-related peptide are generally reflection to be of sensory outcome (Saria *et a*l., 1985). For instance in humans, the sympathetic nerve contribute of the secretory cells is inadequate in the labial glands but affluent in the submandibular glands. Whilst parasympathetic activity suggests a rich flow of saliva, the retort to sympathetic activity is typically petite.

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1. Department of Biochemistry, Crop Improvement Division, Adhiyamaan college of Agriculture and Research, Athimugam, Shoolagiri TK, Krishangiri - 635 105
2. PG & Research Department of Microbiology, Cauvery College for Women, Annamalai nagar, Trichy - 620 018
3. Centre for Pheromone Technology, Department of Animal Science, School of Life Sciences, Bharathidasan University, Trichy - 620 024
4. Department of Neurology, KAPV Medical College, Trichy.

Nevertheless together the stimulation of the parasympathetic nerve and the stimulation of the sympathetic nerve give rise to protein secretion; the protein concentration will be a smaller amount in parasympathetic saliva as an outcome of the large fluid production in response to the parasympathetic innervation. Underneath physiological conditions sympathetic secretory activity is mind to occur at some point in a background of ongoing parasympathetic secretion. Choline and acetylcholine play important roles in many biological processes (Meraz Rios *et al*, 2013). Choline is an essential nutrient, commonly grouped with the B complex vitamins, that plays key roles in many biological processes. Choline is a precursor for the synthesis of acetylcholine (AchE), a critical neurotransmitter in both the peripheral and central nervous systems (Mrak *et al*, 2005). Acetylcholine is a neurotransmitter produced in acetylcholinergic neurons. It plays important roles in skeletal muscle movement, regulation of smooth and cardiac muscles, as well as in learning, memory and mood (Rosenberg, 2005; Ray *et al*, 2007). Cognitive impairment is an exceedingly rare non-motor symptom in AD patients, thus two major types of patients can be distinguished: the patients without cognitive impairment or dementia and AD patients with cognitive impairment or dementia (Yu *et al*, 2014). It may be very difficult to diagnose these cases, therefore, identify a biomarker for severity of cognitive impairment becomes a necessity. The detection and confirmation of molecules implicated in this process could be a good approach for finding new biomarkers.

2. MATERIALS AND METHODS

Subjects

We enrolled 50 AD patients with dementia from the Neurology clinic at the Department of Neurology, GVN Hospital, Trichy. A total of 30 age-matched controls were recruited by GVN Hospital, Trichy. The study was conducted in accordance with the declaration of Helsinki and was approved by the review board of the regional science ethics committee. All subjects provided written consent prior to enrollment in the study. The diagnoses of AD with dementia were made by neurology specialists according to the Trichy Neurology society criteria.

Demographic and clinical investigations

Neuropsychological evaluations

All participants will receive a battery of neuropsychological tests to evaluate common mental status and other cognitive domains. The Mini-Mental State Examination (MMSE) and MCI were used to screen for dementia symptoms. Olfactory function was investigated using the validated 16-item "Sniffin' Sticks" smell-identification battery.

Samples of whole saliva were obtained from AD patients and healthy volunteers using a standardized saliva collection method (Sayer *et al*, 2004; Navazesh and Christensen, 1982). All participants had refrained from food, smoking, and beverages other than water for a minimum of four hours. PD patients were off all PD medication for a minimum of 12 hours. The subjects were seated in a chair and asked to rinse their mouth with still mineral water. They were instructed to spit all produced saliva into a preweighted test tube and not to swallow or talk during the collection. Saliva collected during the first five minutes was discarded and only that obtained during the following 10–50 minutes was used for further analyses. Samples were kept on ice immediately after collection and during subsequent initial processing to minimize degradation of salivary proteins. Flow rate was calculated as g/min, which is nearly equivalent to mL/min (Navazesh and Christensen, 1982). Samples were centrifuged at 3000 rpm for 30 minutes to remove particulate matter. The supernatant was divided into aliquots of 500 μL, which were sonicated for 3x10 seconds, and then immediately frozen at −80°C for a maximum of 7 months until further analyses.

Acetylcholine Assay

Acetylcholine is hydrolyzed by acetylcholinesterase to choline which is oxidized by choline oxidase to betaine and H_2O_2. The resulting H_2O_2 reacts with a specific dye to form a pink colored product. The color intensity at 570nm or fluorescence intensity (530/585 nm) is directly proportional to the acetylcholine concentration in the sample. Use 20 μL samples. Linear detection range: colorimetric assay 10 to 200 μM, fluorimetric assay 0.4 to 10 μM acetylcholine.

Acetylcholinesterase Inhibitor Assay is based on an improved Ellman method, in which thiocholine produced by the action of acetylcholinesterase

forms a yellow color with 5,5'-dithiobis(2-nitrobenzoic acid). Linear detection range 10 to 600 U/L for a 10 minute reaction at 37°C. The intensity of the product color, measured at 412 nm, is proportionate to the enzyme activity in the sample. AChE and cholinesterase activity and biochemical characterization in AD saliva or extracts of salivary glands were determined in micro plate assays using triplicate samples as described previously (Temeyer *et al.* 2010)

Ethics: The study would be carrying out in accordance with the approved guidelines. Written informed consent will be obtained from all participants, and the applicant will be able to depart from the study at any time.

Table 1: Demographic characteristics of patients with AD and controls

Parameter	Control	AD patients	P Value
Age	62.7 ± 9.4	63.7 ± 9.1	0.52
Sex (M/F)	32/28	20/15	0.96
MMSE	32.9 ± 3.4	32.6 ± 2.9	0.5
MCI	28.4 ± 1.2	28.1 ± 1.8	0.5

All values are mean ± SD.

Table 2: Metabolomic profile in patients with AD and controls

Parameter	Control (N=50)	AD patients (N=30)	P Value
Salivary flow (mL/min)	0.42 ± 0.2	0.30 ± 0.2	≤0.001
Sniffin' Sticks	7.8 ± 2.5	12.4 ± 2.3	≤0.001
AChE activity (mmol/ml)	0.32± 0.16	0.24± 0.18	≤0.001
Cholinesterase (nmol /ml saliva)	17.4± 2.5	11.6± 1.8	≤0.05

3. RESULTS

The cut-off chosen for the MMSE varied from 26 to 32. Most studies used a cut-off value of ≥32; MCI score varied from 23-28 proving the progressive decrease with severe cognitive impairment. Odor identification scores were higher in MCI/AD domain. Complementing MCI/AD scores with the Sniffin stick odor test significantly improved diagnostic accuracy of individuals with AD and MCI (P≤0.001). The Stimulated salivary flow rate in the AD type dementia group was significantly lower compared to the control group ($p < 0.001$). Lower levels of acetyl cholinesterase in saliva could indicate increased risk and severity for motor symptoms of AD (P≤0.001). Results from studies in Saliva AChE activity are not very consistent but one showed decreased level of in AD patients compared to control with a sensitivity of 94.8% and specificity of 96.6%. Salivary acetyl cholinesterase concentration was also correlated with MMSE score in patients with MCI and AD ($r = 0.786$; $P < .001$). Furthermore, we found that AD patients status correlated with decreased cholinesterase levels in saliva (Pearson's = −0.198; $P < .05$). Using ROC analysis, confidence interval between AD and control patients are varied from 11- 17 mmol/L. Salivary cholinesterase was obtained in AD patients acquire the sensitivity 85% and specificity 90% through immunoassay. ROC plot is a measure of predictive accuracy. Data analysis was performed using the SPSS 20.0 software. A nominal P value of less than .05 was considered to indicate statistical significance. The statistical power of the study was 85%. Elderly People with AD may develop cognitive impairment. Immunoassays and colorimetric detection from Saliva samples, allowed identifying two major enzymes acetylcholine and esterase associated with cognitive performance, of which the most significant was epidermal growth factor (EGF). Suggesting that salivary acetycholinesterase may be a biomarker for progression to cognitive impairment in AD.

4. DISCUSSION

In this, we present the innovation and validation of salivary acetyl cholinesterase as a novel MCI/AD diagnostic biomarker. According to our preliminary study, salivary acetyl cholinesterase absolutely classifies the entire MCI/AD patients and all cognitively healthy subjects and it showed a very high correlation with validated Saliva biomarkers. Moreover, in our second study control subjects, we found more or less healthy individuals with low levels of saliva acetyl cholinesterase who

were at high risk of converting to MCI/AD dementia (more than 97%). As a consequence, and although more clinical studies are needed, we suggest that salivary acetyl cholinesterase is a precise and robust biomarker for MCI/AD diagnosis and may help to recognize, after a general population screening, those control subjects that endure from underneath diagnosed later on stage preclinical AD or even MCI. Olfaction might serve as helpful biomarkers of early dementia. Here we observe the improvement in diagnostic precision of Alzheimer's disease (AD) and mild cognitive impairment (MCI) when assessing both cognitive functioning and odor identification using Sniffin stick test. Odor identification scores were higher in dementia subjects relative to MCI or AD groups, Sniffin stick test significantly improved diagnostic accuracy of individuals with AD and MCI.

Interestingly, the seromucous cells have been put forward to reflect maturative stages of mucous cells (Riva *et al*., 1999). Secretory cells, categorize as seromucous, show a cell morphology intermediary between true serous cells and mucous cells; the seromucous cells have granule with low electron density. The acinar cells of the labial glands are not only innervated by cholinergic nerves (Rossini *et al*., 1979) but also by vasoactive intestinal peptide-containing nerves (Fehér *et al*., 1999); the acinar cells lack adrenergic innervation (Rossini *et al*., 1979). Customarily, acetylcholine is the parasympathetic transmitter and noradrenaline the sympathetic transmitter. However, in previous report stated that 1970s it became evident that a number of transmission method also the classical cholinergic and adrenergic ones are at work in the neuro-effector region of assorted autonomically innervated organs. The parasympathetic peptidergic transmission mechanisms were instigate to release proteins or, in addition, to stir up fluid secretion and further, to interact positively with each other and with acetylcholine. Moreover, parasympathetic non-adrenergic, non-cholinergic transmitters are implicated in both gland metabolism and growth. The exploration of the field of the parameter of salivary glandular activities by parasympathetic non-adrenergic, non-cholinergic transmission mechanisms not only the rat but also the ferret became convenient trial animals (Ekström *et al*., 1988b; Ekström, 1999a).

Acetylcholine and its synthesizing enzyme, choline acetyltransferase - transferring the acetylgroup from acetyl Coenzyme A to choline, is traditionally associated with nervous structures (Hebb & Ratković, 1962; Wessler & Kirkpatrick, 2008). The acetylcholine formation was due to the specific action of choline acetyltransferase, and denervation experiments showed this enzyme to be confined to the nerves. As a result, no support for an extra-neuronal synthesis of acetylcholine by the activity of choline acetyltransferase was ascertained. The cholinesterase inhibitor physostigmines thwart the breakdown of acetylcholine unconfined from cholinergic nerve endings: acetylcholine accumulates either evokes an effectors response or improve it (Kawashima & Fujii, 2008). Physostigmine is a tertiary amine with lipohilic properties that readily passes biological barriers (Taylor, 1996). Therefore, it has been considered as a therapeutic option in the treatment of Alzheimer's disease (Nordberg & Svensson, 1998).

However, the differences that occur in the early stages of dementia are uncertain. Although the mode of memory defects in patients with MCI and MMSE is different, 5-10 comparisons of the relationship between these abnormal cognitive behaviours; brain structure and function are deficient. Thus, use of positron emission imaging to observe neuroimages in the two subtypes of MCI is related for early diagnosis onset of AD. The preliminary data of the present study suggest that salivary AChE, Cholinesterase activity, values show progressive fall with severity of cognitive impairment lead to cause AD. Further the collection of saliva is a simple, nontraumatic, and suitable method for field areas. This change is unlikely to be due to extra time taken in severe AD as the composition of salivary, ie, AChE, Cholinesterase enzyme reaching the oral cavity, is determined mainly by the secretory activity of the acini . The salivary Acetyl Cholinesterase activity seems to have some important physiological functions. The activity of choline acetyltransferase was responsible for the acetylcholine synthesis in the glands. Denervation experiments showed virtually all of the acetylcholine synthesis, due to the activity of choline acetyltransferase, to be of nervous origin (and not of non-nervous origin). The amount of acetylcholine continuously released

from the cholinergic nerve endings in the salivary glands, in the absence of nerve impulse passage, is concealed for evoking emission of saliva. It may, however, be exposed by the intraductal injection of an acetycholinesterase inhibitor, which prevents the degradation of released acetylcholine, and thus, accumulated acetylcholine in the neuro-effector region reaches suprathreshold levels for evoking secretion as demonstrated in parotid and submandibular glands (Emmelin et al., 1954; Ekström & Emmelin, 1974a,b). Likewise, during on-going nerve stimulation, the cholinergic secretory response will be enhanced by a cholinesterase inhibitor (Månsson & Ekström, 1991). The activity of choline acetyltransferase was responsible for the acetylcholine synthesis in the glands. Denervation experiments showed virtually all of the acetylcholine synthesis, due to the activity of choline acetyltransferase, to be of nervous origin (and not of non-nervous origin). By ELISA and colorimetric analysis in each individual donor sample revealed that salivary acetyl cholinesterase levels were significantly reduced in MCI and AD patients compared with the healthy control group ($P < .001$). Acetylcholinesterase, an important modulator of immune response and inflammation (Dubois *et al*, 2016), represents an important defensive element by inducing a broad spectrum of neurotransmitter and antioxidants effects (Jack *et al*, 2010; Welling *et al*, 2015). A novel role for antioxidants peptides has been proposed in AD pathology as pathogen-targeting mediator and markers of brain disease that are involved in the aggregation of amyloid (Welling *et al*, 2015). The present study showed a novel, straight-forward, and noninvasive approach for assessment of early stages of cognitive decline. We have revealed and validated a novel single saliva biomarker, Acetylcholinesterase, which in our cross-sectional study entirely differentiates clinically diagnosed mild cognitive impairment and AD patients from control group. To promote longitudinal group analyses are needed to concentrate on how salivary acetylcholine, esterase marker may help to differentiate between AD and other neurodegenerative diseases, consist of dementia. However, the evaluation of salivary AChE could be one of the non-invasive biomarkers in diagnosing dementia.

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