Neurodegenerative disorders (NDs) are a group of diseases resulting from a neuronal loss in different regions of the central nervous system (CNS). It is fully accepted that both environmental and intercellular mechanisms are involved in the pathophysiology of NDs. Having been proposed that changes in the lifestyle and nutrition (metabolism) during mid-life could trigger and accumulate cellular and molecular damages resulting in NDs during aging.

Methods: In order to test the hypothesis, we investigated the expression level of two energy metabolism-related [forkhead box O1 (FOXO1) and forkhead box O3 (FOXO3a)] and two pro-inflammatory cytokines [interleukin 1β (IL-1β) and IL-6] genes, using quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Furthermore, changes in the ion concentration of three essential heavy metals [iron (Fe), copper (Cu), and zinc (Zn)] by atomic absorption spectroscopy in patients with NDs, depression, obesity, and diabetes type II, were evaluated and compared with the results of normal individuals.

Results: More than half of the participants in obesity, depression, and ND groups had significant up-regulation of FOXO1 and FOXO3A, down-regulation of IL-1β and IL-6, and higher levels of Fe and Cu in their blood. This pattern of gene expression was not repeated in diabetic patients.

Conclusion: It could be concluded that individuals affected with different levels of obesity and depression have increased the risk of developing NDs later in life, probably through changes in energy metabolism, inflammatory pathways, and ion concentrations.

Introduction
Neurodegenerative disorders (NDs) are a group of diseases resulting from a neuronal loss in different regions of the central nervous system (CNS). It is fully accepted that both environmental and intercellular mechanisms are...
involved in the pathophysiology of NDs.\textsuperscript{3,4,5} Induction of oxidative stress due to increased levels of intercellular metal ions,\textsuperscript{6-7} mitochondrial dysfunction\textsuperscript{8-10} disturbances in energy metabolism and autophagy signaling pathways\textsuperscript{11-13} as well as releasing excessive neuroinflammatory factors in the CNS\textsuperscript{14} are some of the main intercellular pathways involved in neuronal death. Instead, cellular and molecular dysfunctions in neurons are mostly induced by environmental risk factors such as toxic heavy metals,\textsuperscript{15} unregulated homeostasis of essential metal ions\textsuperscript{16} and lifestyle habits.\textsuperscript{17}

We have recently suggested that changes in the normal concentration of essential metal ions such as Iron (Fe), Copper (Cu) and Zinc (Zn) rather than other factors could initiate neurodegeneration processes.\textsuperscript{18} Our proposal was mainly based on increasing/decreasing activity of energy metabolism pathways in neurons due to changes in nutritional habits and lifestyle during mid-life. Basically, changes in our habits have resulted in more energy uptake than its consumption, neurological hormone alterations, and changes in the concentration of essential metal ions. All these conditions, which could be reflected as obesity, diabetes type II, and depression at the clinical level, are directly and indirectly connected to defects in energy metabolism pathways, intracytoplasmic metal ion concentrations, and subsequently cell apoptosis.\textsuperscript{19-22} Changes in lifestyle and nutritional habits have also increased the prevalence of multifactorial diseases such as diabetes type II, obesity, and depression along with NDs.\textsuperscript{23-26}

Moreover, researches have shown that individuals affected by obesity, diabetes type II, and depression have higher risk of developing neurodegeneration during their life.\textsuperscript{27-31} Forkhead box O1 (FOXO1) and forkhead box O3 (FOXO3A) are two important transcription factors that have major roles in promoting autophagy,\textsuperscript{32-34} energy metabolism, and stress responses.\textsuperscript{35-37} Changes in the expression of these genes in CNS could result in neurodegeneration.\textsuperscript{9}

Additionally, elevated levels of inflammatory cytokines and alterations in the concentration of essential heavy metals have been reported in affected tissues of NDs, obesity, and diabetes type II.\textsuperscript{38-41} Although the changes in expression of these two genes have been studied in animal models and are also reported separately in patients suffering from obesity, depression, diabetes type II, and NDs, little is known about the simultaneous pattern of these alterations between all mentioned diseases and healthy individuals.

Therefore, in the present pilot study, we investigated these alterations by measuring the expression of FOXO1 and FOXO3A in combination with two inflammatory genes interleukin 1β (IL-1β) and IL-6 in patients with diabetes type II, obesity, depression, and NDs, and compared them with normal individuals. We also evaluated alterations of the concentration of three metal ions (Fe, Cu, and Zn) in the proposed groups of patients.

### Materials and Methods

Totally, 105 blood samples were collected from patients diagnosed with ND [mild cognitive impairment (MCI), non-familial Alzheimer's and Parkinson's diseases, n = 26], depression (n = 17), obesity (n = 20) and diabetes type II (n = 21). Twenty-one normal individuals with normal body mass index (BMI), fast blood sugar (FBS) and without any neurological complications were analyzed as a control. Samples with a positive family history of diabetes type II, obesity, depressive and NDs were excluded from the control group. Informed consent was obtained from all individual participants included in the study. Consent form has been approved by Ethical Committee of Iran National Science Foundation. General information of patients in different groups has been indicated in table 1.

ND and depression samples were obtained from Imam Khomeini hospital, department of neurology, Tehran University of Medical Sciences, Iran. Obesity, diabetes type II and normal samples

| Group      | Sex     | Age (year) (mean ± SD) |
|------------|---------|------------------------|
| Normal (n = 21) | Man 11 (52) | Woman 10 (48) | 35 ± 3 |
| ND (n = 26)    | Man 11 (42) | Woman 15 (58) | 67 ± 7 |
| Depression (n = 17) | Man 2 (12) | Woman 15 (88) | 42 ± 5 |
| Obesity (n = 20) | Man 8 (40)  | Woman 12 (60) | 48 ± 2 |
| Diabetes type II (n = 21) | Man 8 (38)  | Woman 13 (62) | 54 ± 2 |

SD: Standard deviation; ND: Neurodegenerative disorders [including Alzheimer's disease, Parkinson's disease and MCI (mild cognitive impairment)]
were collected from the Center of Diabetes Screening, Tehran University of Medical Sciences.

Total RNA was extracted from whole blood using the Trizol-chloroform procedure. Briefly, 600 μl of AccuZol (Bioneer, South Korea) was mixed with 1 ml fresh blood. After shaking, 200 μl chloroform (Merck, Germany) was added to the mix, incubated for 15 minutes on ice and centrifuged at 12000 rpm, 15 minutes at 4 °C. The clear supernatant was mixed with 500 μl isopropanol (Merck, Germany), incubated 10 minutes on ice and centrifuged at 11000 rpm, 10 minutes, at 4 °C. The RNA pellet was then washed 2 times with 70% ethanol at 7000 rpm, 5 minutes, at 4 °C and dissolved in 20 μl RNase free ddH$_2$O. The quality of RNA was checked on an agarose gel. The quantity and purity of samples were measured using Nano-drop (Thermo scientific, USA) and A260/280 ratio, respectively. High-quality RNA samples were used for cDNA synthesis.

In order to synthesize cDNA, 500 ng of DNase I-treated (Takara, Japan) RNA was mixed with 1 unit AccuPower® CycleScript reverse transcriptase, 1x reaction buffer, 10 mM dNTPs, 0.5 mM oligo dT and 0.5 mM random hexamer primers, RNase inhibitor and up to 20 μl RNase-free ddH$_2$O. The mix was incubated at 25 °C for 30 seconds for 1 round, and 45 °C for 4 minutes and 55 °C for 30 seconds, for 12 rounds. The reaction was then heat-inactivated at 95 °C for 5 minutes.

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was performed to quantify the expression of FOXO1, FOXO3A, IL-1β, and IL-6. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression was measured as an internal control of the experiment. Primer sequences have been indicated in table 2.

The qRT-PCR reaction was contained 500 ng of cDNA mixed with 25 μl of SYBR green master mix (GeneON, Germany), 300 nM of each reverse and forward primers and up to 50 μl RNase/DNase-free ddH$_2$O. PCR reactions were performed in Corbet research instrument (Rotor-Gene™ 6000, Australia) under the following condition: the one-time initial denaturation at 95 °C for 10 minutes, and 95 °C for 20 seconds, 57 °C for 45 seconds, repeated for 40 cycles and followed by melting curve step.

Serums obtained from 3 ml blood samples were used to measure the concentration of free metal ions including Fe, Cu and Zn. Sample dilution for Cu was 1:50 (serum:ddH$_2$O), for Fe and Zn was 1:10 (serum:ddH$_2$O). Deionized water was used for dilution of samples and standard preparations were prepared with a resistivity of 18.0 MΩ cm (Elga Labwater, Wycombe, Bucks, UK). Working standard solution was freshly prepared in ddH$_2$O in 3 dilutions for each metal: Fe standards (0.5, 1, and 2 mg/l), Cu standards (10, 20, and 40 μg/l), and Zn standards (0.1, 0.2 and 0.4 mg/l).

The elemental determination was done by Varian spectra AA-240FS atomic absorption spectrometer (Varian Australia, Pty Ltd, Mulgrave, Victoria, Australia). Flame atomic absorption was used for detecting Fe and Zn. Furnace atomic absorption was applied to measure Cu.

Seronorm leve2 (SERO AS, Norway) was used in each step to control the qualification of the instrument. Hemolyzed samples were excluded for this test.

This research was designed as a pilot study. qRT-PCR and atomic absorption spectroscopy experiments for each sample were done as triplicate and duplicate, respectively. qRT-PCR data were analyzed using the 2$^{-ΔΔCt}$ method. All data were then analyzed by SPSS software (version 11.5, SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) and Tukey's test were used for data analyses. P less than 0.050 was considered as significant.

Table 2. The sequence of primers used in this study

| Gene name | Forward 5'-3' | Reverse 5'-3' | Reference |
|-----------|--------------|--------------|-----------|
| FOXO1     | TGGACATGCTCAGCAGACATC | TTGGGTACGGCGGTTCAC | 51 |
| FOXO3A    | ATGTGACACTTGGATCCATC | TGTCCACTTGCTGAGAGCAT | 52 |
| IL-1β     | ACAGATGAAGTGCTCCTTCCA | GTCGGAGATTCGTAGCTGGAT | 53 |
| IL-6      | GTGACATCTTCAGCGGCTCTC | GTGCCTCTTGCTGCTTTCAC | 54 |
| GAPDH     | TGCACCACCAACTGCTTTACG | GGCATGGACTGTGGTCTGAG | 55 |

FOXO1: Forkhead box O1; FOXO3A: Forkhead box O3; IL-1β: Interleukin 1β; IL-6: Interleukin 6
Results

Expression of forkhead transcription factors: energy metabolism pathway: In this study, we quantified the expression of FOXO1 and FOXO3A genes in whole blood cells of four groups of patients with NDs, obesity, diabetes type II, and depression and compared them with the normal population. Our data showed that FOXO1 expression was significantly up-regulated (P < 0.050) in more than 50% of obese, depressive, and NDs patients. The number of diabetic patients that showed up-regulation of this gene was not significant. However, the percentage of patients showing down-regulation of FOXO1 was significant (P < 0.011) in all groups of disease (Figure 1A). No significant difference in the pattern of FOXO1 gene expression could be seen between obesity, depression and diabetes type II compared to NDs.

FOXO3A expression was up-regulated in more than 50% of the patients in all four groups of disease. This change was significant for obesity, depression, and NDs (P < 0.050). Down-regulation of this gene which could be seen in less than 42% of the patients was significant (P < 0.011) for obese, diabetic, and neurodegenerative groups (Figure 1B). Similar to FOXO1, the pattern of FOXO3A gene expression was not significantly different between obese, depressive, and diabetic patients versus patients with NDs.
There was no significant correlation between changes in the expression of FOXO1 / FOXO3A and clinical data such as BMI, FBS, low-density lipoprotein (LDL), high-density lipoprotein (HDL), cholesterol, and triglyceride in this study.

Expression of inflammatory factors: In order to check the inflammatory status of our patients, we analyzed the RNA expression of IL-1β and IL-6 (two prominent inflammatory cytokines) in whole blood samples. The results revealed that more than 70% of the patients suffering from NDs, depression, and obesity, had decreased expression of both IL-1β and IL-6 (P < 0.001). However, more than 80% of the diabetic participants showed overexpression of these genes in their blood (Figure 2, A and B).

There was no significant correlation between changes in the expression of IL-1β / IL-6 and clinical data including BMI, FBS, LDL, HDL, cholesterol, and triglyceride in this study.

Determination of essential heavy metal concentration: Measurement of free Fe, Cu, and Zn ions in the serum of patients, as compared with healthy individuals, revealed that the concentration of Fe and Cu increased significantly (P < 0.050) in patients with obesity, depression, and NDs. These changes were not significant between diabetic patients and healthy participants (Figure 3). Changes in the free Zn ion were not significant, as well.
The mean concentration of free iron (Fe) and copper (Cu) in the serum of patients affected with obesity, depression and neurodegenerative diseases (NDs) is significantly higher than the control group. The concentration of zinc (Zn) ions shows no significant change in patients groups. Diabetic patients do not show any change in concentrations of essential metal ions. The graphs present average of metal iron concentration in patients and controls (Y-axis) in each group of study (X-axis). Dark gray bars remark serum level of Cu (µg/l), gray bars present Zn (µg/l) levels and light gray bars show Fe (mg/l) levels. Standard error of the mean (SEM) has been calculated for each bar.

Although Fe and Cu changes in diabetic patients were not significant, we found a significant (P < 0.001) positive correlation between changes in Fe/Cu concentration and FOXO3A gene expression (correlation coefficient of 0.704 and 0.613, respectively) in this group. A positive correlation (P < 0.050, correlation coefficient of 0.457) was also found between the concentration of Cu and FOXO3A gene expression in obese patients.

**Discussion**

In order to test our hypothesis about the relationship between changes in expression of energy metabolism (autophagy) genes and heavy metal concentrations in diabetic, obese, depressive patients and increasing risk of NDs at clinical level, we compared RNA expression of four genes involved in energy metabolism and inflammatory systems as well as determination of essential metal concentrations in blood of two metabolic syndromes (obesity and diabetes type II), an anxiety condition (depression), three NDs (Alzheimer's and Parkinson's diseases and MCI), and normal group. Obesity, diabetes type II and depression are considered as cases with nutritional and lifestyle problems.

Our data showed that more than half of obese and depressed patients had higher levels of FOXO1 and FOXO3A expression (involved in energy metabolism and autophagy signaling pathways), lower levels of IL-1β and IL-6 expression (involved in inflammatory responses) and increased concentrations of free Fe and Cu in their serum, as compared to normal individuals. Interestingly, these results were also repeated in cases with NDs. However, the results were not the same for diabetic patients.

Different studies have highlighted the role of FOXO transcription factors in induction of autophagy in various types of cells. Upregulation of FOXO1 and FOXO3A in the blood (mainly lymphocytes) of patients with obesity and depression might reflect similar changes in the expression pattern of these genes in neural cells. Therefore, increasing forkhead gene expression could cause uncontrolled autophagy events, lead to neural death and increase the risk of neurodegeneration in these patients.

Although increased levels of IL-1β and IL-6 in patients with diabetes type II have also been confirmed in different studies, downregulation of these two pro-inflammatory genes in obesity, depression, and ND groups could be controversial because most studies have highlighted higher levels of inflammatory cytokines in all mentioned disorders. Thus, our results could be explained based on the cells we analyzed in this study and the protective roles of IL-1β and IL-6 in different tissues, especially CNS. Most studies reporting elevated levels of inflammatory cytokines have worked specifically on affected tissues of patients with obesity and neurological problems or have searched for the protein in the serum or cerebrospinal fluid (CSF) of these patients. However, in this study, we focused on the expression of cytokine genes in the blood cells that might respond differently to disease conditions as compared to affected tissues. Additionally, some studies have indicated that IL-1β and IL-6 expression have neuroprotective roles, and therefore a decrease in their expression below the normal levels could result in neural injury and apoptosis. Because of the multifactorial nature of the diseases studied in this research, it is possible that the same disease in different individuals is resulted from the
impairment of different signaling pathways or environmental factors. That is why we also observed higher levels of inflammatory factors and lower levels of energy metabolism genes in fewer cases of obesity, depression, and NDs.

Another hallmark of our study is the age of our participants. In this research, most of the patients with obesity, depression, and diabetes type II as well as normal individuals were below 50 years old and in the mid-life stage, while the mean age of our ND patients was 67 years. Therefore, the similar pattern of molecular changes in obesity, depressive and ND group (with different ages) in this study might indicate that disturbance of different signaling pathways and also ionic concentrations during mid-life could increase the rate of NDs during old age.

To the best of our knowledge, this is the first report directly evaluated FOXO1 and FOXO3A expression along with inflammatory gene expression and metal analysis in the serum of obesity, depression, diabetic type II and NDs simultaneously. In conclusion, we propose that people affected with obesity and depression might have a higher risk of developing NDs mostly through changes in the energy metabolism system and essential metal concentrations. Diabetic patients might be at risk of neuronal loss through inflammatory pathways. Another important point is that obesity, depression, and diabetic conditions in early stages or mild forms are mostly ignored either by affected people or clinicians, while changes in the molecular pathways or ionic concentrations and the consequent cellular damages have already started in their body. Thus, informing negligent people could help them to change their lifestyle and start protective measures.

Conflict of interests
The authors declare no conflict of interest in this study.

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