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Impact of hemolysis on the levels of proteins associated with aging and age-related neurodegenerative diseases in a multicentric clinical research

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ABSTRACT

Introduction: Hemolysis is a known interference factor that has been found to show erroneous effect. Present study analyzes the impact of hemolysis on the concentrations of protein biomarkers of Alzheimer's disease (Aβ42, t-Tau, p-Tau181) along with novel proteins which are currently under investigation (SIRT1,SIRT2,SIRT6,FOXO3A, NFL, Aβ40, GFAP).

Methods: Plasma samples were grouped into two categories: hemolyzed and non-hemolyzed groups. Degree of hemolysis (in percentage) was separately analyzed using Single molecule array (SIMOA) technology. Quantitative analysis for hemolyzed and non-hemolyzed samples were done using surface plasmon resonance (SPR) technology.

Results: The SIMOA analysis indicated that at high levels of hemolysis (1000 mg/dL) there was an increase in NFL protein level up to approximately 30 % whereas p-Tau181 did not show much interference even at higher hemolysate concentration. Aβ40, Aβ42 and GFAP showed modest effect up to hemolysis of 250mg/dL-500 mg/dL. SPR analysis of total Tau (t-Tau), p-Tau181, SIRT1, SIRT6 showed the consistency in the result and there was no significant difference in hemolyzed plasma compared to non-hemolyzed samples. Aβ42 and FOXO3A showed decline in hemolyzed plasma compared to non-hemolyzed samples (4.34 ± 0.18ng/ul; 4.95 ± 0.19ng/ul) and (3.83 ± 0.34ng/ul; 5.12 ± 0.46ng/ul), respectively whereas, a significant increase in the concentration was observed for SIRT2; 2.4 ± 0.10ng/ul in hemolyzed compared to 1.30 ± 0.22ng/ul in non-hemolyzed group.

Conclusions: High grade hemolysis leads to altered protein concentration associated with neurodegeneration. Present study emphasizes the need to have pre-analytical inspection for hemolysis detection especially in a multicentric biomarker study.

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1. Introduction

Circulating proteins are promising candidates for minimally invasive clinical biomarkers for the diagnosis, prognosis and monitoring of many human diseases. The ultimate objective of blood-based protein biomarker research is to establish a minimally invasive clinical test with high specificity and sensitivity, primarily for disease detection and progression. At present, amyloid beta42 (Aβ42) and total tau (t-tau) are the most common CSF (cerebrospinal fluid) biomarkers for AD diagnosis. However, CSF sampling involves lumber puncture which is a painful process. Therefore, blood based biomarker is preferred over CSF as it is minimally invasive and comparatively less painful. In a multicentric project the blood samples collected at distant field sites may need to be transported to a central laboratory. This can result in delayed blood processing or blood processing may be prone to pre-analytical errors, leading to sample rejection due to hemolysis. Previously, few studies had shown the variation in clinical diagnosis and biomarker assessment due to hemolysis [1–3]. The quantification of total protein assessment resulted in falsely elevated levels of total plasma protein concentration. In the presence of haemoglobin, erroneously high values have been reported for: lactate dehydrogenase, aspartate aminotransferase, potassium, acid phosphate, creatine kinase, alanine aminotransferase. Moreover, erroneously low values have been reported for bilirubin, alkaline phosphatase, albumin (by electrophoresis) and gamma-glutamyltransferase [4]. The interference caused by hemolysis in classical cardiac biomarkers has been extensively studied [5–8]. Hemolytic samples are a rather common and unfavorable occurrence in laboratory practice, as they are often considered unsuitable for routine testing due to biological and analytical interference. Hemolysis can result in analyte suppression or enhancement, or can affect the extraction efficiency and analyte stability. Hemolysis has been found to interfere with biomarker analysis in blood-derived specimens [9,10]. Hemolytic samples are often considered unsuitable for routine testing due to biological and analytical interference. A critical evaluation of blood-based biomarkers is important for its robustness and reliability. Protein biomarkers in biological fluids, especially blood samples in particular, have the potential to identify risk of disease or to allow early detection for more effective treatment.

Proteins associated with aging and age-related diseases have been one of the most important analytes to understand the molecular pathophysiology of aging and age-related diseases, specifically neurodegenerative disease. Biomolecules such as amyloid beta40 (Aβ40), amyloid beta42 (Aβ42), neurofilament light chain protein (NFL), Glial fibrillary acidic protein (GFAP) and phosphorylated tau (p-Tau181) are potential biomarkers for dementia and neuro-degenerative diseases [11]. Recent studies has shown the role of Aβ42/Aβ40 as a robust measure for detecting amyloid plaques and it can be utilized to aid in the diagnosis of Alzheimer's disease (AD) and also can identify those at risk for future dementia due to AD [12]. NFL is another relevant molecule that has been reported as a biomarker of disease severity and to predict survival in Parkinson disease [13]. Importantly plasma GFAP, a monomeric intermediate filament protein found in the astroglial cytoskeleton has been recently shown as an early marker associated with brain Aβ pathology but not tau aggregation, even in cognitively normal individuals with a normal Aβ status [14].

Aging is a major risk factor for neurodegenerative diseases. At present, Sirtuins and fork head box classO3A (FOXO3A) are the focus of research for its emerging role in ageing and longevity factors [15,16]. Sirtuins contribute to age-associated cognitive decline through the regulation of synaptic plasticity and adult neurogenesis [17]. Sirtuin1 (SIRT1), the most studied member of this family, plays an important role in several processes ranging from cell cycle regulation to energy homeostasis. SIRT1 has tumor suppression activity in ageing and has been found to reduces the functions of PGC-1α and FOXO1, thereby resulting in the reduction of mitochondrial biogenesis, oxidative metabolism, and anti-oxidant defense pathways. Sirtuin2 (SIRT2) has been established that SIRT2 level increases with aging, and a growing body of evidence shows that the activity of SIRT2 mediates various processes such as Parkinson disease pathogenesis, including aggregation of α-synuclein (α-syn), microtubule function, oxidative stress, inflammation, and autophagy [18]. Sirtuin6 (SIRT6), is another important protein with anti-aging effects. It inhibits aging via four main pathways: promotion of DNA damage repair, maintenance of the normal telomere structure of chromosomes, regulation of glucose and NAD + metabolic balance, and regulation of the senescence-associated secretory phenotype [19]. Moreover, FOXO has been found to act as a molecular link between aging and disease risks including type 2diabetes and AD. Genetic variation in FOXO3 has been shown to be associated with health span as well as longevity in humans [16,20–22]. All the protein molecules studied here are being investigated as a potential biomarker candidate in different aging and neurodegenerative diseases to achieve the harmonized reference values by conducting multi-centric studies.

In the present study, we have analyzed the degree of hemolysis interference with the different analytes and quantified the difference in the plasma concentration of proteins (Aβ40, Aβ42, NFL, GFAP, t-Tau and p-Tau 181) in age-related neurodegenerative disorder and novel neuronal proteins (SIRT1, SIRT2, SIRT6 and FOXO3A) that are most strongly associated with aging, using two most sensitive technologies; Single molecule arrays (SIMOA) which is an ultra-sensitive immunoassay technology that allow detection of proteins and nucleic acids at lowest possible levels and Surface plasmon resonance (SPR) which is an optical-based, label-free detection technology for real-time monitoring of binding interactions between two or more molecules. The throughput, flexibility and sensitivity of the SPR platform gives researchers the potential to characterize biomolecular interactions in any binding study.

2. Materials and methods

2.1. Study participants

Venous blood samples were collected (5 mL) from 27 healthy volunteers into different spray-coated K_2EDTA tubes (BD Vacutainer®, United States) to study the effect of in vitro hemolysis, after the participants had given the informed consent to participate in the study. The study was performed as per the rules and regulations of the Institute Ethics Committee (IECPG-251/March 24, 2021), All India Institute of Medical Sciences, New Delhi.

2.2. Blood processing, plasma isolation and storage

The collected blood samples from each participant were divided equally into two micro-centrifuge tubes. The first set of tubes was taken for plasma isolation to obtain non-hemolyzed plasma while the other set underwent a freeze-thaw cycle at −80 °C to cause hemolysis (Supplementary Table S 1).

Plasma Isolation: The collected blood samples were centrifuged to remove cells from plasma. Centrifugation was done for 10 min at 3000×*g* using a refrigerated centrifuge. Repeat centrifugation was given for 15 min at 2000×*g* to deplete platelets in the plasma sample. The resulting supernatant was collected as plasma into a clean micro-centrifuge tube. The plasma supernatant from hemolyzed and the non-hemolyzed samples were harvested after the first centrifugation with care not to disturb the underlying blood cell layer.

Storage: Multiple aliquots of the isolated plasma samples were prepared and it was carefully stored at −80 °C for the SIMOA HD-X as well as SPR quantification analysis.

2.3. Preparation of hemolytic blood samples

Hemolytic blood plasma samples were prepared using ASSURANCE™ Interference Test Kit (INT-01H, Sun Diagnostics, USA). The procedure was followed as per the manufacturer's instruction. Briefly, different amounts of hemolysate were added to the isolated plasma to simulate various degrees of hemolysis as described below:

- 1000 mg/dl: 2 ml of base plasma +0.143 ml of hemolysate
- 500 mg/dl: 1:2 dilution of 1000 mg/dl
- 250 mg/dl: 2 ml of base plasma +0.036 ml of hemolysate
- 100 mg/dl: 2 ml of base plasma +0.014 of hemolysate
- 50 mg/dl: 1:2 dilution of the 100 mg/dl
- 25 mg/dl: 1:4 dilution of the 100 mg/dl
- 0 mg/dl: No hemolysate added

2.4. Hemolysis interference analysis by SIMOA HD-X analyzer

The hemolysate added blood plasma samples were quantified for different analytes (i.e., NFL, Aβ40, Aβ42, GFAP) using the Simoa Neurology 4-Plex E kit (N4PE, Ref; 103670). p-Tau181 interference was measured using the Simoa p-Tau181 Advantage Kit (Ref; 104111, Simoa p-tau-181 V2 Advantage Kit). All measurements were performed on the Simoa HD-X analyser, according to manufacturer's instructions (Quanterix, Billerica, Massachusetts, USA). Samples were diluted 1:4 using the instrument's on-board dilution protocol and run in duplicate from a single well each on a 96-well plate. Eight-point calibration curves and sample measurements were determined on Simoa HD-X analyzer software using a weighting factor $1/Y^2$ and a four-parameter logistic curve fitting algorithm.

2.5. Hemoglobin (HGB) estimation

Aliquots of hemolyzed plasma were taken for estimation of HGB concentrations using automated hematology analyzer XN1000sysmex. The concentration of hemoglobin was measured in an increasing gradation of plasma sample colour (N = 8). Hemolyzed plasma samples were analyzed to set the cut off value. Samples containing more than 100 mg/dL of hemoglobin were set as a cut-off value and the sample was considered as hemolyzed for the study (Reference ranges: 0–100 mg/dL for plasma HGB).

Samples were categorized according set cut off HGB concentrations into 2 groups:

Group I (Less than or equal to 100 mg/dL), non-hemolyzed (N = 27);
Group II (Greater than 100 mg/dL) hemolyzed (N = 27);

The results were analyzed to determine whether hemolysis had a significant impact on the analyte concentrations.

2.6. Evaluation of proteins analytes by SPR

Quantitative alterations in the concentration of well-known neurodegenerative as well as age associated protein molecules such as Aβ42, t-Tau p-Tau181, SIRT1, SIRT2, SIRT6, FOXO3A, were analyzed between hemolyzed and non-hemolyzed plasma (groups I & II) using SPR technology.

Quantification of proteins in plasma were performed by SPR, using the BIAcore-3000 (Wipro, GE Healthcare, Sweden) which is a biosensor-based system for real time label free monitoring of specific interaction analysis. All SPR measurements were performed at 25 °C. Primary antibodies used were mouse anti-human SIRT1 monoclonal IgG (Sc-74504,Santa Cruz Biotechnology Inc. USA), goat anti-human SIRT2 polyclonal IgG (Sc-31912, Santa Cruz Biotechnology Inc. USA), rabbit anti-human SIRT6 polyclonal IgG (Sc-517196,Santa Cruz Biotechnology Inc. USA), mouse anti-human FOXO3A IgG (Sc-48348,Santa Cruz Biotechnology Inc. USA), goat anti-human t-Tau IgG (Sc-32274, Santa Cruz Biotechnology Inc. USA) and rabbit anti-human p-Tau181 IgG (12885S (D9F4G), Cell Signaling Technology Inc. MA, USA) were immobilized in different flow cells of CM5 sensor chip via amine coupling kit (Wipro GE Healthcare, Sweden). The system was equilibrated with a running buffer; HBS-EP buffer and maintained at a flow rate of 5 ml/min. The

experimental flow cell dextran was activated using 1:1 vol mixture (110 μl each) of N-ethyl-N′-3 diethylaminopropylcarbodiimide (EDC) (75 μg/μl) and N-hydroxysuccinimide (NHS) (11.5 μg/μl). The antibody was diluted to 100 μg/ml in 10 mM sodium acetate (pH 5.0) and injected over the activated chip surface. Unreacted groups were blocked by ethanolamine (pH 8.5).

Different concentrations of purified recombinant human SIRT1, SIRT2, SIRT6, FOXO3A, Aβ42, t-Tau, and p-Tau181 proteins were passed over the immobilized antibodies on respective flow cells and corresponding resonance units (RU) were obtained. The standard curve was prepared by plotting different concentration of the proteins versus corresponding RU obtained.

Similarly, plasma samples of the participants from group I and group II were diluted (1:80) with HBS-EP buffer and passed over the corresponding immobilized antibodies in respective flow cells. The RU for each sample was recorded and the concentrations of proteins in the plasma of hemolyzed vs non-hemolyzed samples were calculated from the standard curve.

2.7. Biased percentage calculation: to compare the concentrations of the hemolyzed samples with non-hemolyzed samples, bias percentage was calculated by the formula

$$[(CX - C1) / C1)]x\ 100$$

C1: Concentration of non-hemolyzed sample,
CX: Concentration of hemolyzed sample.

2.8. Statistical analysis

The data were analyzed by Graph pad prism5 (GraphPad Software, Inc, La Jolla, USA) software. The results were presented in mean with standard deviation and percentage. The Student t-test was used to compare the continuous variables between the groups. A *p*-value <0.05 was considered to be statistically significant.

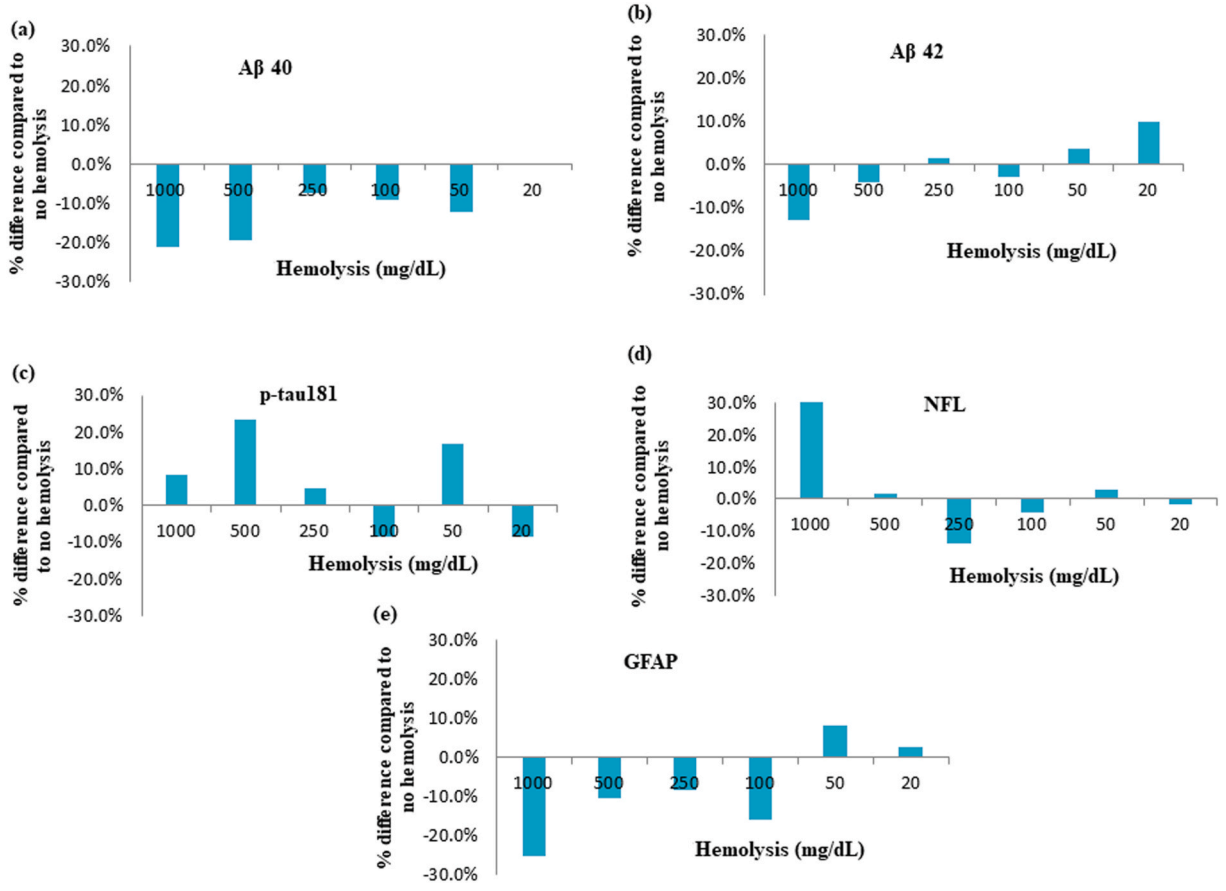

Fig. 1. Effect of hemolysis on different proteins involved in neurodegenerative diseases. (a) Aβ 40, (b)Aβ 42, (c) p-tau181 (d) NFL (e) GFAP. Varying degrees of hemolysis interference can be observed at different hemolysate concentrations. High levels of hemolysis increase NFL levels. Increasing levels of hemolysis decrease levels of Aβ 42, Aβ 40 and GFAP, the overall effect is found to be modest up to hemolysis of 250mg/dl-500 mg/dl. Ptau181 shows minimal effect.

3. Results

3.1. Hemolysis interference analysis by SIMOA HD-X

Aβ 40: Low grade hemolysis up to 250 mg/dL showed minor effect on Aβ 40 concentrations (Fig. 1a) whereas high hemolysate concentration (500–1000 mg/dL) showed interference, leading to quantitative decline approximately 20 %.

Aβ 42: No difference was observed in Aβ42 proteins at low grade hemolysis. At high hemolysate concentration (1000 mg/dL), a decrease of approximately 10 % was detected for Aβ 42 (Fig. 1b).

p-Tau181: p-Tau181 showed minimal hemolysis interference effect. No consistent interference effect was observed both at low grade as well as high grade hemolysis (Fig. 1c).

NFL: Varying degrees of interference can be observed at different concentrations of hemolysate. No substantial changes were observed at low level of hemolysis. High level (1000 mg/dL) of hemolysis increases levels up to approximately 30 % (Fig. 1d).

GFAP: A considerable decline can be observed in the concentration of GFAP with the increased hemolysis. High level of hemolysis (1000 mg/dL) leads to quantitative decline (25 %) in the concentration of protein in plasma samples (Fig. 1e).

3.2. Estimation of hemoglobin concentration

The concentration of hemoglobin varied from 100 to 1600 mg/dL (Supplementary Fig. S1). Hemoglobin concentration less than or equal to 100 mg/dL were considered as non-hemolyzed whereas samples having hemoglobin concentration above 100 mg/dL were taken as hemolyzed.

3.3. Quantitative estimation of analyte concentration in hemolyzed vs non-hemolyzed samples by SPR

The SPR analyses of all the proteins were determined using the standard plot obtained through the known protein concentrations. The SPR signal obtained after immobilization of antibodies were noted. The standard curves were plotted between RU and the obtained different concentrations of respective pure proteins. The binding of the ligands (proteins) were in the linear range. The RU increased linearly as the concentration of proteins were increased which provided evidence of sensitivity of the proteins. The concentrations of proteins in plasma were determined from the standard curve using RU obtained from binding of plasma over the respective antibodies. One RU corresponds to immobilized protein concentration of 1 pg/mm^2. Table 1 summarizes the quantitatively altered concentrations of all the proteins investigated in this study to analyze the impact of hemolysis.

Aβ42: Aβ protein concentration in non-hemolyzed samples was found to be 4.95 ± 0.19ng/ul whereas in hemolyzed samples it was 4.34 ± 0.18ng/ul (Fig. 2a). The decrease in concentration of Aβ42 in hemolyzed sample was found to be a very small change (0.62ng/ul).

t-Tau: The concentrations of t-Tau in the plasma of hemolyzed and non-hemolyzed samples were found to be 67.63 ± 4.89 ng/μl and 66.58 ± 2.81 ng/μl respectively (Fig. 2b). No significance difference was observed in the blood plasma t-Tau protein concentration with respect to hemolyzed versus non-hemolyzed plasma samples (p-value = 0.8538).

p-Tau: The concentration of pTau protein in the blood plasma of hemolyzed and non-hemolyzed samples were found to be 0.74 ± 0.05 ng/μl and 0.73 ± 0.04 ng/μl respectively (Fig. 2c). No difference was observed with respect to hemolyzed versus non-hemolyzed plasma samples (p-value = 0.8122).

SIRT1: The concentrations of SIRT1 in the plasma of hemolyzed and non-hemolyzed samples were found to be 6.84 ± 0.27 ng/μl and 6.95 ± 0.65 ng/μl respectively. Hence, no significance difference was observed with respect to hemolyzed versus non-hemolyzed plasma samples (p-value = 0.8768) (Fig. 3a).

SIRT2: The estimated concentration of SIRT2 was found to be significantly increased (p-value <0.0001) in hemolyzed samples by 85 %. It was found to be 1.30 ± 0.22ng/ul in non-hemolyzed sample whereas in hemolyzed samples, 2.4 ± 0.10ng/ul (Fig. 3b).

SIRT6: The estimated concentrations of SIRT6 protein in the blood plasma of hemolyzed and non-hemolyzed samples were found to be 7.54 ± 0.45 ng/μl and 5.62 ± 0.91 ng/μl respectively (Fig. 3c). The difference was marginal with respect to hemolyzed versus non-hemolyzed plasma samples.

FOXO3A: The estimated concentration of FOXO3A was found to be decreased in hemolyzed samples by 25 %. In non-hemolyzed samples it was 5.12 ± 0.46ng/ul whereas in hemolyzed samples it was 3.83 ± 0.34ng/ul (Fig. 3d).

Table 1
Quantitative comparison of proteins in Hemolyzed and Non Hemolyzed plasma samples by SPR.

Proteins	Concentration (Non-Hemolyzed) (ng/ul)	Concentration (Hemolyzed) (ng/ul)	p -Value
SIRT1	6.95	6.84	0.8768
SIRT2	1.30	2.41	<0.0001
SIRT6	5.61	7.53	0.0564
FOXO3A	5.12	3.83	0.0272
t-Tau	66.58	67.63	0.8538
p-Tau	0.728	0.744	0.8122
Aβ 42	4.95	4.34	0.0206

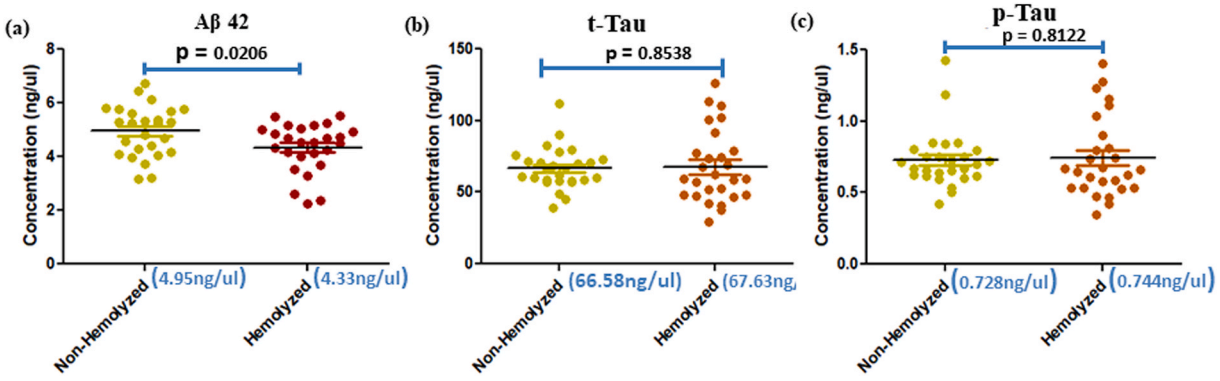

Fig. 2. Variation in age-associated neurodegenerative protein concentrations in hemolyzed samples. (a) A significant decline of 0.616ng/ul was observed for Aβ 42 protein in hemolyzed blood plasma compared to non-hemolyzed samples ($p = 0.0206$). Comparable protein concentrations were observed for both (b) t-Tau and (c) p-Tau protein $p = 0.8538$ and $p = 0.8122$ respectively.

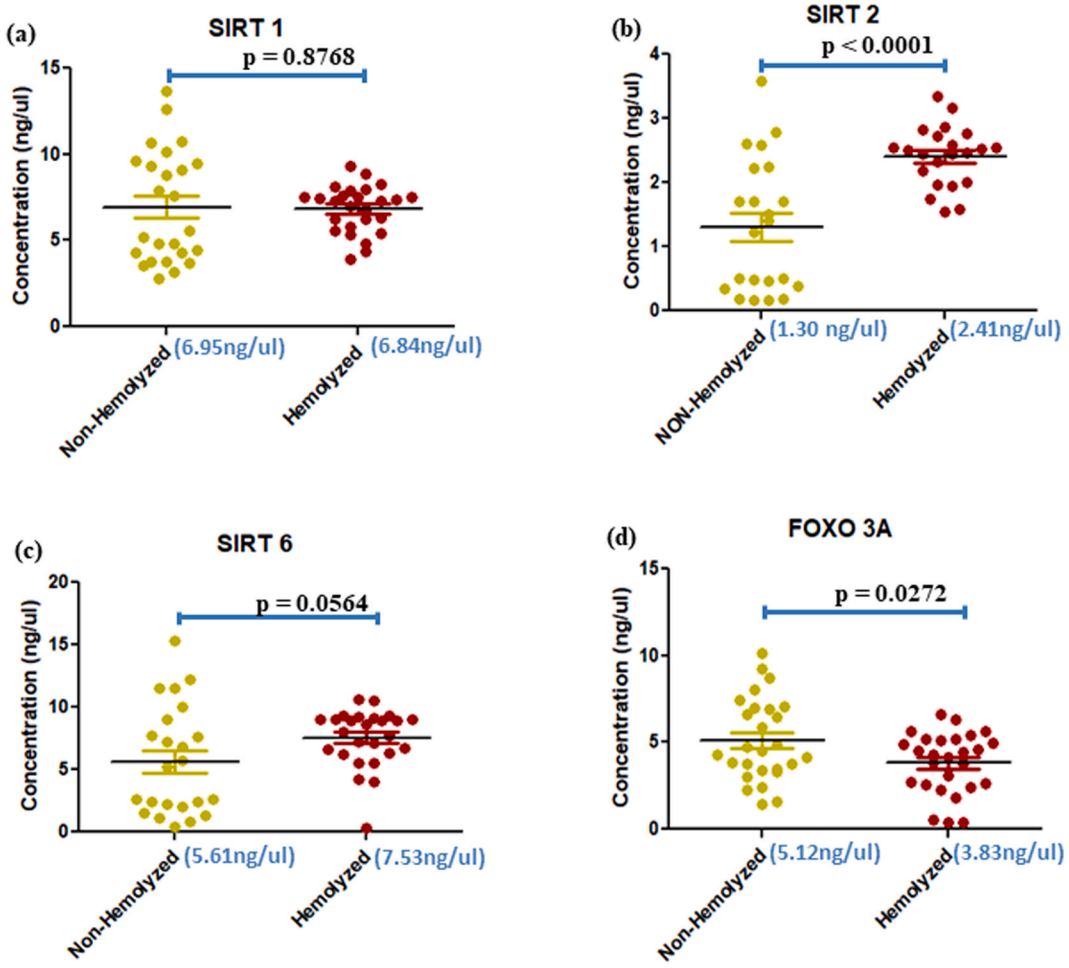

Fig. 3. Difference in protein concentrations in Non-Hemolyzed versus Hemolyzed samples: (a) Comparable concentration can be observed for SIRT1 protein. (b) A significant increase of 1.11ng/ul was observed for SIRT2 protein in Hemolyzed blood plasma compared to non-hemolyzed samples ($p < 0.0001$). (c) Increase in SIRT6 protein was not found to be significantly affected due to hemolysis ($p = 0.0564$). (d) A significant decline of 1.29ng/ul was observed for FOXO3A protein in hemolyzed blood plasma compared to non-hemolyzed samples ($p = 0.0272$).

4. Discussion

Translation of biomarkers into disease diagnostics requires a comprehensive understanding of the impact of sample handling to avoid false prediction of biomarkers. Proteins contamination caused by RBC due to hemolysis impacts the quantitation assessment of biomarker study [23]. It has also been found that the stored blood samples leads to the changes in the viability of the RBC and accumulation of contaminating proteins and cells. These changes in RBC ultimately lead to hemolysis, and consequently, a release of the cytosolic contents into solution [24,25]. To our knowledge, this study first time provides comprehensive information regarding the impact of hemolysis on the concentration of proteins associated with aging and age-related neurodegenerative diseases in the human blood plasma.

In our assessment of degree of hemolysis interference on neuronal proteins, we found high grade hemolysis impacted the protein concentration. NFL showed interference at 1000 mg/dL hemolysate, whereas at low grade hemolysis, the interference was found to be minimal. Aβ40 and Aβ42 exhibited variable effect, both at low as well high hemolysate concentrations. Kiko et al. had previously found that the RBC Aβ levels increases with age and suggested that plasma Aβ40 and Aβ42 bind to RBCs, implying a pathogenic role of RBC Aβ. On the other hand, providing an antioxidant supplement (astaxanthin, a polar carotenoid) to humans, it has been shown to decrease RBC Aβ as well as oxidative stress marker levels [26]. RBC-Aβ interference might lead to alterations in the concentration of Aβ40 and Aβ42.

This study found GFAP protein interference analysis indicates at high grade of hemolysis, (1000 mg/dL) leads to increased GFAP concentration. The release of intracellular analytes into serum/plasma in hemolyzed specimens is known to cause bias in test results. This bias is dependent on the intracellular concentration of the analyte which is being investigated, the method/instrument used, and the degree of hemolysis [27].

In our study of age-associated proteins, SIRT1 and SIRT6 did not show any variation in hemolytic versus non-hemolytic plasma samples whereas SIRT2 showed two-fold increase in blood plasma concentration of hemolyzed samples. FOXO3A showed a decline of 1.29ng/ul in hemolyzed samples compared to non-hemolyzed. FOXO3A is also critical for hematopoietic self-renewal. In vivo experiments have established that FOXO3A deletion in hematopoietic stem cells increases ROS and impairs their hematopoietic capacity [28,29]. The analysis of microtubule-associated protein t-Tau and p-Tau exhibited no significant difference in the concentration of hemolyzed versus non-hemolyzed plasma samples. Aβ42 showed a minor decline in hemolyzed plasma compared to non-hemolyzed samples. Aβ42 and Tau are considered as the trigger and bullet in AD pathogenesis [30]. Aβ has been shown to play a key role by oxidatively impairing the capacity of red blood cells (RBCs) to deliver oxygen to the brain [26]. The integrity of RBCs plays a crucial role in blood based biomarker research. Delayed centrifugation or temperature variation during the plasma/serum isolation protocol severely impacts the analytes concentrations. Previous study had found that the introduction of ice after 1 h at room temperature causes a significant reduction in the concentration of eotaxin, G-CSF compared to room temperature for 1 h alone. This reduction in concentration suggests that ice can also negatively impact specific analyte levels and the lowest measurable value may not necessarily be the closest to in vivo value [31]. Thus, it can be inferred from the study that the presence of high degree of hemolysis leads to interference in the concentration of analytes which are under investigation for the biomarker study.

5. Conclusion

Present study concludes that high grade hemolysis has severe implication on biomarker study. Varying impact may be observed at low grade hemolysis. This study specifically highlights the effect of hemolysis on proteins associated with aging and age-related disorder. The study also emphasizes on the need to ensure the pre-analytical factors specially hemolysis in a multicentric biomarker research. This will ensure the sample quality for proper quantitative measurements of the analytes, its reproducibility and validation. Based on hemolysis colour change, severely hemolyzed samples may directly be excluded from the study which might interfere with the concentration of biomarker predictor. Incorporation of the present information on the impact of hemolysis on level of protein will provide an efficient way to obtain valid inference for accurate detection of biomarker candidate in a multicentric study.

CRediT authorship contribution statement

Masroor Anwar: Writing – original draft, Validation, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Km Renu:** Methodology, Formal analysis. **Abhinay Kumar Singh:** Formal analysis, Data curation. **Abhilasha Nayal:** Formal analysis. **Bharat Thyagarajan:** Formal analysis. **Peifeng Hu:** Formal analysis. **Jinkook Lee:** Formal analysis. **Sharmistha Dey:** Writing – review & editing, Validation, Supervision, Conceptualization. **A.B. Dey:** Formal analysis.

Data availability

All materials used in this study will be made available on request.

Ethics approval

All India Institute of Medical Sciences Ethics Committee (AIIMS) approved the study protocol (IECPG-251/March 24, 2021)).

Consent for publication

The manuscript contains no individual person's data in any form.

Declaration of competing interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plabm.2025.e00455.

Data availability

Data will be made available on request.

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