Blood circulatory level of seven sirtuins in Alzheimer's disease: Potent biomarker based on translational research

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Research Article

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Abstract

Alzheimer's disease (AD) is an accelerating neurodegenerative disorder. Dysfunction of mitochondria and oxidative stress contributes to the pathogenesis of AD. Sirtuins play a role in this pathway and can be a potential marker to study neurodegenerative changes. This study evaluated serum levels of all seven sirtuins (SIRT1 – SIRT7) proteins in three study groups: AD, Mild Cognitive Impairment (MCI), and Geriatric Control (GC) by Surface Plasmon Resonance (SPR) technique. Further, it was validated by the Western blot experiment. ROC analysis was performed to differentiate the study group based on the concentration of serum SIRT proteins. Out of seven sirtuins, serum SIRT1, SIRT3, and SIRT6 levels (mean $\pm$ SD) were significantly decreased in AD (1.65 ± 0.56 , 3.15 ± 0.28 , 3.36 ± 0.32 ng/ μ l); compare to MCI (2.17 ± 0.39 , 3.60 ± 0.51 , 3.73 ± 0.48 ng/ μ l) and GC (2.84 ± 0.47 , 4.55 ± 0.48 , 4.65 ± 0.55 ng/ μ l). ROC analysis showed the cut-off value with high sensitivity and specificity for cognitive impairment (AD and MCI). The concentration declined significantly with the disease progression. No specific difference was observed in case of other SIRTs between the study groups. This study reveals an inverse relation of serum SIRT1, SIRT3, and SIRT6 concentration with AD. ROC analysis showed that these serum proteins have greater accuracy in diagnosing of AD. This is the first report of estimation of all seven serum sirtuins and the clinical relevance of SIRT3 and SIRT6 as serum protein markers for AD.

Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder, characterized by progressive loss of memory, executive function, thinking, and behavior leading to a comprehensive cognitive dysfunction. The AD symptoms progressed slowly and worsened over time, which later interfered with the activities of daily living [1]. Mild Cognitive Impairment (MCI) is defined as impairment of one or more cognitive domains, is the earliest stage of disease, and is associated with an increased risk of AD. At present, AD is un-curable as there is no effective therapy that can delay the onset or slow AD progression. It is crucial to explore novel and effective treatment strategies for AD. There is a need to understand the fundamental molecular and cellular involvement in the disease, translating into clinical diagnosis and therapies.

The neuropathological hallmarks of AD are comprised of extracellular aggregation of β -amyloid peptide and intracellular precipitations of insoluble protein aggregates of phosphorylated microtubule-associated tau protein. It is also pathologically characterized by oxidative stress, mitochondrial damage, altered mitochondrial distribution, neuro inflammation, calcium deregulation, metal dis homeostasis, neurofibrillary tangle formation and amyloid- β (A β) oligomerisation and fibrillation [2]. Neurodegeneration in AD is associated with a significant increase in neuronal ROS production [3]. Factors such as cell senescence, oxidative stress and ageing are all crucial factors in the pathogenesis of AD [4].

The research on the sirtuin pathway is very challenging for bio-gerontologists due to its promising results in laboratory models of ageing and age-related diseases. There are seven members of the sirtuin (SIRT1-SIRT7) family. Sirtuins (silent information regulator) are a conserved family of NAD-dependent proteins found in all domains of life [5]. The sirtuin protein family has been linked to ageing and age-related diseases. Sirtuins have been implicated in influencing ageing and regulating transcription, apoptosis, stress resistance and energy efficiency, and alertness during low-calorie restriction. Many studies have shown the potential role of sirtuins in AD and other neurodegenerative disorders.

SIRT1 has been most extensively studied in AD. The animal and cell culture models showed that SIRT1 plays a neuroprotective role in the brain. A significant reduction in RNA and protein expression levels of SIRT1 was reported in the parietal cortex of post-mortem AD patients' brains in compared to controls. The loss of SIRT1 is strongly associated with the accumulation of amyloid-beta and tau in the cerebral cortex of AD patients [6]. In our previous study, SIRT1 was downregulated in the blood of AD patients compared to normal older individuals [7]. The meta-analysis has found an association between a polymorphism in an intron of SIRT2 gene and AD susceptibility [8].

SIRT3 is present in the mitochondrial matrix. Mitochondria is the main source of ROS and energy production within neurons thus are the critical mediator of age-related diseases such as neurodegeneration [9]. Mitochondrial ROS production increases in the brains of aged mice [10]. Similarly, there is a progressive trend toward a pro-oxidant state as human's age; thus, ROS increases with age [11]. Progressive ROS accumulation in neurons poses a significant threat, leading to exacerbated protein aggregation, cell death, and the onset of the pathogenesis of AD, as well as several other neurodegenerative diseases [12, 13]. The overexpression of SIRT1, SIRT3 reduced ROS generation, thereby prevent neuronal death [14, 15]. In the absence of SIRT3, there is hyperacetylation of SOD2 (Superoxide dismutase2) and Cyp-D (Cyclophilin D); thus, it restores resistance to stress [16]. Both SIRT1 and SIRT3 levels were found to be reduced in the entorhinal cortex and hippocampal sub-regions in the post-mortem human AD brain [17, 18].

The role of SIRT3 in mitochondrial function and its high expression levels in the brain [19] lead to a potential target for therapeutics to prevent neurodegenerative disease. SIRT3 and SIRT1 overexpression have been shown to protect neurons from cellular stresses and ROS formations [20, 14], mitochondrial dysfunction, and apoptosis [21]. In both AD and PD animal models, CR reduces neurodegeneration, possibly via up-regulation of SIRT3 [22]. SIRT3 plays a significant role in neuroprotection and improvement of mitochondrial function by mediating antioxidant and metabolic effects, which results in enhanced neuronal survival and lessen ageing effects. The expression of SIRT3 and SIRT6 changes the ageing brain in a region-specific manner [23, 24]. SIRT3 is expressed in multiple tissues, including the kidney, heart, liver, adipose tissue and brain [25]. SIRT6 is present in the nucleus. It is known as a longevity gene. It is a NAD-dependent histone deacetylase that has multiple roles such as telomere maintenance, DNA repair, genome integrity, energy metabolism and inflammation, which ultimately regulate life span [26]. Defects in DNA repair and glucose metabolism have been shown to contribute to the manifestation of late-onset and age-related AD [27]. Ageing is the primary risk factor for neurodegenerative diseases and also associated with the accumulation of unrepaired DNA damage [28, 29].

SIRT6 deficient mice showed DNA damage in a tissue-specific manner, particularly in the brain [30]. Reduced level of SIRT6 found in all brain regions of ageing rats [31]. SIRT6 regulated stability and phosphorylation of Tau protein through increased activation of GSK3 α / β . SIRT6 mRNA and protein levels are found to be decreased in AD patients. Defects in DNA repair and glucose metabolism have been shown to cause age-related cognitive impairment in mice and humans [32, 33]. Therefore, defects in SIRT6 levels or function may be intricately linked to late-onset or age-related AD. SIRT6 expression was reported to be decreased

in the brains of an AD mouse model and post-mortem brains of AD patients compared to cognitively normal subjects. It was found that A β 42 significantly decreased SIRT6 expression, which was associated with a decrease level of p53 [34].

Study on brain-specific SIRT6–knockout (SIRT6KO) mouse model, exhibit accelerated ageing and causes pathological changes in behaviour, presented with accumulated DNA damage, and increased apoptosis and hyperphosphorylated Tau levels. There was an increase in Tau stability and phosphorylation through GSK3 activation when SIRT6 depleted in cells. These findings indicate that SIRT6 protects the brain from naturally accumulating DNA damage, protecting against neurodegeneration [35]. The potential roles of SIRT6 in AD have remained unexplored. SIRT4, SIRT5 and SIRT7 were shown to have different roles in various tissues, but their role in brain was unexplored.

The identification of biomarker molecules in blood would be more widely applicable than cerebrospinal fluid (CSF) due to non-invasiveness, less expensive and less time consuming. Sirtuin protein can be the next target for AD for early diagnosis. Based on several findings on the involvement of sirtuin proteins in neurodegenerative diseases and evidence from our previous work, this study focused on quantifying all sirtuins in blood of AD, MCI and GC to identify specific sirtuin to establish it as potential blood-based marker for AD.

Material And Methods

Study Participants

A total of 138 subjects were recruited for the present study. All participants underwent clinical testing, biochemical testing, and cognitive assessment at the Memory Clinic, Department of Geriatric Medicine of the All India Institute of Medical Sciences, New Delhi, India. The Ethics Committee of AIIMS approved the study protocol (IESC/T-28/03.01.2014). After providing informed consent, each participant completed a brief questionnaire about their medical history, health behaviours such as addiction, demographic, education profile, occupation, living status, co-morbidities and disease duration. Among them, 47 patients were diagnosed with AD, 43 patients with MCI and 48 subjects were Geriatric Controls (GC). All diagnoses were made as per the existing diagnostic criteria by the team of geriatricians and a neuropsychologist. All participants with MCI met Petersen's 2011 criteria: 1) concern about their cognitive deficit in comparison to their previous level; 2) performance that is lower than their age- and education- matched normative; 3) no or minimal impairment in their basic and instrumental activities of daily living (ADL); and 4) not meeting DSM-V-2013 criteria for dementia. A basic protocol was completed, which included the HMSE, Barthel Activities of Daily Living (BADL), Instrumental Activities of Daily Living (IADL) and Geriatric Depression Scale (GDS).

Older subjects above 60 years of age, with no reported major systemic or psychiatric illness, were recruited as geriatric controls (GC). Also, consent was taken from volunteers and informants who accompanied the patients to the Out Patient Geriatric Department. Further, any participant with delirium or any chronic systemic illness and major psychiatric diseases were excluded from the study. It was made sure that all possible reversible clinical causes were ruled out at the diagnosis by the geriatrician. HMSE scores considered for these three groups were: Normal: 26 and above, MCI: 17–25 and AD: less than 17. AD was diagnosed as per NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association criteria) [36]. Normal control group comprised of subjects with no apparent or disability and HMSE score of 26 or more. Further, each case underwent imaging evaluation of Magnetic Resonance Imaging (MRI) of the brain and positron emission tomography (PET) scan of Tau and fluorodeoxyglucose (FDG).

Two milliliters of venous blood samples were collected from all subjects and were allowed to stand for 1 hour. Serum samples were extracted after centrifugation at 3000 rpm for 10 minutes and stored in aliquots at -80°C until the time of experimental work.

Estimation of serum sirtuin levels by SPR technology

Estimation of serum proteins was performed by SPR, using the BIAcore-3000 [Wipro GE Healthcare, Sweden], a biosensor-based system for real-time monitoring of specific interaction analysis. Primary antibodies; mouse anti-human SIRT1 monoclonal IgG (Santa Cruz Biotechnology), goat anti-human SIRT2 polyclonal IgG (Santa Cruz Biotechnology), rabbit anti-human SIRT3 polyclonal IgG (Santa Cruz Biotechnology), rabbit anti-human SIRT4 polyclonal IgG (Santa Cruz Biotechnology), mouse anti-human SIRT5 monoclonal IgG (Santa Cruz Biotechnology), rabbit anti-human SIRT6 polyclonal IgG (Sigma) and rabbit anti-human SIRT7 polyclonal IgG were immobilized on different flow cells of CM5 sensor chip via amine coupling kit (Wipro GE Healthcare, Sweden). To prepare the standard curve six different known concentrations of the purified recombinant proteins of SIRT1 (0, 3.18, 5.31, 10.62, 15.94, 21.25 ng/ μ l), SIRT2 (0, 1.94, 3.42, 6.84, 11.42 ng/ μ l), SIRT3 (0, 2.1, 4.2, 6.4, 8.5, 10.7 ng/ μ l), SIRT4 (0, 1.40, 4.21, 8.43, 11.25, 14.06 ng/ μ l), SIRT5 (0, 2.7, 4.5, 6.3, 9.12 ng/ μ l) SIRT6 (0, 2.14, 4.28, 6.42, 8.56, 10.7 ng/ μ l) and SIRT7 (0, 1.55, 3.87, 5.42, 7.75, 11.62 ng/ μ l) passed over the respective immobilized antibodies on sensor chip and RU values were obtained.

Serum was passed over the immobilized antibodies. For each sample, the RU value was recorded and the serum concentration of sirtuins in different subjects was derived from the standard curves.

Estimation of serum sirtuin levels by Western blot

For further validation, the expression level of proteins in serum was performed by Western blot experiment. Albumin was removed from Albumin out kit (G-Biosciences) according to the manufacturer's protocol, followed by BCA (Pierce™ BCA Protein Assay Kit) to determine the concentration of serum proteins. The standard curve was plotted using different dilutions of BSA (Bovine Serum Albumin). The samples were diluted empirically such that the approximate concentration of the protein would fall in the linear range of the standard curve. Diluted samples were mixed with 200 μ l working BCA reagent in 96 well plates and incubated for 30 minutes at 37°C. At 562 nm, the absorbance was measured by ELISA reader. The protein concentrations of the samples were calculated from the standard curve. Randomly, three samples were selected, from each group and 30 μ g of total protein were loaded on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). Then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore Membrane Technologies), after

that membranes were blocked with 5% nonfat dry milk (NFM) which was made in TBS (10 mM Tris pH 7.5, 150 mM NaCl), and incubated for 2 h at room temperature and after that incubated with primary antibodies which were diluted with TBS, containing 1% NFM at 4°C overnight. Following primary antibodies were used: mouse anti-human SIRT1 monoclonal IgG (1:300), rabbit anti-human SIRT3 polyclonal IgG (1:500) and rabbit anti-human SIRT6 polyclonal IgG (1:2000). Then washing was done with TBS-T (20 mM Tris pH7.5, 500 mM NaCl, 0.05% Tween 20) and membranes were incubated with HRP-conjugated secondary antibodies; goat anti-mouse IgG (1:5000, Santa Cruz Biotechnology, USA), goat anti-rabbit IgG (1:4000, Santa Cruz Biotechnology, USA) and goat anti-rabbit IgG (1:4000, Santa Cruz Biotechnology, USA) at room temperature for 1 hour. After washing with TBS-T, the PVDF membranes were visualized by Enhanced Chemiluminescent System (Pierce ECL Western Blotting Substrate, Thermo Scientific, Rockford, IL) and my Image Analysis Software (Thermo scientific) was used to observe the density of bands.

Statistical Analysis

Data analysis was performed using Stata/SE version 9 and Graph pad prism5 (College Station, TX, USA and GraphPad Software, Inc, La Jolla, USA). Descriptive analysis was done for all variables with mean and standard deviation. Statistical tests: chi-square test for categorical variables and continuous variables for baseline comparison among groups (AD, MCI and GC) were performed using one-way ANOVA followed by post hoc comparison using the Bonferroni test. Correlation between two continuous variables was analysed using Cary Pearson correlation coefficient. For detecting optimum diagnostic cut-off value Receiver operating characteristics (ROC) analysis was executed. Analysis of covariance was done to check the difference in the levels of Sirtuins between controls and patients after adjusting age, gender, education, occupation, and duration. The p-value of < 0.05 was considered as statistically significant.

Results

Demographic and clinical data

Descriptive analysis of various demographic and clinical parameters of study subjects are presented in Table 1 and Table 2. The age of each study group (AD, MCI and GC) was categorised into three subgroups: 60-65 years, 66-75 years and ≥ 76 years. The mean age of AD, MCI and GC subjects were 70.74, 68.62 and 68.23, respectively. Mean HMSE scores were 13.63, 22.44, 28.31 in AD, MCI and GC groups, respectively. With respect to sex, age category, location, education, addiction, family history of AD and number of co-morbidities, there were no significant differences between groups. While significant differences were observed with respect to occupation, duration of disease, BADL, hypertension, joint disease, MTA score and parietal score between all three groups. With the increase duration of the disease, the incidence of cognitive impairment also increases and was significant among the groups ($p < 0.0001$).

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Quantification by SPR

The concentrations of sirtuins in serum were determined from the standard curves using RU obtained from the binding of serum over the sirtuin antibodies. The immobilized protein concentration of 1 pg/mm² corresponds to one RU. Out of seven sirtuins the concentration of serum SIRT1, SIRT3 and SIRT6 was found in GC group (SIRT1: 2.84 ± 0.47 ng/ μ l, 95% CI: 2.70-2.97 ng/ μ l), (SIRT2: 4.55 ± 0.48 ng/ μ l, 95% CI: 4.42-4.69 ng/ μ l), (SIRT3: 4.65 ± 0.55 ng/ μ l, 95% CI: 4.48 – 4.81 ng/ μ l) significantly higher than MCI group (SIRT1: 2.17 ± 0.39 ng/ μ l, 95% CI: 2.05–2.29 ng/ μ l), (SIRT2: 3.60 ± 0.51 ng/ μ l, 95% CI: 3.45 – 3.76 ng/ μ l), (SIRT3: 3.73 ± 0.48 ng/ μ l, 95% CI: 3.58– 3.88 ng/ μ l) , and AD group (SIRT1: 1.65 ± 0.56 ng/ μ l, 95% CI: 1.48–1.82 ng/ μ l), (SIRT2: 3.15 ± 0.28 ng/ μ l, 95% CI: 3.07 – 3.24 ng/ μ l), (SIRT3: 3.36 ± 0.32 ng/ μ l, 95% CI: 3.26 – 3.45 ng/ μ l) (**Fig.1**). SIRT1 (nonfrail– 4.67 ± 0.48 ng/IL; frail – 3.72 ± 0.48 ng/IL; $P < 0.0001$), SIRT2 nonfrail– 15.18 ± 2.94 ng/ IL; frail – 14.19 ± 2.66 ng/IL; $P = 0.016$), and SIRT3 (nonfrail– 7.72 ± 1.84 ng/IL; frail – 6.12 ± 0.97 ng/IL; $P < 0.0001$) levels were significantly lower among frail patients compared with the non-frail. SIRT1 (nonfrail– 4.67 ± 0.48 ng/IL; frail – 3.72 ± 0.48 ng/IL; $P < 0.0001$), SIRT2 (nonfrail– 15.18 ± 2.94 ng/IL; frail – 14.19 ± 2.66 ng/IL; $P = 0.016$), and SIRT3 (nonfrail– 7.72 ± 1.84 ng/IL; frail – 6.12 ± 0.97 ng/IL; $P < 0.0001$) levels were significantly lower among frail patients compared with the non-frail.

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SIRT4 (GC– 2.66 ± 1.16 ng/ μ l, 95% CI: 2.33-3.00 ng/ μ l; MCI– 3.49 ± 0.66 ng/ μ l, 95% CI: 3.29 – 3.70 ng/ μ l; AD– 2.56 ± 0.76 ng/ μ l, 95% CI: 2.34 – 2.78 ng/ μ l; AD vs GC $p = 0.6133$, AD vs MCI $p < 0.0001$, MCI vs GC $p = 0.0001$).

SIRT5 (GC-1.25 ±0.44 ng/μl, 95% CI: 1.12-1.37 ng/μl; MCI-1.22±0.45ng/μl, 95% CI: 1.08–1.37 ng/μl; AD- 2.18±0.59 ng/μl, 95% CI: 2.01–2.35 ng/μl; AD vs GC p<0.0001, AD vs MCI p<0.0001, MCI vs GC p=0.8069).

SIRT7 (GC-2.31±0.63 ng/μl, 95% CI: 2.13-2.49 ng/μl; MCI- 1.81±0.37 ng/μl, 95% CI: 1.69 –1.93 ng/μl; AD-1.87±0.49 ng/μl, 95% CI: 1.73– 2.02 ng/μl; AD vs GC p<0.0003, AD vs MCI p= 0.5114, MCI vs GC p<0.0001).

Hence, SIRT2, SIRT4, SIRT5 and SIRT7 were not significant among different groups. (Fig.1).

In the analysis of covariance, after adjustment of occupation, duration of disease, joint disease, co-morbidities between three groups, the serum protein levels of SIRT1, SIRT3 and SIRT6 were found to be significantly lower (p<0.0001) in the case of AD group as compared to MCI (p<0.0001) and GC group (p<0.0001). The values of all three proteins were significantly different among the three categories (Table 3). A significant difference was observed in serum protein concentration with respect to age, sex, education, duration of disease, occupation, joint disease, HTN and DM in different groups (Table 4).

ROC analysis was carried out based on SPR data to measure the utility of SIRT1, SIRT3 and SIRT6 out of seven sirtuins as protein markers for AD and MCI (Fig.2). The threshold for detecting AD and MCI was selected based on the distribution of specificities and sensitivities. In case of SIRT1, the area under curve (AUC) for distinguishing AD vs GC was 0.9275 (cut-off -2.31 ng/μl; sensitivity – 93.75 %; specificity – 87.23 %), AUC for distinguishing MCI vs GC was 0.8697 (cut-off – 2.52 ng/μl; sensitivity – 79.17 %; specificity – 76.74%) and AUC for distinguishing AD vs MCI was 0.7905 (cut-off – 1.90 ng/μl; sensitivity – 76.74 %; specificity – 74.47 %). For SIRT3, the area under curve (AUC) for distinguishing AD vs GC was 0.9969 (cut-off -3.68 ng/μl; sensitivity – 97.92 %; specificity – 95.74 %), AUC for distinguishing MCI vs Control was 0.9169 (cut-off – 3.99 ng/μl; sensitivity – 91.67 %; specificity – 79.07 %) and AUC for distinguishing AD vs MCI was 0.7840 (cut-off – 3.33 ng/μl; sensitivity – 74.42 %; specificity – 78.72 %). Similarly, in the case of SIRT6, the AUC for distinguishing AD vs GC was 0.9818 (cut-off – 3.97 ng/μl; sensitivity – 93.75 %; specificity – 93.62 %), AUC for distinguishing MCI vs GC was 0.897 (cut-off – 4.1 ng/μl; sensitivity – 81.25 %; specificity – 76.74 %) and AUC for distinguishing AD vs MCI was 0.7288 (cut-off – 3.45 ng/μl; sensitivity – 74.42 %; specificity – 68.09 %). This result shows that lower serum SIRT1, SIRT3 and SIRT6 levels can be a distinctive marker of AD.

Pearson correlation analyses evaluated the correlation between protein concentrations (SIRT1, SIRT3 and SIRT6) and HMSE score. We found that lower HMSE scores were associated with lower concentration of proteins with significant positive correlation (Pearson correlation coefficient for SIRT1 r=0.294, p-value <0.0010, SIRT3 r=0.373, p value<0.0001 and for SIRT6 r= 0.307, p-value <0.0001) (Fig.3). With respect to age, sex, years of education and duration of disease, no significant difference was observed in serum sirtuin concentration among AD, MCI and GC subjects.

By Western blot

Western blot analysis of serum samples was used to validate differential expression of SIRT1, SIRT3 and SIRT6 in AD, MCI and GC groups obtained by SPR data. The result showed a low band density of SIRT1, SIRT3 and SIRT6 proteins in AD and MCI patients compared to GC subjects, as observed in SPR data (Fig. 4).

Discussion

Dementia and specifically AD in older people, represent a booming public health problem. AD is one of the most commonly underestimated and under-diagnosed geriatric syndrome in India. It is challenging for early diagnose AD and MCI due to distinct and subjective clinical features. There is no definitive treatment for AD, which implies that the conditions need to be detected early to stop further dependence. Most of the molecules failed to prove them as a potent blood-based biomarker because of the lack of sensitivity and specificity. Several promising researches are under process which include brain imaging, cerebrospinal fluid proteins, and substances in blood towards establishing biomarkers for dementia, especially AD, but none showed promising marker for early clinical diagnosis.

Previous studies have analysed serum levels of SIRT1 and SIRT3 protein with ageing and found that the expression was downregulated with age [7, 37]. SIRT2, SIRT4, SIRT5 and SIRT7 are largely unexplored for neurodegenerative diseases and the brain. These proteins are new therapeutic targets for neurodegenerative diseases in recent time due to a critical role in the nervous system. With this goal, we are here for the first time quantified serum levels of seven sirtuin proteins in AD and MCI patients and compared them with GCs.

The age category of 66–75 years was more in this study subjects. It is as per the existing literature and reflects the progressive nature of cognitive decline which becomes increasingly apparent during this stage with a transformation into frank dementia in the late 70s and 80s. Male subjects were more predominant than female subjects in our study, which may be due to low health-seeking awareness among females and the structured socio-economic and socio-cultural issues. Different Evaluation Methods (clinical score - HMSE and imaging-MRI Scores) were independently assessed to the defined criteria for the groups (AD, MCI, GC) they were found to be significant (p < 0.0001) with the disease. The standard cut-offs (26 and above) for HMSE scores were applied to our subjects in our setup. The 50% out of total subjects were considered normal using HMSE, a highly sensitive scoring system in detecting a cognitive decline in our setup. When structural imaging (MRI) using MTA scores was used to classify the subjects, the values were statistically significant among the groups.

This study explored serum protein levels that change in association with AD, MCI patients, and GC subjects. Pair-wise comparisons between the three groups showed significantly lower SIRT1, SIRT3 and SIRT6 protein expression in AD patients than in MCI and GC subjects (p < 0.0001, p < 0.0001 and p < 0.0001, respectively). ROC curves for SIRT1, SIRT3 and SIRT6 protein levels were plotted separately and showed reasonably good sensitivity and specificity at the cut-off value for detecting AD and MCI patients compared to GC subjects. A significant correlation (p < 0.0001) existed between serum SIRT1, SIRT3 and SIRT6 concentration with HMSE scores. A positive correlation of protein with the HMSE score indicates that these serum protein levels decrease with poorer cognitive impairment (HMSE score).

The previous literature supports our present finding, which correlates these proteins and their involvement in AD pathology. A comparative immunoblotting and immune histochemical study of SIRT1, SIRT3 and SIRT5 in the AD brain grouped according to Braak and Braak stages of neurofibrillary degeneration in a study. The decrease in SIRT1 expression in neuronal subcellular redistribution with stepwise loss of neuroprotection dependent on the neuronal population and SIRT1 and SIRT3 decrease simultaneously with AD progression, while expression of SIRT5 increases during the progression of AD [38].

It was noted that SIRT3 was expressed at high levels in the normal brain and other nervous system tissues [39, 40]. However, most previous studies showed downregulation of SIRT3 in AD [41, 42]. Many studies reported the potential role of sirtuins in AD and other neurodegenerative disorders. SIRT1 and SIRT3 mRNA/protein levels were reduced in AD brain and were correlates with the stage/duration of the disease [43, 41]. In turn, up-regulation of SIRT3mRNA that followed the spatial and temporal profiles of A β accumulation has been shown in mice, and higher SIRT3mRNA was observed in the temporal cortex of AD cases [44, 45].

SIRT6 depletion is linked to DNA damage and ageing, reported in a recent study [29]. SIRT6 loss led to tau hyperphosphorylation *via* the increase in GSK3 activity, with the latter restored with SIRT6 re-expression. SIRT6 levels are reduced in human AD brain samples and mouse models [35]. These studies suggested that decrease in SIRT6 expression during AD may further enhance neuronal death and degeneration.

In our study, ROC results indicate that serum SIRT1, SIRT3 and SIRT6 have great potential to be diagnostic protein markers for AD for its accuracy in the study group. This is the first study to report the circulatory level of all seven sirtuins in serum of AD, MCI and GC subjects and the clinically diagnostic relevance of SIRT3 and SIRT6 as serum protein markers for Alzheimer's disease.

Conclusion

For the first time, in the present study, we detected and evaluated all serum Sirtuins concentrations in AD, MCI patients and GC to explore the clinical relevance's. SIRT1, SIRT3 and SIRT6 protein expressions in serum were significantly low in AD compared to those in MCI and GC subjects. These protein markers can be potential sensitive diagnostic markers of AD.

A strong inverse correlation between SIRT1, SIRT3 and SIRT6 with cognition (HMSE) was identified in the study groups. These results suggest that SIRT1, SIRT3 and SIRT6 may be novel blood-based markers for AD. This study may open up the new vision of blood-based biomarkers for clinical practice and a therapeutic target for AD.

Declarations

Future prospective

A larger sample size and longitudinal studies are required to validate the potential role of serum sirtuin levels in AD.

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Data Availability: All materials used in this study will be made available.

Code Availability: Not applicable.

Ethics Approval: All India Institute of Medical Sciences Ethics Committee (AIIMS) approved the study protocol (IESC/T-28/03.01.2014).

Consent for Publication: The manuscript contains no individual person's data in any form.

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Authors Contribution:

RP: major part of the experiment and wrote the manuscript, AKS: run the sample in Biacore, PK, PC, ABD: diagnosed the patients and provided blood samples; MP, SB: performed statistical analysis, SD: concept and design of work, wrote and edited the manuscript.

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Tables

Table 1: Demographic data of AD, MCI and GC study subjects

Variable	Total subjects	AD	MCI	GC	p value
N	138	47	43	48	
Sex					
Male, n (%)	102 (73.91)	32 (66.67)	34 (79.07)	36 (76.60)	0.354
Female, n (%)	36 (26.09)	16 (33.33)	9 (20.93)	12 (23.4)	
Age category, n (%)					
60-65 Yrs	46 (33.33)	11 (23.4)	18 (41.86)	17 (35.42)	0.355
66-75 Yrs	66 (47.83)	24 (51.06)	19 (44.19)	23 (47.92)	
≥76 Yrs	26 (18.84)	12 (25.53)	6 (13.95)	8 (16.67)	
Age (mean±SD)		70.74±7.02	68.627±7.23	68.23±5.78	0.277
Urban (n,%)	73 (53.28)	26 (56.52)	25 (58.14)	22 (45.83)	0.434
Rural (n,%)	64 (46.72)	20 (43.48)	18 (41.86)	26 (54.17)	
Education [(Years) n, %]					
Illiterate (0)	18 (13.04)	5 (10.64)	7 (16.28)	6 (12.5)	0.482
Primary (Class1-5 th)	29 (21.1)	9 (19.15)	10 (23.26)	10 (20.83)	
Secondary (Class 6 th -12 th)	48 (34.78)	19 (40.43)	14 (32.56)	15 (31.25)	
Graduate & above (≥13 th)	43 (31.16)	14 (29.78)	12 (27.9)	17 (35.42)	
Occupation					
Unemployed	35 (25.37)	13 (17.66)	7 (16.28)	15 (31.25)	0.0001
Employed	5 (3.62)	0 (0)	0 (0)	5 (10.42)	
Retired	63 (45.65)	24 (51.06)	19 (44.19)	20 (41.67)	
Business or Farming	35 (25.36)	10 (21.28)	17 (39.53)	8 (16.67)	
Addiction					
Yes	40 (29.20)	19 (40.43)	11 (25.58)	11 (22.92)	0.184
No	97 (70.80)	28 (59.57)	32 (74.42)	37 (77.08)	

Table 2: Clinical data of AD, MCI and GC study subjects

Variable	Total subjects	AD	MCI	GC	p value
N	138	47	43	48	
Family history of AD					0.876
Yes	7 (5.07)	3 (6.38)	2 (4.65)	2 (4.17)	
No	131 (94.93)	44 (93.62)	41 (95.35)	46 (95.83)	
Duration of disease (Years)					0.0001
Nil, n (%)	23 (16.67)	0	0	23 (47.92)	
Upto 2 Yrs, n (%)	76 (55.07)	19 (40.43)	33 (76.74)	24 (50.0)	
2-4 Yrs, n(%)	27 (19.57)	18 (38.30)	8 (18.6)	1 (2.08)	
≥ 5 Yrs, n(%)	12 (8.7)	10 (21.28)	2 (4.65)	0	
HMSE (mean±SD)	-	13.63±6.35	22.44±4.27	28.31±1.01	0.0001
MOCA (mean±SD)	-	7.21±5.93	14.79±8.05	24.04±5.81	0.0001
BADL (mean±SD)	-	17.51±4.22	19.34±1.37	19.48±2.89	0.0035
GDS (mean±SD)	-	3.62±3.18	4.74±3.17	3.87±3.35	
BADL					0.001
Normal	106 (76.81)	28 (59.57)	34 (79.07)	44 (91.67)	
Impaired	32 (23.19)	19 (40.43)	9 (20.93)	4 (8.33)	
Hypertention					0.001
Yes	54 (39.13)	19 (40.43)	25 (58.14)	10 (20.83)	
No	84 (60.87)	28 (59.57)	18 (41.86)	38 (79.17)	
Joint disease					0.019
Yes	17 (12.32)	4 (8.51)	2 (4.65)	11 (22.92)	
No	121 (87.68)	43 (91.49)	41 (95.35)	37 (77.08)	
No. of co-morbidities					0.149
0	52 (37.68)	19 (40.43)	11 (25.58)	22 (45.83)	
1	44 (31.88)	14 (29.79)	15 (34.88)	15 (31.25)	
2	27 (19.57)	10 (21.28)	8 (18.60)	9 (18.75)	
≥3	15 (10.87)	4 (8.51)	9 (20.93)	2 (4.17)	
MRI (MTA Score)					0.0001
0	48 (36.92)	0	3 (7.69)	45 (97.83)	
1	34 (26.15)	5 (11.11)	28 (71.79)	1 (2.17)	
2	25 (19.23)	17 (37.78)	8 (20.51)	0	
≥3	23 (17.69)	23 (51.11)	0	0	
MRI (Parietal Score)					0.0001
0	68 (52.31)	13 (28.89)	15 (38.46)	40 (86.96)	
1	40 (30.77)	18 (40.03)	16 (41.03)	6 (13.04)	
2	20 (15.38)	12 (26.67)	8 (20.51)	0	
3	2 (1.54)	2 (4.44)	0	0	

HMSE -Scores <26 out of 30 is cognitively impaired. GDS- Scores >9 out of 15 is suggestive of depression. BADL- Total possible scores range from 0 – 20, with lower scores indicating increased disability. MTA- Medial Temporal Atrophy.

Table 3: Unadjusted and adjusted values (ng/μl) represented as mean ± SE of serum Sirtuinproteins by SPR in AD, MCI and GC subjects

Protein	Unadjusted/ Adjusted	GC	MCI	AD	p value	Post-hoc Comparison	p value
SIRT1	Unadjusted	2.84±0.06	2.17±0.06	1.654±0.08	0.0001	NC/MCI	0.0001
						NC/AD	0.0001
						MCI/AD	0.0001
	Adjusted	2.92±.14	2.26±.24	1.52±.27	0.0001		
SIRT3	Unadjusted	4.55±0.15	3.60±0.51	3.15±0.28	0.0001	NC/MCI	0.0001
						NC/AD	0.0001
						MCI/AD	0.0020
	Adjusted	4.42±0.15	4.02±0.46	2.83±0.24	0.0001		
SIRT6	Unadjusted	4.64±0.55	3.72±0.48	3.37±0.34	0.0001	NC/MCI	0.0001
						NC/AD	0.0001
						MCI/AD	0.0300
	Adjusted	4.67±0.13	4.14±0.42	2.83±0.32	0.0001		

Adjusted for occupation, duration of disease, joint disease, no. of co-morbidities

Table 4: Concentration of serum SIRT1, SIRT3 and SIRT6 (ng/μl) proteins with respect to Demographic and Clinical parameters by SPR

Figures

Fig. 1

Figure 1

Estimation of serum concentration of sirtuin proteins by SPR. Scatter diagram showing the difference in the serum level of SIRT1 (a) and SIRT2 (b) SIRT3 (c)

Category	Subcategory	SIRT1				SIRT3				SIRT6		
		AD (Mean ± SD)	MCI (Mean ± SD)	GC (Mean ± SD)	p-value	AD (Mean ± SD)	MCI (Mean ± SD)	GC (Mean ± SD)	p-value	AD (Mean ± SD)	MCI (Mean ± SD)	
Age (Years)	60-65	1.88±0.67	2.20±0.41	3.20±0.54	0.0001	3.04±0.23	3.46±0.53	4.50±0.37	0.0001	3.21±0.22	3.57±0.59	
	66-75	1.56±0.52	2.12±0.39	2.65±0.31	0.0001	3.16±0.39	3.66±0.52	4.51±0.55	0.0001	3.27±0.33	3.77±0.38	
	≥ 76	1.62±0.51	2.21±0.39	2.59±0.15	0.0001	3.34±0.28	3.45±0.84	4.54±0.46	0.0001	3.7±0.44	3.56±0.73	
Gender	Male	1.58±0.56	2.13±0.42	2.82±0.49	0.0001	3.21±0.36	3.56±0.59	4.54±0.47	0.0001	3.38±0.39	3.64±0.55	
	Female	1.87±0.51	2.3±0.23	2.86±0.44	0.0001	3.0±0.23	3.55±0.37	4.47±0.47	0.0001	3.19±0.22	3.80±0.28	
Duration of disease (Years)	<2 Years	1.66±0.53	2.15±0.38	2.92±0.48	0.0001	3.23±0.32	3.56±0.52	4.51±0.44	0.0001	3.12±0.29	3.67±0.50	
	≥2 Years	1.49±0.54	1.91±0.39	2.76±0.46	0.0016	2.97±0.34	3.52±0.78	-	0.0052	3.43±0.36	3.62±0.57	
Education (Years)	0	1.93±0.51	2.13±0.43	2.88±0.30	0.0039	3.11±0.15	3.74±0.38	4.30±0.35	0.0001	3.31±0.15	3.78±0.27	
	1-12	1.60±0.54	2.25±0.32	3.08±0.56	0.0001	3.25±0.38	3.42±0.55	4.55±0.53	0.0001	3.43±0.36	3.59±0.61	
	≥13	1.35±0.53	2.08±0.38	2.75±0.45	0.0001	2.99±0.26	3.75±0.66	4.64±0.39	0.0001	3.22±0.42	3.73±0.45	
Occupation	Unemployed	1.73±0.53	2.27±0.24	3.05±0.41	0.0007	3.11±0.15	3.74±0.38	4.30±0.35	0.0001	3.08±0.12	3.48±0.45	
	Retired	1.53±0.53	2.10±0.45	2.61±0.24	0.0001	3.25±0.38	3.42±0.55	4.55±0.53	0.0001	3.15±0.38	3.82±0.52	
	Business & Farming	2.02±0.76	2.33±0.34	2.74±0.39	0.0318	2.99±0.26	3.75±0.66	4.64±0.39	0.0002	3.28±0.35	3.28±0.52	
Joint disease	No	1.65±0.58	2.18±0.38	2.78±0.41	0.0001	3.16±0.29	3.56±0.57	4.53±0.52	0.0001	3.35±0.77	3.52±0.11	
	Yes	1.67±0.27	1.85±0.48	3.01±0.63	0.0021	3.25±0.94	3.44±0.13	4.44±0.23	0.0001	3.34±0.35	3.67±0.53	
HTN	No	1.68±0.55	2.27±0.49	2.82±0.44	0.0001	3.14±0.32	3.60±0.50	4.51±0.47	0.0001	3.43±0.44	3.61±0.42	
	Yes	1.60±0.57	2.10±0.28	2.87±0.59	0.0001	3.18±0.37	3.50±0.62	4.51±0.46	0.0001	3.25±0.26	3.72±0.60	
DM	No	1.65±0.51	2.16±0.43	2.83±0.49	0.0001	3.17±0.31	3.55±0.56	4.48±0.47	0.0001	3.47±0.57	3.58±0.42	
	Yes	1.66±0.79	2.18±0.26	2.89±0.26	0.0045	3.13±0.44	3.56±0.59	4.81±0.34	0.0001	3.29±0.25	3.68±0.53	

SIRT4 (d) SIRT5 (e) SIRT6 (f) SIRT7 (g) in different groups (AD, MCI and GC).

Fig. 2

Figure 2

ROC analysis showing the AUC for SIRT1 (a) SIRT3 (b) and SIRT6 (c) to distinguish AD vs GC, MCI vs GC and AD vs MCI.

Fig. 3

Figure 3

Scatter diagrams showing correlation between serum sirtuin protein concentration with HMSE (Score), (a) SIRT1 (b) SIRT3, (c) SIRT6 by Pearsons's correlation analysis.

Fig. 4

Figure 4

Western blot analysis to confirm the presence of serum (a) SIRT1 (b) SIRT3 and (c) SIRT6 in AD, MCI and GC subjects.