

# 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±SD) were significantly decreased in AD ( $1.65\pm 0.56$ ,  $3.15\pm 0.28$ ,  $3.36\pm 0.32$ ng/ $\mu$ l); compare to MCI ( $2.17\pm 0.39$ ,  $3.60\pm 0.51$ ,  $3.73\pm 0.48$ ng/ $\mu$ l) and GC ( $2.84\pm 0.47$ ,  $4.55\pm 0.48$ ,  $4.65\pm 0.55$ ng/ $\mu$ 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 behaviour 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 explained as impairment of one or more cognitive domains, is the earliest stage of disease, and 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 hallmark of AD comprises of extracellular aggregation of  $\beta$ -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- $\beta$  ( $A\beta$ ) oligomerisation and fibrillation [2]. Neurodegeneration in AD leads to 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].

Many studies have shown the potential role of Sirtuins in ageing and age-related diseases such as AD and other neurodegenerative disorders. 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 in parietal cortex of post-mortem AD patients' brains compared to controls reported in the literature. Accumulation of amyloid-beta and tau in the cerebral cortex of AD patients is strongly associated with the loss of SIRT1 [5]. In our previous study, SIRT1 was downregulated in the blood of AD patients compared to normal older subjects [6]. Study has found an association between AD susceptibility and a polymorphism in an intron of SIRT2 gene [7].

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 [8]. Mitochondrial ROS production increases in the brains of aged mice [9]. Similarly, there is a progressive trend toward a pro-oxidant state as human's age; thus, ROS increases with age [10]. 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 [11, 12]. The overexpression of SIRT1, SIRT3 reduced ROS generation, thereby prevent neuronal death [13, 14]. Absence of SIRT3 causes hyperacetylation of SOD2 (Superoxide dismutase2) and Cyp-D (*Cyclophilin D*) [15]. Both SIRT1 and SIRT3 levels were found to reduce in the entorhinal cortex and hippocampal sub-regions in the post-mortem human AD brain [16, 17]. SIRT3 regulates mitochondrial function and highly express in the brain [18]. It also mediated antioxidant and metabolic effects, which results in enhanced neuronal survival. Overexpression of SIRT3 and SIRT1 protect neurons from cellular stresses and ROS formations [19, 13], mitochondrial dysfunction, and apoptosis [20]. Calorie restriction reduced neurodegeneration by up-regulating SIRT3 [21] in AD and PD animal models. SIRT3 and SIRT6 expressions change the ageing brain in a region-specific manner [22, 23]. SIRT3 is also expressed in the tissues of kidney, heart, liver, adipose tissue and brain [24].

SIRT6 is present in the nucleus known as a longevity gene. It is a NAD-dependent histone deacetylase that has various roles such as telomere maintenance, DNA repair, genome integrity, energy metabolism, inflammation and life span regulation [25]. Accumulation of senescent cells in ageing due to the defective DNA repair and accumulation replication is one of the causes of for neurodegenerative diseases such as AD. SIRT6 were deficit in aged mice brain [26] and damaged DNA [27]. It is also downregulated in post-mortem brains of AD patients compared to cognitively normal subjects. It was found that  $A\beta$  42 significantly decreased SIRT6 expression, which was associated with a decrease level of p53 [28].

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 [29]. The potential roles of SIRT6 in AD have remained unexplored. SIRT4, SIRT5 and SIRT7 showed to have different roles in various tissues, but their role in brain was not explored.

Identification of biomarker molecules in blood would be more widely applicable than cerebrospinal fluid (CSF) due to non-invasiveness, less expensive and very rapid. Sirtuin protein may be the next target for AD for early diagnosis. Based on several findings on 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.

# 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 diagnosed with AD, 43 patients with MCI and 48 subjects were Geriatric Controls (GC). All were diagnosed 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 recruit as geriatric controls (GC). All the volunteers and informants who accompanied the patients to the Outpatient Geriatric Department gave the consent. Further, any participant with delirium or any chronic systemic illness and major psychiatric diseases were not included in the study. All possible reversible clinical causes were rule 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) [30]. Normal control group consist 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).

## **Estimation of serum sirtuin levels**

Two millilitres collected venous blood samples 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.

## *By SPR technology*

SPR was used for estimation of serum proteins, 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/μl), SIRT2 (0, 1.94, 3.42, 6.84, 11.42 ng/μl), SIRT3 (0, 2.1, 4.2, 6.4, 8.5, 10.7 ng/μl), SIRT4 (0, 1.40, 4.21, 8.43, 11.25, 14.06 ng/μl), SIRT5 (0, 2.7, 4.5, 6.3, 9.12 ng/μl) SIRT6 (0, 2.14, 4.28, 6.42, 8.56, 10.7 ng/μl) and SIRT7 (0, 1.55, 3.87, 5.42, 7.75, 11.62 ng/μl) passed over the respective immobilized antibodies on sensor chip and to obtained RU values.

The 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.

## *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 μl 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 μg 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**

Statistical analyses were performed in Stata/SE version 9 and Graph pad prism5 (College Station, TX, USA and GraphPad Software, Inc, La Jolla, USA). Descriptive analysis was done and presented with mean and standard deviation for all variables. Two groups were compared by using the Chi-square test for

categorical variables and student's t test for continuous variables. Three groups (AD, MCI and GC) were compared for continuous variables by using one-way ANOVA followed by post hoc comparison using the Bonferroni test. Correlation between two continuous variables was assessed using the Cary Pearson correlation coefficient. Receiver operating characteristics (ROC) analysis was performed for detecting optimum diagnostic cut-off value of Sirtuin proteins. Analysis of covariance was done to check the difference in the levels of Sirtuins between controls (GC) and patients (AD, MCI) after adjusting age, gender, education, occupation, and duration. The p-value of < 0.05 was considered as statistical tests were 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  $\geq 76$  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 ( $p < 0.0001$ ).

### *Estimation 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/mm<sup>2</sup> corresponds to one RU. Out of seven sirtuins concentration of serum SIRT1, SIRT3 and SIRT6 was found in GC group (SIRT1:  $2.84 \pm 0.47$  ng/ $\mu$ l, 95% CI: 2.70-2.97 ng/ $\mu$ l), (SIRT2:  $4.55 \pm 0.48$  ng/ $\mu$ l, 95% CI: 4.42-4.69 ng/ $\mu$ l), (SIRT3:  $4.65 \pm 0.55$  ng/ $\mu$ l, 95% CI: 4.48 - 4.81 ng/ $\mu$ l) significantly higher than MCI group (SIRT1:  $2.17 \pm 0.39$  ng/ $\mu$ l, 95% CI: 2.05-2.29 ng/ $\mu$ l), (SIRT2:  $3.60 \pm 0.51$  ng/ $\mu$ l, 95% CI: 3.45 - 3.76 ng/ $\mu$ l), (SIRT3:  $3.73 \pm 0.48$  ng/ $\mu$ l, 95% CI: 3.58- 3.88 ng/ $\mu$ l) , and AD group (SIRT1:  $1.65 \pm 0.56$  ng/ $\mu$ l, 95% CI: 1.48-1.82 ng/ $\mu$ l), (SIRT2:  $3.15 \pm 0.28$  ng/ $\mu$ l, 95% CI: 3.07 - 3.24 ng/ $\mu$ l), (SIRT3:  $3.36 \pm 0.32$  ng/ $\mu$ l, 95% CI: 3.26 - 3.45 ng/ $\mu$ l) (**Fig.1**).

SIRT1 (nonfrail-  $4.67 \pm 0.48$  ng/IL; frail -  $3.72 \pm 0.48$  ng/IL;  $P < 0.0001$ ), SIRT2 (nonfrail-  $15.18 \pm 2.94$  ng/IL; frail -  $14.19 \pm 2.66$  ng/IL;  $P = 0.016$ ), and SIRT3 (nonfrail-  $7.72 \pm 1.84$  ng/IL; frail -  $6.12 \pm 0.97$  ng/IL;  $P < 0.0001$ ) levels were significantly lower among frail patients compared with the non-frail.

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Serum sirtuin levels of other four sirtuins in Mean $\pm$ SD:

SIRT2 (GC- $11.28 \pm 1.71$  ng/ $\mu$ l, 95% CI: 10.79-11.78 ng/ $\mu$ l; MCI- $11.76 \pm 1.94$  ng/ $\mu$ l, 95% CI: 11.16 -12.36 ng/ $\mu$ l; AD-  $11.33 \pm 1.62$  ng/ $\mu$ l, 95% CI: 10.85-11.80 ng/ $\mu$ l; AD vs GC  $p=0.897$ , AD vs MCI  $p=0.2507$ , MCI vs GC  $p=0.2145$ ).

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

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

SIRT7 (GC- $2.31 \pm 0.63$  ng/ $\mu$ l, 95% CI: 2.13-2.49 ng/ $\mu$ l; MCI-  $1.81 \pm 0.37$  ng/ $\mu$ l, 95% CI: 1.69 -1.93 ng/ $\mu$ l; AD- $1.87 \pm 0.49$  ng/ $\mu$ l, 95% CI: 1.73- 2.02 ng/ $\mu$ l; 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/ $\mu$ l; sensitivity - 93.75 %; specificity - 87.23 %), AUC for distinguishing MCI vs GC was 0.8697 (cut-off - 2.52 ng/ $\mu$ l; sensitivity - 79.17 %; specificity - 76.74%) and AUC for distinguishing AD vs MCI was 0.7905 (cut-off - 1.90 ng/ $\mu$ l; 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/ $\mu$ l; sensitivity - 97.92 %; specificity - 95.74 %), AUC for distinguishing MCI vs Control was 0.9169 (cut-off - 3.99 ng/ $\mu$ l; sensitivity - 91.67 %; specificity - 79.07 %) and AUC for distinguishing AD vs MCI was 0.7840 (cut-off - 3.33 ng/ $\mu$ l; 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/ $\mu$ l; sensitivity - 93.75 %; specificity - 93.62 %), AUC for distinguishing MCI vs GC was 0.897 (cut-off -4.1 ng/ $\mu$ l; sensitivity -81.25 %; specificity - 76.74 %) and AUC for distinguishing AD vs MCI was 0.7288 (cut-off - 3.45 ng/ $\mu$ l; 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 detect 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 includes 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 [6, 31]. 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.

In the present study, subjects were more in the age category of 66-75 years. As per the literature, a cognitive decline and the transformation into dementia which becomes more progressive during this stage. Male subjects were more dominant than female subjects in our study, which may be due to the social context and lower economic status. Evaluation Methods such as a clinical score - HMSE and an imaging-MRI Scores were independently assessed for the study groups (AD, MCI, GC) and found significant ( $p < 0.0001$ ) with the disease. We have used standard cut-offs (26 and above) for HMSE scores for our subjects in our setup. The 50% out of total subjects were normal using HMSE, a highly sensitive scoring system in detecting a cognitive decline in our setup. All subjects were classified by using structural imaging (MRI) using MTA scores, the values were statistically significant among the groups.

This study explored the association of serum protein level that changes 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 and the stage/duration of the disease [32, 33], while expression of SIRT5 increases during the progression of AD [34].

It was reported that SIRT3 decreases in AD [33, 36]. Study in mice showed up-regulation of SIRT3 mRNA that followed the spatial and temporal profiles of A $\beta$  accumulation [38]. In the temporal cortex of AD patient's higher level of SIRT3 mRNA was found [39].

SIRT6 depletion causes DNA damage and ageing, reported in a recent study [40]. 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 [29]. 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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**Code Availability:** Not applicable.

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**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.

**Consent to participate:** Informed consent in writing was obtained from the controls, patients or their attendants (if a patient is incapable of making signature)

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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 <sup>th</sup> )	29 (21.1)	9 (19.15)	10 (23.26)	10 (20.83)	
Secondary (Class 6 <sup>th</sup> -12 <sup>th</sup> )	48 (34.78)	19 (40.43)	14 (32.56)	15 (31.25)	
Graduate & above (≥13 <sup>th</sup> )	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	
<b>Family history of AD</b>					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)	
<b>Duration of disease (Years)</b>					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	
<b>BADL</b>					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)	
<b>Hypertention</b>					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)	
<b>Joint disease</b>					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)	
<b>No. of co-morbidities</b>					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)	
<b>MRI (MTA Score)</b>					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	
<b>MRI (Parietal Score)</b>					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

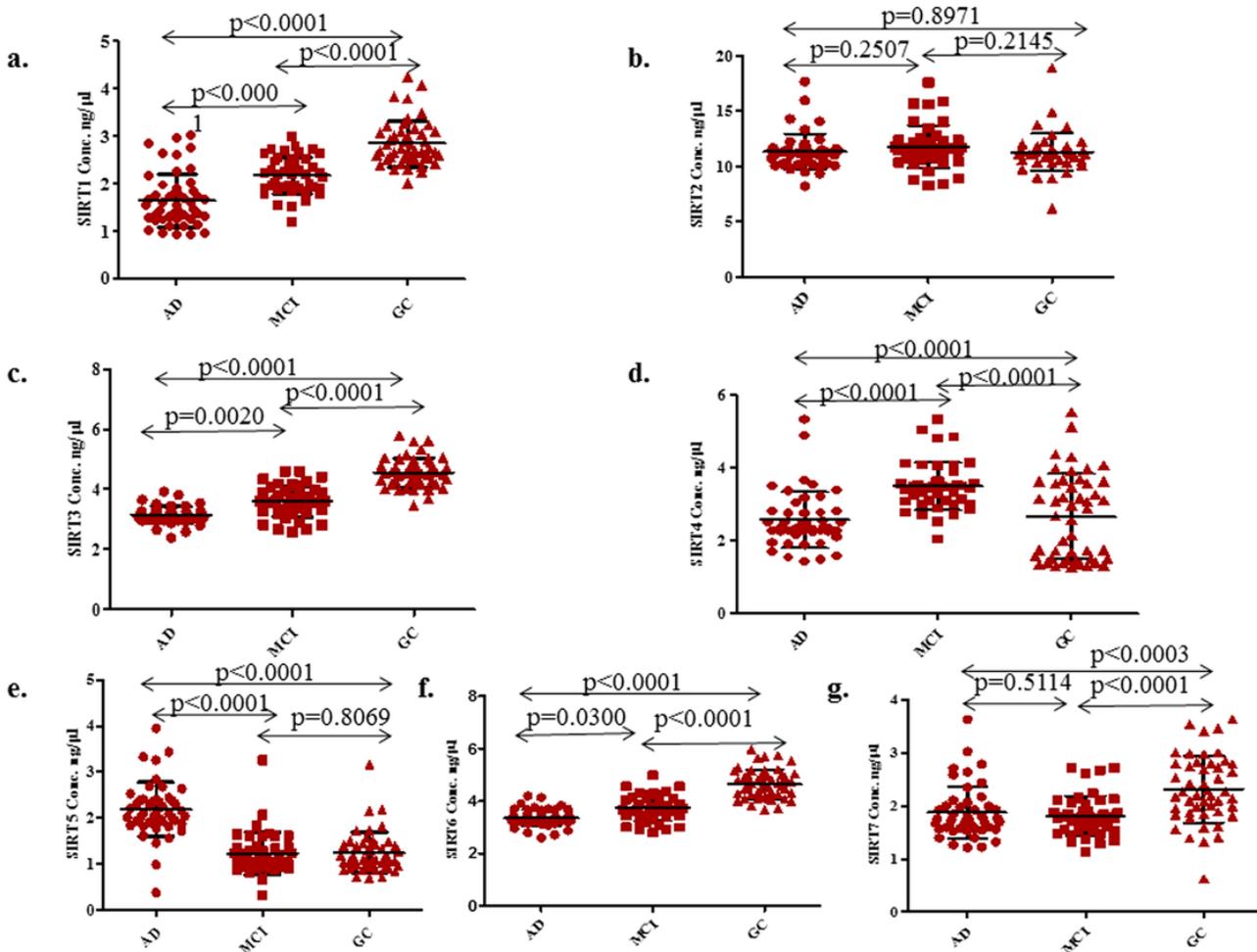
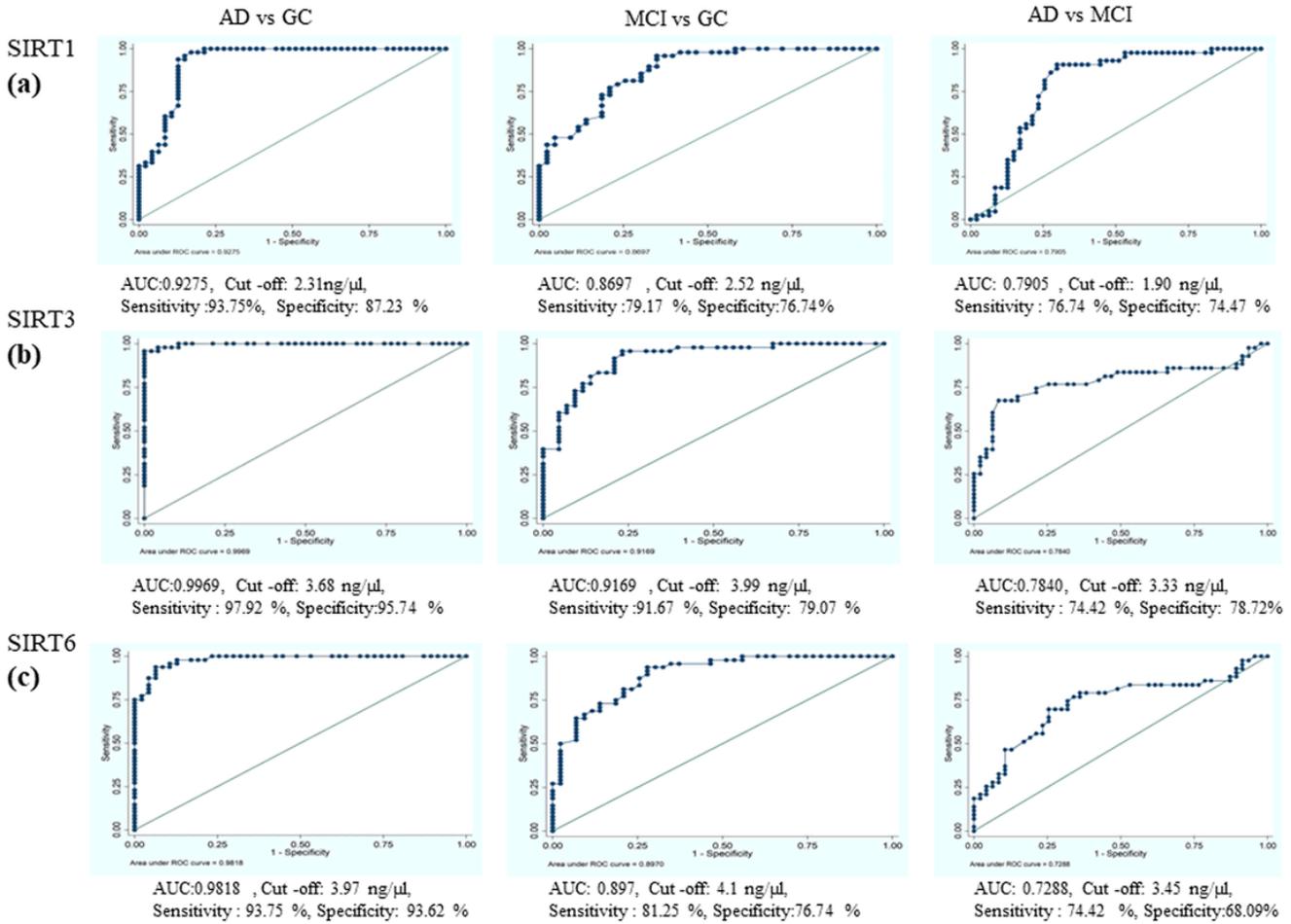


Fig. 1

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

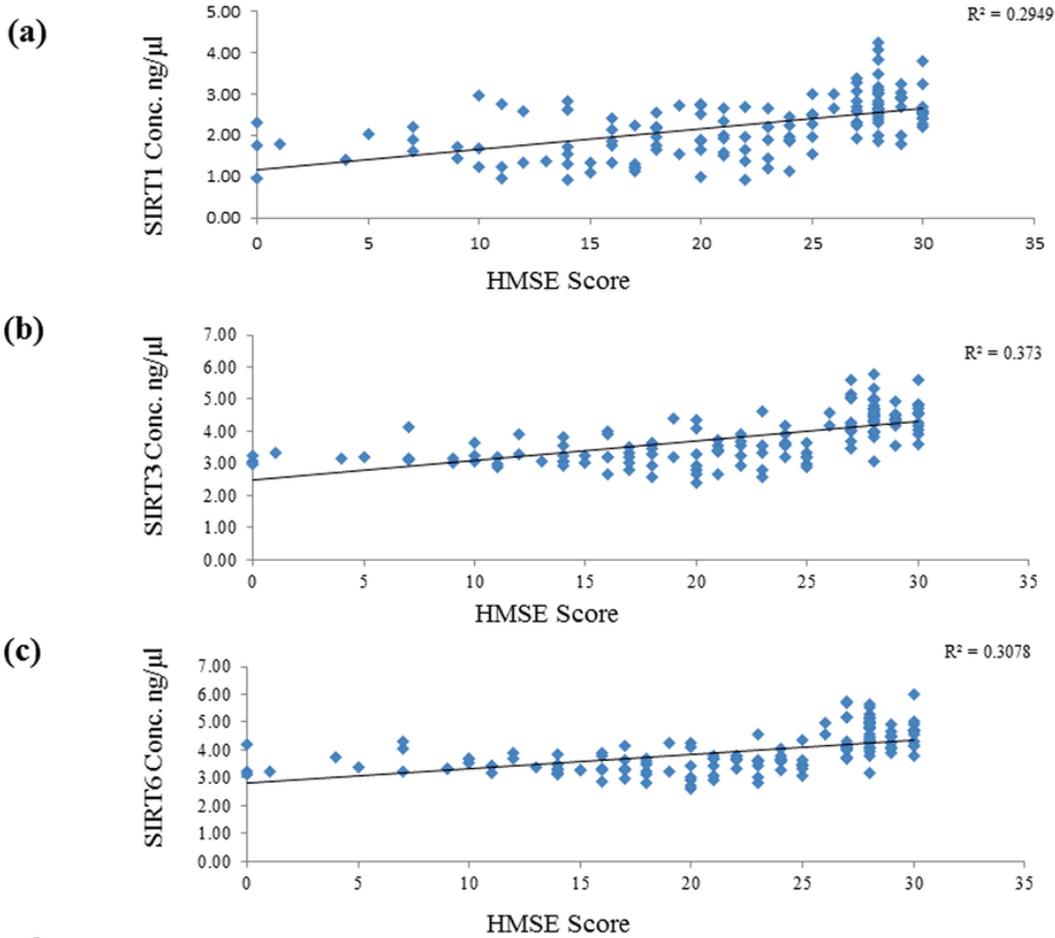
SIRT4 (d) SIRT5 (e) SIRT6 (f) SIRT7 (g) in different groups (AD, MCI and GC).



**Fig. 2**

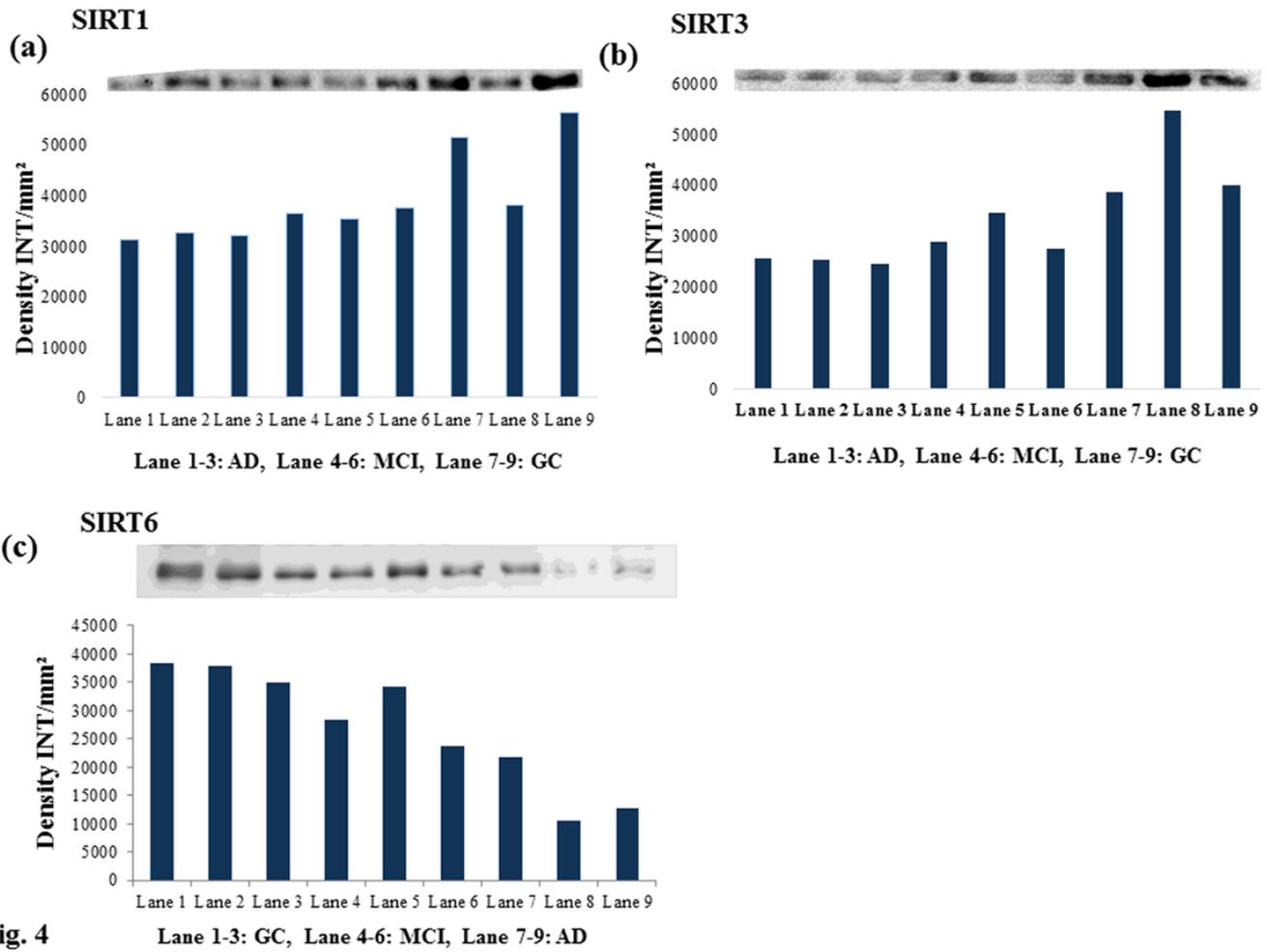
Figure 2

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 Pearson'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.