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ORIGINAL ARTICLE

Metals toxicity and its correlation with the gene expression in Alzheimer's disease

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Abstract

Alzheimer's disease is a common neurodegenerative disease in the elderly population and a leading cause of dementia. Genetics and environmental risk factors were considered to play a major role in the onset of the disease. This study aimed to examine the correlation between different metals levels and the gene expression in Alzheimer's patients with age-matched control subjects. Non- essential metals were measured in the whole blood due to its higher concentration in red blood corpuscles (RBCs) and essential biometals in the serum samples of Alzheimer's disease (AD) by using Inductively coupled plasma optical emission spectroscopy (ICP-OES) that allows the analysis and detection of the different elements at low levels. Gene expression level was performed by quantitative real-time PCR (qRT-PCR). In this study, the levels of Lead and Arsenic metals were not detected in the AD patient samples. Cadmium, Mercury, and Aluminum were found higher in cases as compared to controls with 0.009240 ± 0.0007707 ($P = < 0.0001$), 0.02332 ± 0.001041 ($P = < 0.0001$), and 0.09222 ± 0.02804 ($P = 0.0087$) respectively. Essential biometal like copper was higher 0.1274 ± 0.02453 ($P = 0.0254$) in cases, while iron 0.1117 ± 0.009599 ($P = 0.0304$) and zinc 0.03800 ± 0.003462 mg/L were found significantly lower as compared to controls. All targeted genes such as APP, PSEN1, PSEN2, and APOE4 were found up-regulated in AD patients. We concluded that there was no significant correlation between metals dyshomeostasis and gene expressions in this study.

Keywords Neurodegenerative disease · Gene expression · Non- essential metals · Apolipoprotein · Amyloid-beta plaque · Alzheimer's disease

Introduction

Alzheimer's disease is characterized by neuronal cell death and synaptic loss of the brain with the accumulation of insoluble amyloid-beta (Aβ) protein and hyperphosphorylated tau protein [1, 2]. Many studies have focused on finding out reliable biomarkers in the peripheral blood for early clinical diagnosis, but facing problems due to the multifactorial nature of the disease onset [3, 4].

Genetics and environment, both may play an important role in an early and late-onset of the disease [5]. Metal ions such as zinc and copper have a key role in brain

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neurobiology. Its altered homeostasis may contribute to protein misfolding and influence protein energetics, with specific attention on conformational and structural changes [6–8]. Essential metals like Zn, Cu, and Fe were found to be excessively bound with amyloid-beta and nearby extracellular senile plaques [9, 10]. Some non-essential metals (As, Pb, Cd, and Hg) have also been reported to induce cognition impairment in the brain [11]. The four most common genes that have been associated with more than half of the genetic risk of Alzheimer's disease are amyloid precursor protein (APP) on chromosome 21, presenilin 1 on chromosome 14, presenilin 2 on chromosome 1, and apolipoprotein E on chromosome19. Apolipoprotein (ApoE) has the most important genetic role in the late-onset of the disease; this protein is important for the proper growth, maintenance, and transportation of cholesterol in synapses [12]. PSEN2 gene has been found significantly downregulated in the auditory cortex region of the brain by using real-time PCR [13]. However, many studies have used a blood sample to estimate the level of heavy metals for many years [14–16]. Furthermore, the inductively coupled plasma-optical emission spectrometry technique (ICP-OES) is the most commonly used technique for multi-element analysis in biological samples [17, 18]. In this context, wet digestion of various biological samples using mineral acids has been followed by many studies [19–21]. The microwave digestion technique is more reliable and efficient, currently been used widely for biological sample preparation [15, 22, 23]. This study was conducted to investigate the correlation of metals toxicity with gene expression levels in AD patients.

Material and method

This study was approved by the Ethics committee of King George's Medical University, Lucknow. Written informed consent forms were collected from all the subjects or primary caregivers. Probable Alzheimer's patients mainly from the Indian population n = 50 (32 females and 18 males) were enrolled with 50 age-matched non-demented control subjects from the department of the Geriatric Mental Health department. Four ml peripheral blood sample was collected from each patient into a plane and EDTA vial. The patients were clinically diagnosed by criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer Disease and Related Disorders Association (NINCDS-ADRDA). Patients were enrolled by following Mini-Mental State Examination criteria (MMSE ≤ 20) to understand the disease progression; the P-value was < 0.0001, which is statistically significant. Subsequently, the MMSE score for the non-demented control subject was between 26 and 30. The mean age for the Alzheimer's patient was 74.13 ± 1.683. The person suffering from Diabetes mellitus, hypertension, and other chronic illness were excluded from this study.

Metals estimation

Inductively coupled plasma optical emission spectroscopy (ICP-OES) was used for metals analysis such as lead, mercury, cadmium, aluminum, and arsenic (Pb, Hg, Cd, Al, and As) from blood samples of Alzheimer's disease. Blood samples were digested by using the Multiwave Reaction System (Multiwave 3000, Anton Paar, Perkin Elmer) with the rotor 16HF100 (100 ml PFA vessels, 40 bars) and pressure, temperature (p/T) sensor. All samples were appropriately homogenized by vortexing for 2 min before pipetting and then transferred into a closed vessel for the acid digestion following the (Hseu 2004) method [24]. The reaction mixture was: 0.5 ml whole blood, 1.5 ml of nitric acid, 0.5 ml perchloric acid, 1 ml hydrogen peroxide (Thermo Fisher Scientific), and 1 ml ultra-pure water from a milli-Q purification apparatus with one blank sample at each set simultaneously, according to the digestion program shown in the electronic supplementary material (Appendix S1).

Essential metals zinc, copper, and iron (Zn, Cu, and Fe) were analyzed from the serum samples diluted in a 1:3 ratio. The serum was isolated from the whole blood centrifuged at 2000 rpm for 15 min. Multi-element standard (Perkin Elmer Pure Plus, USA) stock solution 1000 mg/L was used for the preparation of calibration standard solution of different concentration levels 0.005, 0.05, 0.5, and 1 mg/L. The obtained clear solution after acid digestion was kept into fresh tubes after sufficient cooling of the vessels. The sample was analyzed in triplicate by ICP-OES (Optima 8000, Perkin Elmer) inbuilt with winlab32 software for elemental analysis at given instrumental conditions in Table 1.

Instrumentation

The instrument was equipped with a cyclonic spray chamber and concentric high solid nebulizer for the determination of metal ions. Samples were aspirated by a manual analysis control process.

Table 1 Operating conditions of the ICP-OES instrument

S. no.	Instrumental conditions	Value
1	Plasma Gas Flow (L/min)	8
2	Auxiliary Gas Flow (L/min)	0.2
3	Carrier Gas Flow (L/min)	0.55
4	RF Power [W]	1300
5	Plasma view	Axial
6	Sample flow rate (ml/min)	1.0
7	Read delay/s	30

Instrument detection limits were determined by measuring the emission intensities of seven blanks. The limits of detection (LOD) and limits of quantification (LOQ) were calculated from the formula $C_{LOD} = 3sb/m$ and $C_{LOQ} = 10sb/m$ respectively. Where Sb is the standard deviation of seven replicate blank measurements, and m is the slope of the calibration curve. The figures of merit of the instrumental conditions are available online in the supplementary material (Appendix S2). Before sample analysis, the linear calibration curve for the standard was done. The determined correlation coefficient (R^2) means for all the metals was 0.997. The instrument was adjusted to measure the samples in triplicate, and the mean concentration of analyte and relative standard deviation were automatically calculated.

Isolation of total RNA and cDNA synthesis

Total RNA was isolated from whole blood by using the TRIZOL (Invitrogen) extraction method. The yield and quality of the isolated RNA were assessed by using a nanodrop (Thermo scientific nanodrop 2000 spectrophotometer).

Complementary DNA (cDNA) was synthesized by using Thermo scientific verso cDNA synthetic kit (AB-1453/B) by following reaction mixture preparation: 5 × cDNA synthesis buffer 4 µl, dNTP mixture 2 µl, RNA primer 1 µl, RT Enhancer 1 µl, Verso Enzyme mix 1 µl, RNA template 1 ng, and nuclease-free water for total 20 µl final reaction volume at the following PCR conditions: 1 cycle at 42 °C for 30 min and inactivated at 95 °C for 2 min as prescribed in the protocol. 10× diluted cDNA was used as a template for real-time PCR.

Oligonucleotide primers

Primers were designed from The National Center for Biotechnology Information (NCBI) by using the Primer Quest tool. The details of the primer sequence used in this study have been mentioned in the online supplementary material (Appendix S3).

Quantitative real-time PCR

Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems™, USA) instrument was used for the quantitative gene expression study by using SYBR- Green chemistry. All reactions were carried out in duplicate containing 10 µl volume per well with No Template control (NTC). Beta Actin (β-actin) was used as an endogenous control. Amplification conditions for PCR reaction were: 50 °C for 20 s, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. A dissociation curve was generated to distinguish the specific amplicon from the unspecific amplicon. The amplified real-time PCR product was loaded onto agarose gel containing ethidium bromide dye and run by using the electrophoresis technique. Appeared bands were captured using image lab ™ software (version 5.1) associated with Gel Doc™ XR + and ChemiDoc™ XRS + System.

Ct value was calculated by using Data assist software version 3.01. The fold change was calculated by using the comparative Ct method ($2^{-\Delta\Delta CT}$), where ΔΔCT was the difference between ΔCT and the ΔCT calibrator value.

Statistical calculations

Graph pad Prism software 8.4 version was used for statistical analysis. Heatmapper tool was used for the gene expression data. The non-parametric test was used to perform the data analysis. All results were expressed as Mean ± SEM for all the parameters. P-value < 0.05 was considered as statistically significant.

Results

In this study, the levels of Lead and Arsenic metals were not detected (ND*) in the blood sample of AD patients. The levels of cadmium, mercury, and aluminum were found higher in cases as compared to controls. Non- essential metals such as cadmium and mercury were estimated significantly higher with a P-value < 0.0001 in AD.

At the same time, the levels of essential biometal like copper were higher in the serum samples of cases. In contrast, iron and zinc were found lower in cases as compared to controls. Essential metals analysis suggested that zinc was significantly lower with a P-value < 0.0001 in serum samples of Alzheimer's disease (Fig. 1, Table 2). Metals concentration was measured by ICP-OES in the mg/L unit.

The expression levels of selected genes such as APP, PSEN1, PSEN2, and APOE4 were found up-regulated in AD patients. PSEN2 gene was found significantly up-regulated with a P-value of 0.0002 in cases as compared to controls (Fig. 2, Table 3). Normalized gene expression of AD patients has been shown in (Fig. 3) by using a heatmap. The correlation between metals level and gene expression was not found significant in Alzheimer's disease (Fig. 4).

Discussion

Essential biometals like Cu, Zn, and Fe were also found concentrated in and around amyloid-beta plaques in the AD brain [9]. Many studies reported a decreased level of Zn^{2+} in serum and blood but increased in the cerebrospinal fluid [25–27]. Increased Cu^{2+} level may enhance the dimerization

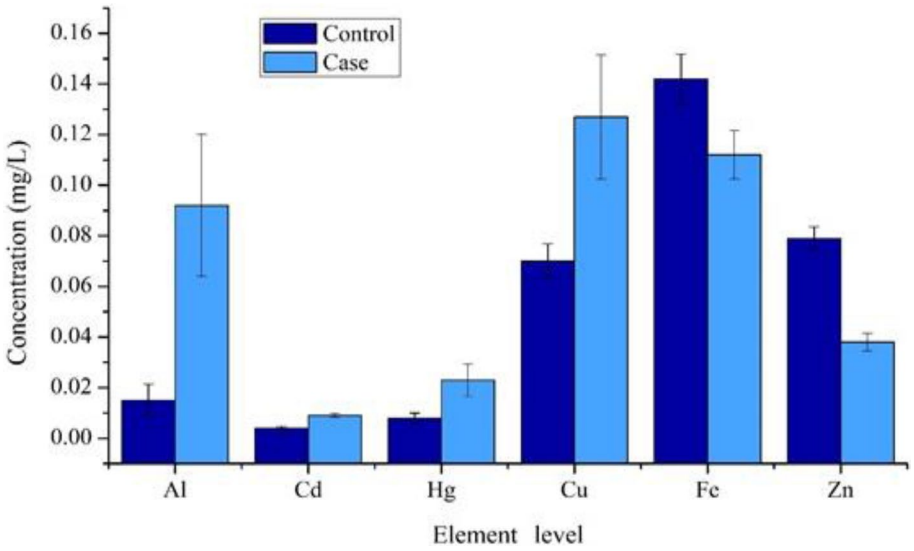

Fig. 1 Bar graph illustrating essential and non-essential metals level in AD cases and controls

Table 2 Result of metals concentration in cases and controls

S. no.	Name of metals	Mean ± SEM (cases) mg/L	Mean ± SEM (Controls) mg/L	P-value
1	As	ND	ND	–
2	Pb	ND	ND	–
3	Cd	0.009240 ± 0.0007707	0.004192 ± 0.0006247	< 0.0001***
4	Hg	0.02332 ± 0.001041	0.00834 ± 0.001954	< 0.0001***
5	Al	0.09222 ± 0.02804	0.01525 ± 0.006378	0.0087**
6	Zn	0.03800 ± 0.003462	0.0785 ± 0.004561	< 0.0001***
7	Fe	0.1117 ± 0.009599	0.1418 ± 0.009810	0.0304*
8	Cu	0.1274 ± 0.02453	0.06959 ± 0.006861	0.0254*

Levels of significance were set to *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001

Table 3 Result of the gene expression in cases and controls

S. no.	Gene name	Mean ± SEM (cases)	Mean ± SEM (controls)	P-value
1	APP	1.292 ± 0.1213	0.8960 ± 0.06331	0.0047**
2	PSEN1	4.784 ± 1.095	1.938 ± 0.2973	0.0138*
3	PSEN2	1.098 ± 0.07128	0.7800 ± 0.04219	0.0002***
4	APOE4	1.046 ± 0.09770	0.7140 ± 0.05813	0.0043**

Levels of significance were set to *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001

of amyloid-beta precursor protein that leads to the overproduction of extracellular Aβ [28].

In our study, we have also found a higher level of Cu and lower levels of Zn and Fe in the serum samples of AD. Similarly, a low level of zinc has been found in serum samples of the patients [29].

Moreover, it has been found that Apo E isoform can bind to metals such as iron, copper, and zinc that are commonly involved in the onset of AD showed a maximum affinity with copper and minimum for zinc [30]. Zinc and apolipoprotein E (Apo E) have been associated with the amyloid-beta pathology involved in Alzheimer's disease. Apolipoprotein E4 (apoE4) is the most prevalent genetic risk factor expressed in more than half of patients and is thus considered as an important possible therapeutic target in AD [31, 32].

Different studies have shown the association among non-essential metals such as Cd, Pb, and MeHg as a risk factors for cognitive functions [33, 34]. A growing amount of evidence has shown that Cd is able to initiate neuronal apoptosis [35, 36]. We have found a higher level of Cd, Al, and Hg in this study. Pb and As were not detected in the blood

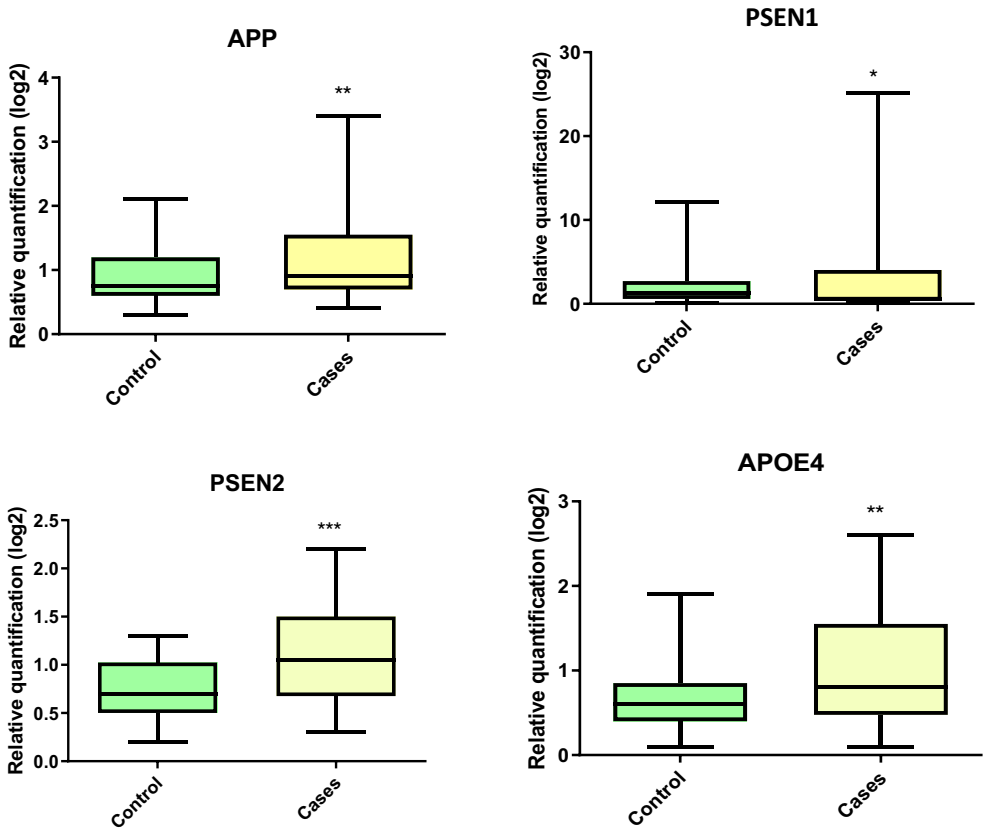

Fig. 2 Box plot showing the gene expression level of APP, PSEN1, PSEN2, and ApoE 4 in AD with controls. Symbol (*) for $P \leq 0.05$, (**) for $P \leq 0.01$ and (***) for $P \leq 0.001$

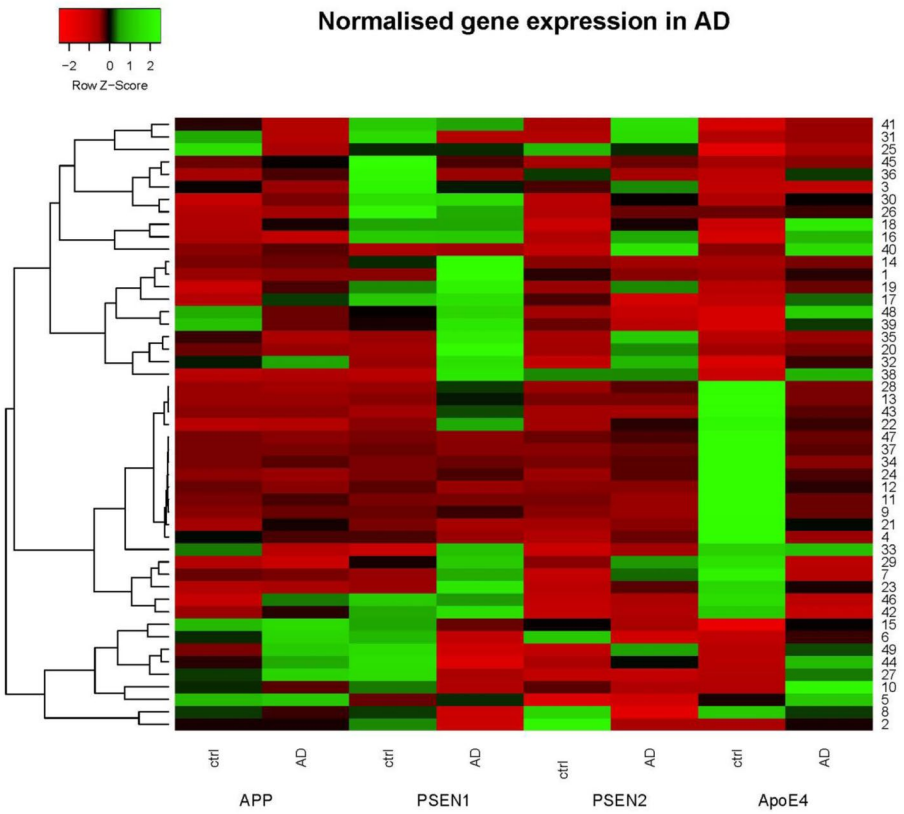

Fig. 3 Heatmap of APP, PSEN1, PSEN2, and ApoE4 genes. Red color represents that the expression of a gene is relatively up-regulated, green represents the particular gene is downregulated, and black indicates normal expression of the genes in AD disease. (Color figure online)

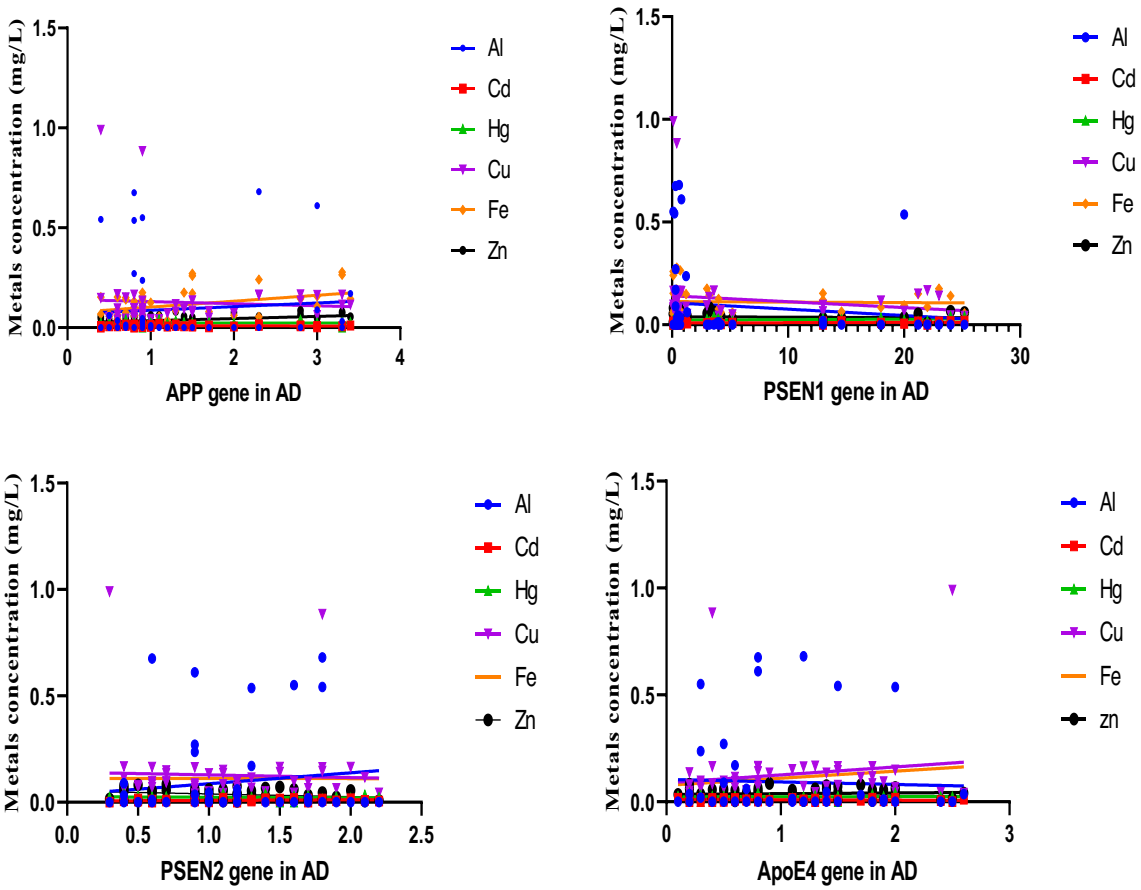

Fig. 4 Multiple linear regression graph illustrated an insignificant correlation between different metals level and gene expression level (APP, PSEN1, PSEN2, and ApoE4) in AD

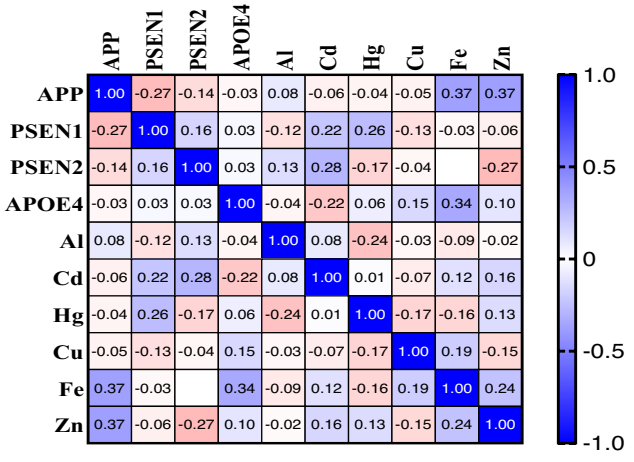

Fig. 5 Correlation matrix plot illustrating correlation coefficients between Metals and Gene expression level in AD. Correlation coefficient + 1 indicates a perfect positive relationship, Correlation Coefficient 0 indicates No relationship, and Correlation Coefficient − 1 indicates a perfect negative relationship

samples of AD. The correlation between metals level and gene expression was not found significant in Alzheimer's disease (Fig. 5).

A higher concentration of mercury levels has been reported in isolated subcellular fractions of AD brains [37]. One study proposed that inorganic arsenic associated with cognitive impairment may be due to activation of pro-inflammatory responses, which may further contribute to neurodegenerative disease [38].

A higher level of aluminum accumulation was found in AD and more pronounced in the hippocampus and entorhinal cortex region of the brain [39–42]. Aluminum may induce conformational changes in amyloid-beta and increased its aggregation in vitro [43]. Several in vivo studies, showed that aluminum might involve in the up-regulation of the APP gene that leads to the deposition of amyloid-beta plaque in the cerebral cortex and hippocampus region of the brain [44, 45]. Aluminum is also able to accelerate the aberrant splicing isoform of PSEN2 that occurs in the neural tissue of AD [46].

Presenilin 1 (PSEN1) and presenilin 2 (PSEN2) genes are the major components of ϒ-secretase. Any mutation in these

genes can alter the amyloid-beta protein synthesis [47–49]. One study conducted on PSEN1 and PSEN2 genes by using 150 postmortem tissue samples from the different regions of the brains showed no difference in PSEN1 expression, but PSEN2 was significantly downregulated in AD patients [13]. Furthermore, contrary to this increased PSEN1 expression alone can increase the activity of ϒ secretase and elevate the accumulation of Aβ in vivo [50].

Nevertheless, no correlation between metals level and gene expression was established by this study. It may be that only 50 AD cases were examined in this study compared to the many millions of AD cases worldwide that have not been studied.

Conclusion

Environmental exposure and genetic risk factors may play the role of disease onset independently in AD. Essential biometals such as copper were higher, while iron and zinc were found lower in the serum sample of AD, and its imbalance may lead to neurodegeneration. Further studies are required using a large sample size of AD patients in the future. We concluded that there was no significant correlation between metals dyshomeostasis and gene expressions in this study.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-021-06386-x.

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Author contributions Conception or design of the work by Prof. Anoop Kumar Verma, Data collection, Data analysis and interpretation by Jyoti Yadav, Drafting the article and Critical revision of the article by Dr. MD. Kaleem Ahmad, Prof. R. K. Garg, Dr. Shiuli, and Prof Abbas Ali Mahdi. Final approval of the version to be published by Prof. Shrikant Srivastava.

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Data availability The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest All authors declare no conflict of interest.

Ethical approval This study was approved by the Ethics committee of King George's Medical University, Lucknow by the Ref. code- 95th ECM II B IMR-F/P2. Written informed consent forms were collected from all participating subjects.

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