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Serum Multivalent Cationic Pattern: Speculation on the Efficient Approach for Detection of Alzheimer's Disease

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Alzheimer's disease (AD) is increasingly becoming one of the greatest medical challenges. Due to the social and financial burden of AD, detection of AD in its early stages is a topic of major research interest. Thus, emergence of well-validated screening methods for fast detection of AD in the early stages would be of great importance. It is now recognized that the homeostasis and serum bioavailability of multivalent cations (*e.g.* zinc, copper, and iron) are disturbed in AD. Using a standard chemometric approach (hierarchical clustering analysis), we find that the serum concentrations of an array of such multivalent cations can be a fingerprint for identification of AD patients. This may pave the way for a reliable, efficient, and inexpensive method for early detection and treatment of AD.

A lzheimer's disease (AD) was named for Alois Alzheimer, a neuropathologist, who in 1906 detected amyloid plaques, neurofibrillary tangles, and arteriosclerotic changes in the brain of his patient (*i.e.* Auguste D, who died after a 5-year history of progressive cognitive destruction, hallucinations, delusions, and severely impaired social performance). Nowadays, AD is recognized as the most common cause of dementia in the world, with huge patient maintenance costs (*e.g.* in the United States, 172 billion dollars in 2010[1] and the prediction of around a trillion dollars by 2050 without emergence of effective treatment approaches[2–5]). AD pathology begins many years before the first symptoms appear[6,7]. Thus, well-validated screening methods for AD are needed to discover new drugs and improve the design of clinical trials, and offer the opportunity for preventive treatment[8].

One of the most well-recognized characteristics of AD is the accumulation in the brain of insoluble amyloid beta (Aβ) peptides, which are derived from the amyloid precursor protein[9]. These aggregates lead to the formation of amyloid fibrils[10–12]. These in turn go on to form senile plaques and contribute to neuronal cell death. Many AD therapies being developed target various amyloid β-peptide (Aβ) states (production, oligomerization, aggregation, and fibrillation). However, clinical results have not to date demonstrated the effectiveness of Aβ-based therapeutic methods, thus it is possible that other aspects of AD may contribute to its etiology.

It is increasingly being accepted that excesses or deficiencies of multivalent cations (*e.g.* Cu^{2+}, Fe^{3+}, Fe^{2+}, and Zn^{2+}) have crucial roles in several commonly known neurodegenerative disorders[13–19]; for instance, some multivalent cations can specifically and saturably bind to human Aβ (as Aβ is a metal binding protein), inducing tinctorial amyloid formation[13–19]. The brains of AD patients suffer from metallostasis, or fatigue of metal trafficking, leading to the redistribution of metals into inappropriate compartments[20]. Furthermore, it was shown that AD patients have significant changes in biologically functional metals in cerebrospinal fluid (CSF), serum, and plasma[17]. For instance, it was shown in some studies that the levels of copper, zinc, and iron in serum, plasma, and CSF were increased or decreased in AD patients compared with age-matched controls[21–28]. There may be a correlation between levels of multivalent cations in serum and their status in the brain[29,30]. It is noteworthy that deficiency of individual multivalent cations (such as iron) has a profound effect on how the brain barrier systems transport other cations (such as copper) between the blood, brain interstitial fluid and CSF[31,32]. Interestingly, disruption of homeostasis of individual multivalent cations may affect the amount of other multivalent cations[32–35]; for instance it was shown that excess zinc ingestion is among the causes of copper

deficiency[34]. As another example, systemic iron levels altered the transport of copper across the brain barriers; more specifically, in an iron-deficient rat model, a considerable increase (+55%) of copper levels in the CSF, brain parenchyma and the choroid plexus was observed, while no effect was detected on CSF iron levels[31]. Here, we will check our hypothesis that an array of changes in levels of multivalent cations (*e.g.* zinc, copper, iron, magnesium, and calcium) could provide a unique marker for detection of AD.

Results

The concentrations of multivalent cations were measured in serum of AD and control subjects (Table S1 of the supporting information (SI)), and the patterns of their variations were evaluated by a standard chemometric approach, hierarchical clustering analysis (HCA). It is notable that people with specific diseases which affect levels of multivalent cations in blood (*e.g.* pneumonia, tuberculosis, acute kidney failure, chronic kidney failure, cirrhosis, hepatitis C, men-
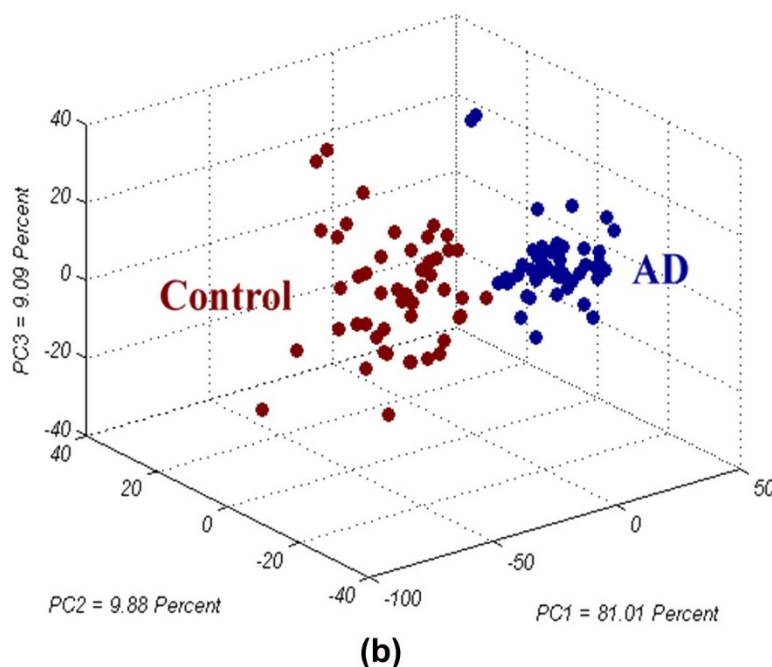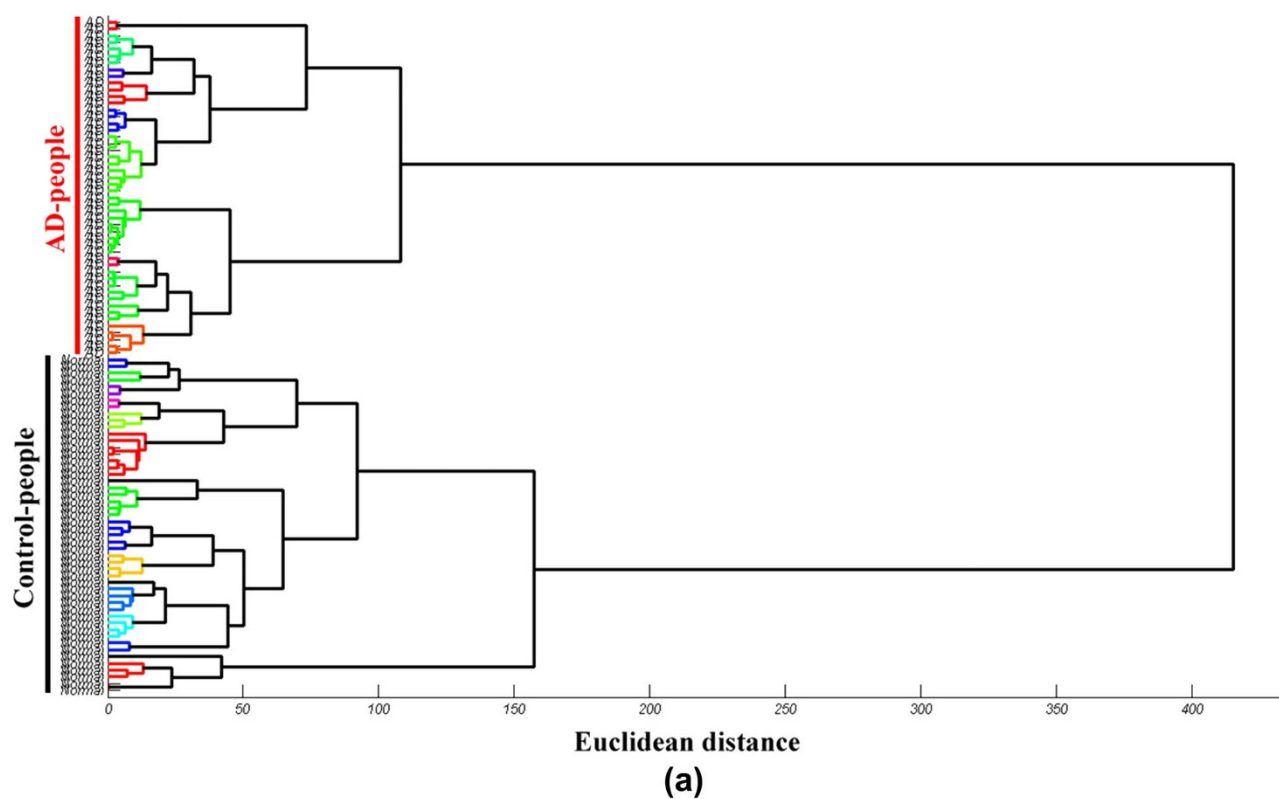

Figure 1 | (a) Hierarchical cluster analysis of 100 Iranian subjects (50 AD and 50 controls) in the full five-dimensional space comprising levels of five multivalent cations (Zn^{2+}, Fe^{2+}, Cu^{2+}, Mg^{2+}, and Cu^{2+}) in human serum. As shown, all 100 subjects were correctly classified as AD or control. HCA used minimum variance (Ward's Method) for clustering. (b) PCA score plot using the three most important principal components from all 100 subjects.

Figure 2 | (a) Hierarchical cluster analysis of 50 Chinese subjects (30 AD and 20 controls) in the full eight-dimensional space comprising levels of multivalent cations in human serum. As shown, the AD and control subjects were accurately classified with very few (just 2) misclassifications. HCA used minimum variance (Ward's Method) for clustering. (b) PCA score plot using the three most important principal components from all 50 subjects.
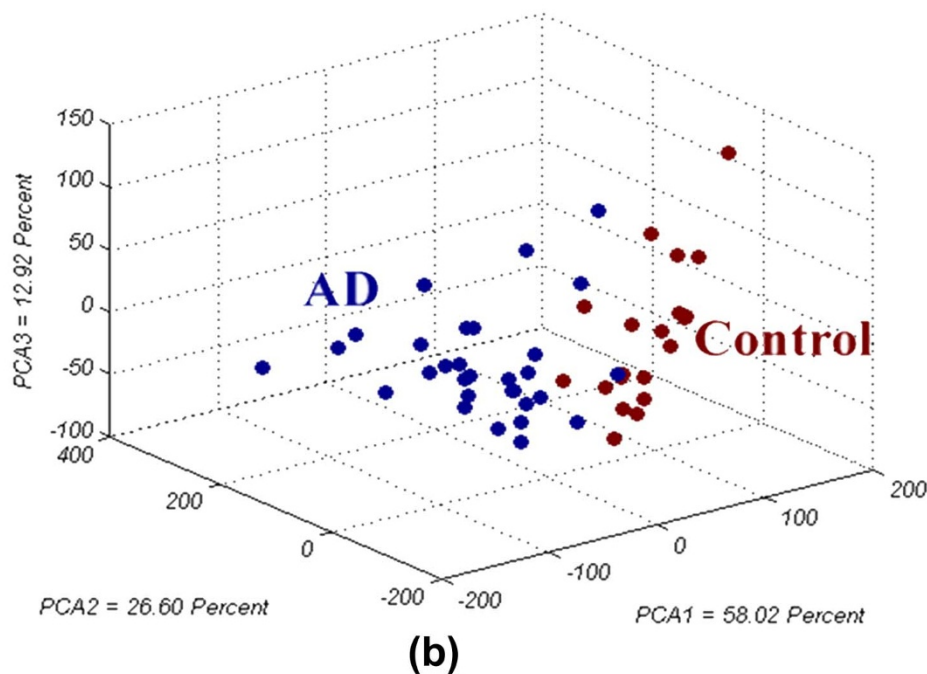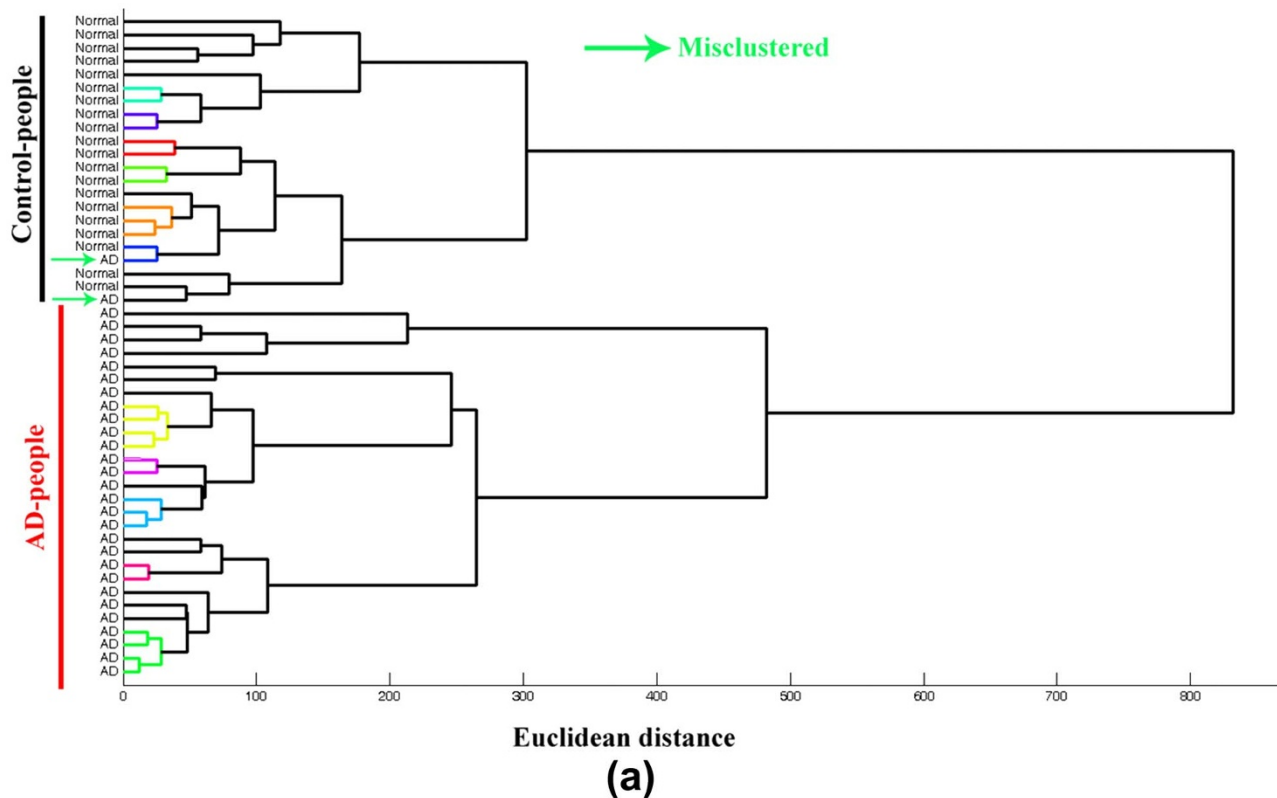
ingitis, psychosis, vitamin D3 deficiency, rheumatoid arthritis, Lyme disease, acute cerebral damage, anemia, or infection caused by parasites or worms) were removed from this study. Heavy smokers (exceeding 20 cigarettes per day) were also removed, as were subjects with high cadmium levels. In addition, people who were using specific drugs which could disturb the balance of multivalent cations were asked to stop using the drugs 4 days before extraction of their serum. These drugs were calcium carbonate (inducing high calcium),

furosemide (depleting magnesium and calcium level), lisinopril (decreasing blood zinc level), and nifedipine (increasing copper level).

In order to check the discrimination capability of the identified multivalent cations, the HCA method, which is a classification scheme based on the Euclidean distance between data points in their full dimensionality[42], has been employed. The advantage of HCA compared with other model-dependent statistical analyses (*e.g.* lin-
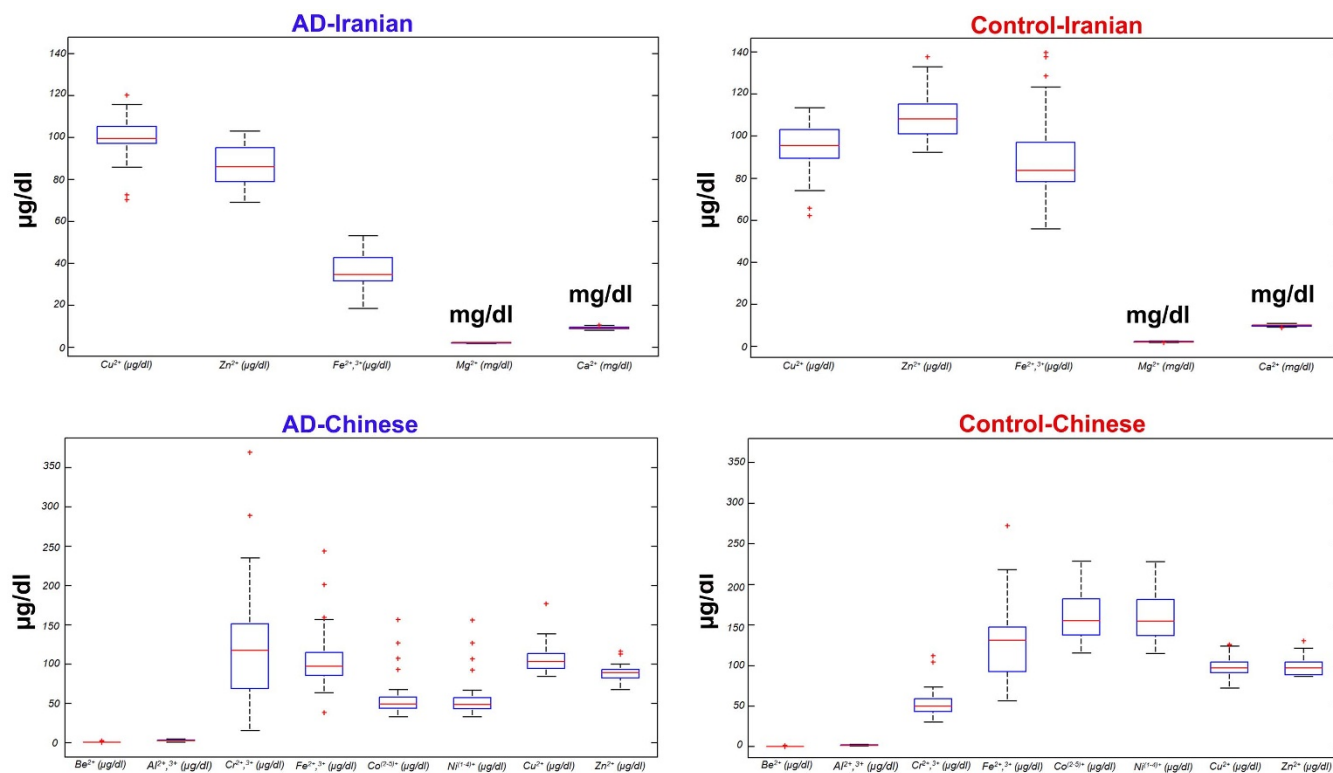
Figure 3 | Box plots showing the different patterns of multivalent cations of AD and control cases for Iranian and Chinese subjects; on each box, the central mark is the median, the edges of the box are the 25th and 75th percentiles, the whiskers extend to the most extreme data points not considered outliers, and outliers are plotted individually.

ear discriminant analysis) is that it makes no postulations considering the classification of results one is trying to establish. In this case, we have defined a model using the levels of five multivalent cations–zinc, copper, iron, magnesium, and calcium–in serum of Iranian subjects. The achieved dendrograms, for Iranian subjects, as shown in Figure 1, correctly classified all 100 subjects as either AD or control.

Beyond the Euclidean distance of the array response, there is much greater information available in the variance of the specific points of the array of data. In this case, we have employed principal component analysis (PCA), which uses the variance in the array to evaluate the relative contributions of independent dimensions. Using standard PCA, all AD and control subjects from Iran were analyzed, and the first three dimensions in PCA space are presented in Figure 1b, showing very good clustering of the AD patients and accounting for 99.98% of the total variance.

Discussion

The variation of individual cations cannot be recognized as a hallmark of AD; however, we hypothesized that an array of changes in levels of multivalent cations (*e.g.* zinc, copper, iron, magnesium, and calcium) could be unique. Using HCA and PCA approaches, we found a good discrimination between AD and normal cases. Interestingly, the familial AD subjects (young people aged 30 years and above), in whom AD was caused by genetic problems and not by cationic abnormalities, were classified as controls (Figure S1 for details). We further employed the method to check whether it has capability to define the stage of AD. Of our patients, 13, 20, and 17 suffered from mild cognitive decline, moderate cognitive decline, and severe cognitive decline, respectively. Severe misclassification between various stages occurred, showing that patterns of multivalent cations in serum could not define AD stages.

In order to probe the accuracy of our method on a population with different genetics and environment, data were analyzed from a published study[24] of Chinese living in Hong Kong. In these subjects, information on eight different multivalent cations–cadmium, beryllium, aluminium, chromium, cobalt, nickel, zinc, copper, and iron–was provided (Table S2). The HCA method was employed to produce a new model for the predetermined Chinese subjects, and the achieved dendrograms worked well (see Figure 2a), however, several subjects were misclassified. Some of the misclassification might be due to lack of information on other diseases of the participants. A three-dimensional PCA score plot of the Chinese subjects showed very good clustering of 50 experimental trials of AD and control subjects. More specifically, the data have exceptionally high dispersion, requiring 6 dimensions to capture >97% of the total variance (Figure S2).

In order to check the validity of the method, 40 subjects from Iran (20 AD and 20 control) were examined by the HCA algorithm developed to classify the Iranian subjects; in this case, an individual person conducted the analysis blind to the diagnoses, then another individual revealed the correct diagnoses and checked the accuracy of the method. The obtained results demonstrated that our clustering method had more than 85% accuracy (3 subjects were incorrectly assigned) for detection of AD (Figure S3).

In summary, we have proposed a simple, inexpensive, and fast approach for detection of AD (based on variation of multivalent cationic patterns in AD and Control subjects of human serum specimens; see Figure 3). Classification analysis reveals that the AD subjects have high dimensionality and, consequently, may be accurately discriminated from non-AD subjects. The findings may provide an insight into the early detection of AD and illumination of potentially new therapeutic avenues. We call upon active researchers in the field to perform similar experiments in various parts of the world so that the resulting database can be transferred to user friendly software for fast screening of AD, according to geographic and ethnic features.
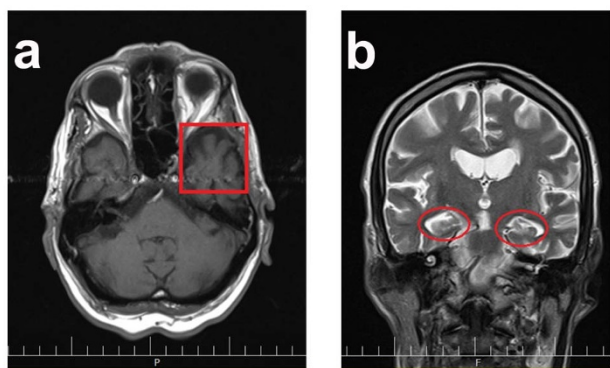
Figure 4 | MRI image of patient with FAST 4. (a) Axial section of brain showing considerable shrinkage and volume loss in left medial temporal lobe. (b) Coronal section of brain showing atrophy of hipocampus.

One should note that specific diseases, which can induce variations in the levels of multivalent cations in blood (*e.g.*, pneumonia, tuberculosis, acute kidney failure, chronic renal failure, cirrhosis, hepatitis C, meningitis, psychosis, vitamin D3 deficiency, rheumatoid arthritis, Lyme disease, cerebrovascular accident, anemia, infection caused by parasites or worms), cannot be used in the proposed method. Furthermore, heavy smokers should be asked to quit smoking until the concentration of cadmium in their blood decreased to the normal range since cadmium can enter blood during smoking[31], and it is known that cadmium is able to displace Fenton metal ions (*e.g.*, Fe^{2+}, Zn^{2+}, and Cu^{2+}) from their carriers[36–41]. Using additional factors, such as more multivalent cations in human serum together with variation of several marker proteins (*e.g.*, melanotransferrin)[42], one can expect to have more accurate results.

Methods

Iranian subjects and analysis. The written informed consent by caregivers were signed. The necessary management was done for the patients after laboratory results. For taking blood samples from subjects, they were fasted at least 4 hours, which is a sufficient period of time for our analysis not to be influenced by dietary intake. Human serum was extracted from a total of 50 healthy individuals (they are age matched with AD patients) without neurological disease (25 males and 25 females) serving as controls and 50 participants with clinical diagnosis of AD (22 males and 28 females) from the expert neurologist in dementia at Roozbeh Hospital (Tehran, Iran), according to NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association) Alzheimer's criteria. Early onset familial AD was diagnosed by mutation of PSEN1, PSEN2 and APP genes by using clinical test according to NINCDS/ADRDA criteria. Furthermore, for diagnosis as early onset familial AD, the patients have a strong familial history of AD and the ages of onset were before 65 years. Neurological tests that were used by expert neurologist included problem solving test, a Farsi version of Mini-Mental Status Examination (MMSE) as a cognitive performance test and the Isaacs Set Test as assessing verbal fluency. For Issacs Set Test[43] patients were asked to name subjects in four categories (city, fruit, animal, food) in a 15 seconds interval. The stages of Alzheimer's disease for subjects were classified according to Functional assessment staging (FAST) ,which includes 7 stages. Furthermore, confirmation of AD diagnosis was done by MRI (Standard protocol of 1.5 tesla MRI with Coronal section). For example, Figure 4 shows different brain sections of an AD with FAST 4. Figure 4a belongs to axial section of brain, which demonstrates noticeable shrinkage and volume loss of left medial temporal lobe; Figure 4b illustrates hippocampus atrophy and loss of volume which are convincing markers of AD that confirm our diagnosis for the patient. Subjects were evaluated by an expert neurologist and their stages and scores for cognitive test were determined. For example, one of our AD patients whose FAST stage[44] was recognized as 4 has a MMSE score and Isaacs Set Test score of 23 and 14, respectively. All subjects gave written informed consent, except that in AD patients who could not give consent, surrogate consent was obtained from their guardians. The study was approved by the ethics committee of Roozbeh Hospital. For preparation of serum, blood was collected in clot activator tubes (B–D Vacutainer System, Franklin Lakes, NJ, USA), and centrifuged at 3,000 g at 4°C for 10 min. The aliquots were immediately frozen at −70°C and stored until assayed. The concentrations of multivalent cations in serum samples were assayed by inductively coupled plasma-mass spectrometry using an ICP-MS, Thermo X7 instrument from Thermo Elemental, Winsford, UK.

Chinese subjects. Subjects were ethnic Chinese living in Hong Kong. All subjects gave written informed consent, except that in AD patients who could not give consent, surrogate consent was obtained from their guardians. Human serum was extracted from a total of 20 healthy individuals (5 males and 15 females) serving as controls and 30 participants with clinical diagnosis of AD (8 males and 22 females) from the NINCDS-ADRDA criteria. The local Clinical Research Ethics Committees approved study of these subjects. Serum was collected from all subjects. Cation analysis was performed using an ICPMS7500c from Agilent Technologies, Palo Alto, CA, USA. Samples were pre-treated with diluents containing 0.05% tetra-methyl ammonium hydroxide and a mixture of internal standards containing rhodium, yttrium and iridium before analysis. An on-line reaction cell filled with helium was used to eliminate polyatomic interference due to compounds with similar mass-charge ratio. The inter-assay coefficients of variation were generally <9%.

Principal component analysis. In order to systematically compare data between AD and control subjects, principal component analysis (PCA) was used. The goals of PCA are to (a) extract the most important information, (b) compress the size of the data set by keeping only the important information, (c) simplify the description of the data set, and (d) analyze the structure of the observations and the variables.

In performing this method, PCA computes new variables called *principal components* which are obtained as linear combinations of the original variables. The first principal component is required to have the largest possible variance. The second component is computed under the constraint of being orthogonal to the first component and to have the largest possible inertia. The other components are computed likewise. The values of these new variables for the observations are called *factor scores*. These factors scores can be interpreted geometrically as the *projections* of the observations onto the principal components.

Hierarchical cluster analysis. Dendrograms of HCA were based on all dimensions of data, hence representing 100% of the total variance. For hierarchical cluster analysis, the Euclidean distances between pairs of objects were computed in an input data matrix followed by calculation of a hierarchical cluster tree from the Euclidean distance matrix of input by the inner squared distance method (minimum variance algorithm). Finally, the dendrogram plot of the hierarchical binary cluster tree was prepared by the generated input. A dendrogram consists of many U-shaped lines connecting objects in a hierarchical tree. The height of each U represents the distance between the two objects being connected.

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Acknowledgements

Author contributions

M.A., M.N. and H.A. prepared Iranian serum samples; L.B. provided Chinese serum information; S.M.A. performed HCA and PCA analysis; M.M. designed the experiments and wrote the paper.

Additional information

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