



## Research article

## Gene expression of serotonergic markers in peripheral blood mononuclear cells of patients with late-onset Alzheimer's disease

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## ABSTRACT

Serotonin or 5-hydroxytryptamine (5-HT) is primarily involved in the regulation of learning and memory. Pathological changes in metabolism or functional imbalance of 5-HT has been associated with Alzheimer's disease (AD). The hypothesis tested is that in peripheral blood, markers of the serotonergic pathway can be used as a diagnostic tool for AD. The current study measured the relative expression of 5-HT receptors (5-HTR<sub>2A</sub> and 5-HTR<sub>3A</sub>) as well as the 5-HT catalytic enzyme, Monoamine oxidase A (MAO-A) mRNA in Peripheral Blood Mononuclear Cells (PBMCs) of patients with late-onset Alzheimer's disease (LOAD) and age-matched controls. 5-HTR<sub>2A</sub>, 5-HTR<sub>3A</sub>, and MAO-A mRNA expressions were examined in PBMCs of 30 patients with LOAD and 30 control individuals. Real-time quantitative PCR was used to measure mRNA expression. The dementia status of patients in this study was assessed using a Mini-Mental State Examination (MMSE). Mean data of relative mRNA expression of 5-HTR<sub>2A</sub>, 5-HTR<sub>3A</sub> and MAO-A were significantly lower in PBMCs of patients with LOAD compared with controls. Based on the down-regulation of serotonergic markers in PBMCs, our findings may be another claim to the systemic nature of LOAD. The role of peripheral serotonergic downregulation, in the pathogenesis of AD, needs to be further studied. Given the extremely convenient access to PBMCs, these molecular events may represent more complete dimensions of AD neuropathophysiology or possibly lead to a new direction in studies focused on blood-based markers.

## 1. Introduction

Alzheimer's disease (AD) is the most common type of dementia, which is caused by the demise of neurons in the aged brain [1]. It is progressive and causes patients to be deprived of memory, communication skills and the ability to live independently [2, 3]. The high prevalence of AD (10% of people over the age of 65), and its heavy social and economic burden have created major challenges to public health in the 21st century [2, 4, 5]. Previous studies have shown that beta-amyloid (A $\beta$ ) and tau proteins contribute to the clinical development of the disease [1, 6]. In sporadic AD (also known as late-onset AD, LOAD), the age at which the disease starts is over 65 years old and accounts for more than 95% of cases of AD. LOAD is considered as a multifactorial (or complex) disease, and several environmental and genetic factors contribute to its pathogenesis [7, 8]. It is estimated that genetic abnormalities occur in 70% of LOAD patients [9].

There are several hypotheses regarding the complex pathogenesis of AD, in which many of the various factors are interconnected. However, none of the hypotheses has been able to recapitulate all of the pathological aspects of AD and further studies are warranted [8]. 5-HT is a biogenic monoamine that acts as a neurotransmitter, neuromodulator and also a peripheral neurocrine hormone [10, 11]. 5-HT is involved in regulating learning and memory processes in adulthood. Pathological changes in the metabolism of 5-HT, or the imbalance in the transmission of serotonergic messages, have been associated with the etiology of a variety of pathophysiological conditions in the brain including AD [12]. In the brain of AD patients, the reduction in the number of serotonergic neurons in the raphe nucleus, as well as the reduction of 5-HT and its metabolites in post-mortem tissue has been observed [13, 14]. Several 5-HT receptors including 5-HT<sub>2A</sub>, 5-HT<sub>2C</sub> and 5-HT<sub>4</sub> modulate amyloid precursor protein (APP) processing which is necessary for A $\beta$  formation. Several reports also emphasized the beneficial effects of 5-HT<sub>6R</sub> receptor

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inhibition in promoting recognition [15, 16]. There is indirect evidence that the serotonergic system may be involved in tau hyperphosphorylation, which is another hallmark of AD [17].

Monoamine oxidase-A (MAO-A) is an enzyme that acts as a 5-HT degradation catalyst (oxidative deamination) and plays an important role in regulating intracellular levels of the neurotransmitter [18, 19]. Studies have shown that MAOs are associated with multiple neurological and psychiatric disorders, including AD [20]. It has also been noted that in different regions of the brain of AD patients, the level of activity of MAO-A and its expression is up-regulated [21]. Therefore, it has been thought that MAO inhibitors increase amine neurotransmitters, decrease ROS and have neuroprotective effects [22]. Although AD was commonly considered as a brain disorder, it has now become clear that AD is a systemic disease that also affects peripheral tissues outside of the central nervous system [23]. In confirmation of this concept, a number of studies have shown that in 80% of cases, blood- and brain-derived cells have similar transcriptome [24]. In this regard, blood cells and several brain tissues have remarkable similarity for gene expression that encodes neurotransmitter receptors [11].

Based on previous findings that suggest both 5-HT and MAO systemic disorders play a role in the pathophysiology of AD, the present study aimed to determine the relative expression patterns of related genes in Peripheral Blood Mononuclear Cells (PBMCs) in AD patients and compare them with control individuals. The goal was to establish if peripheral markers can be developed to aid in the clinical diagnosis of AD. We examined the expression of 5-HTR2A, 5-HTR3A and MAO-A mRNA extracted from PBMCs simultaneously. It was hypothesized that there would be a significant difference between the mean relative expression of selected genes in the control and LOAD groups. In addition, we examined the relationship between the expression levels of 5-HT receptors (5-HTR2A, 5-HTR3A) and MAO with demographic characteristics of Alzheimer's patients. This part of the study hypothesized that there is a correlation between age, duration of AD and MMSE total score with the relative expression of 5HTR2A, 5HTR3A and MAO genes.

## 2. Materials and methods

### 2.1. Study population

Study participants were recruited from a residential aged-care facility located in Mehriz County, Yazd, Iran. The population of the study was the residents 65 years or older. Thirty participants (15 females, 15 males) were recruited for each of the control and LOAD groups. The participants were evaluated by a geriatrician dispatched from the memory clinic of Hazrat-Rasoul-Akram Hospital (affiliated with Iran University of Medical Sciences) to determine the memory status of the study participants. The patients were diagnosed by the Diagnostic and Statistical Manual of the American Psychiatric Association (DSM-IV). All patients with LOAD underwent conventional neuropsychiatric testing. Mini-Mental State Examination (MMSE) was performed for all the participants [25, 26, 27]. The control group (non-Alzheimer's volunteer) were selected from a cohort of people aged 65 years and over in the same residential center and two daycare centers located in the same

city. Detailed medical information was obtained from the records of the residential aged-care facility. Subsequently, all individuals or their caregivers completed a questionnaire that asked for personal and family history of AD, medical pedigree, demographic characteristics, laboratory test results, and medication use. Subjects with AD and less than 65 years of age, familial AD, non-Alzheimer's dementia (secondary to other central nervous system or systemic conditions), psychotic disorders and patients under supportive drug therapy in the treatment of AD were excluded. Written informed consent was obtained from all participants or their primary caregivers before the enrolment into the study. The study protocol was approved by the ethical committee of the National Institute of Genetic Engineering and Biotechnology Research (NIGEB). The demographic characteristics of the study groups are summarized in Table 1.

### 2.2. Mononuclear cell separation, RNA isolation, and reverse transcription

Complete blood samples were collected in 5 ml volume from all participants by phlebotomy from the antecubital vein. The samples were then poured into tubes containing 0.5 ml of ethylenediaminetetraacetic acid (EDTA) with anticoagulant activity. PBMCs were isolated from the rest of the blood components using a gradient density centrifuge technique using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). RNA extraction from PBMCs was performed by the High Pure RNA Isolation Kit (Roche, Germany) according to the manufacturer's instructions. The quantity and quality of the extracted RNA samples were confirmed by optical density ratio of 260 nm–280 nm using NanoDrop 2000 instrument (Wilmington, USA) and agarose gel analysis, respectively. For reverse transcription polymerase chain reaction (RT-PCR), 400 ng of total RNA was reverse transcribed into First Strand cDNA complementary DNA (cDNA) by cDNA synthesis kit (Fermentase, Germany) based on the manufacturer's protocols. Finally, the cDNA samples were kept at -70 °C until used for PCR.

### 2.3. PCR and real-time PCR analysis

The beta-actin gene was used as a housekeeping gene to normalize expression of target genes. Oligo7 software (version 7.56) was used to design the primers. To eliminate amplification of genomic DNA and pseudogenes, primers were validated using Primer-BLAST online software (Table 2). In all samples, a PCR reaction was performed to confirm transcription of target genes and to determine the contamination of the DNA using master mix PCR (Cinnagen, Tehran, Iran), specific gene primers and cDNA. Then the samples were loaded on a 1.5% gel. Real-time PCR was carried out for relative quantification of the mRNA expression of all Targeted genes using SYBR Green Real-time PCR Master Mix (Light Cycler Fast Start DNA Master Plus SYBR Green I, Roche, Germany), previously Designed primers, and cDNA. Detection of fluorescent intensity in each sample was accomplished using a Thermocycler Rotor-Gene 6000 instrument (Corbett Research/Australia). Melting curve analysis indicated only one peak per reaction that was further confirmed by observing a band by electrophoresis of PCR products.

Table 1. Demographic and clinical characteristics of participants.

	LOAD patients (n = 30)	Controls (n = 30)	Independent t-test (p-value)
Male/female, n	15/15	15/15	-
Age (years), mean ± SD	88.7 ± 6.4	82.5 ± 5.1	p < 0.001
MMSE total score, mean ± SD	9.1 ± 3.1	24.8 ± 3.2	p < 0.0001
Age of disease onset, mean ± SD	78.9 ± 8.8	-	-
Duration of illness (years), mean ± SD	9.8 ± 6.5	-	-

SD, Standard Deviation; LOAD, Late-onset Alzheimer's disease; MMSE, Mini-Mental State Examination. Interpretation of the scores on the MMSE (out of 30 points) includes the categories of severe (9 or less), moderate (10–18 points), mild (19–23 points) and normal cognition (24 or more points) [25].

**Table 2.** The primer data for 5-HTR2A, 5-HTR3A, MAO-A and  $\beta$ -actin.

Genes	OligoName	Primers Sequence (5' → 3')	Amplicon Size (bp)	Annealing Tm (°c)	Genbank Accession Number
5-HTR2A	5HTR2A- F	CCATCCAGAATCCCATCCACC	180	56	NM_001165947.2
	5HTR2A-R	GGACAAAGTTATCATCGGCGAG			
5-HTR3A	5HTR3A-F	GGTACCGGCAGTACTGGA	466	63	NM_001161772.3
	5HTR3A- R	CGGCGGATGACCACATAG			
MAO-A	MAO-A- F	GCTGGACAAAGACTGCTAGGCGG	225	63	NM_000240.3
	MAO-A- R	GCTTCACTTGGTCTCCGAGGAGGT			
ACTB	$\beta$ -actin- F	AGACGCAGGATGGCATGGG	161	62	NM_001101.3
	$\beta$ -actin- R	GAGACCTTCAACACCCAGCC			

5-HT2A, 5-hydroxytryptamine receptor 2A; 5-HTR3A, 5-hydroxytryptamine receptor 3A; MAO-A, Monoamine oxidase A;  $\beta$ -actin, Beta-actin.

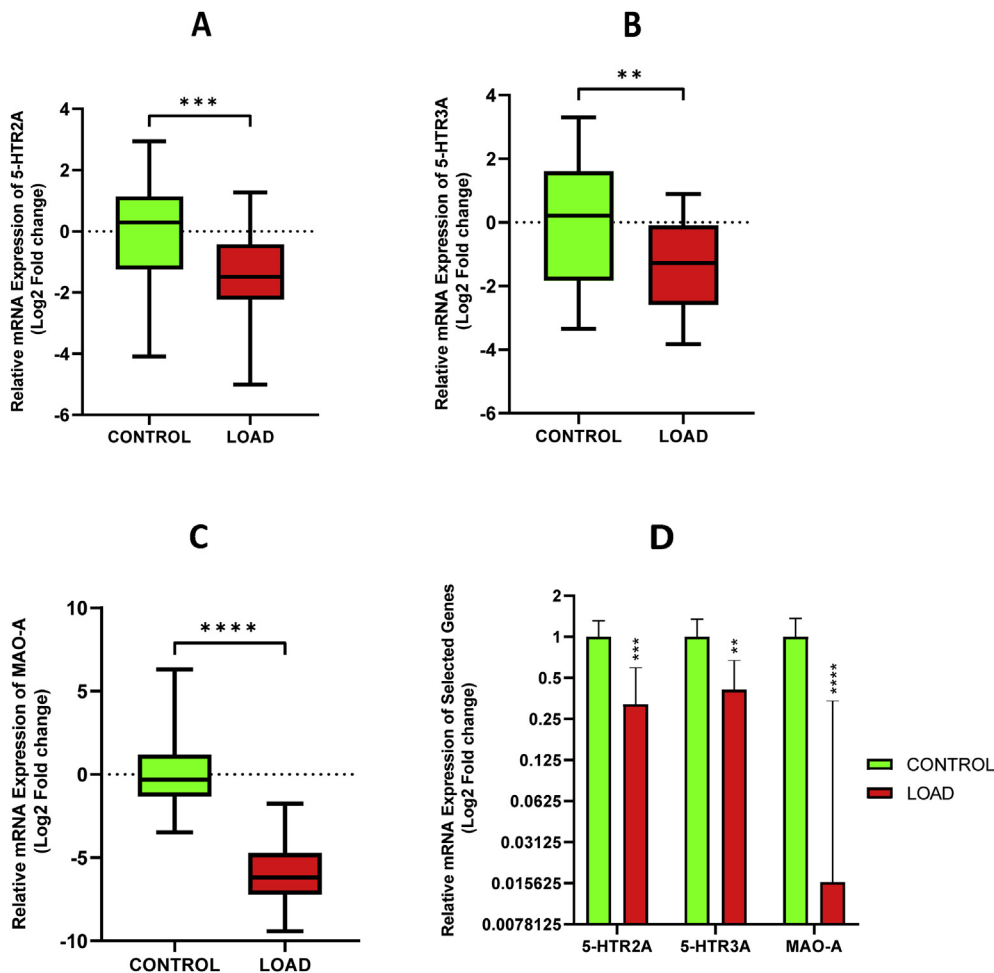
**2.4. Data analysis**

The LinRegPCR Software (Version 11.0) was used to calculate PCR efficiency (E) and crossing point deviation ( $\Delta$ CP) based on Real-time PCR data of each sample. Subsequently, Relative Expression Software Tool (REST © 2009, Version 2.0.13) was used for statistical analysis and the calculation of relative gene expression. GraphPad Prism Software (version 8.0.2.263; San Diego, CA) was used to determine the normal distribution of variables using the Kolmogorov-Smirnov test. Comparisons have been calculated by SPSS software (version 23; IBM SPSS Statistics). To compare the mean of each group, independent t-test was used and the Pearson correlation coefficient was used to determine the relationship between the variables. P-value of less than 0.05 was considered statistically significant.

**3. Results**

**3.1. Comparison of demographic characteristics between LOAD and control groups**

Means and standard deviations for the age and MMSE total score in both groups and age of disease onset and duration of illness in the LOAD group are presented in Table 1. Based on these results, the majority of patients in the LOAD group were in severe cognitive impairment, and the majority of the control group were in normal cognitive status. To assess the differences between the two groups in terms of age and MMSE score, Levene's test for equality of variances was initially performed. In the age variable, the statistical value of F = 0.731 and p-value = 0.396 and in the MMSE score variable, the statistical value of F = 0.449 and p-value =



**Figure 1.** Comparison of fold changes in mRNA expression levels of 5-HTR2A, 5-HTR3A, and MAO-A in PBMCs samples among control individuals and LOAD patients. A: 5-HTR2A is DOWN-regulated in LOAD patients (in comparison to control individuals) by a mean factor of 0.376 (p = 0.0008). B: 5-HTR3A is DOWN-regulated in LOAD patients (in comparison to control individuals) by a mean factor of 0.418 (p = 0.0046). C: MAO-A is DOWN-regulated in LOAD patients (in comparison to control individuals) by a mean factor of 0.014 (p < 0.0001). D: The mRNA expression ratios plotted for 5-HTR2A, 5-HTR3A, and MAO-A were significantly decreased in LOAD patients in comparison to the control individuals. Error bars represent standard Error of Mean. The number of subjects in each group = 30. Statistical significance is denoted by an asterisk: \*\*, P < 0.01; \*\*\*, P < 0.001; \*\*\*\*, P < 0.0001. PBMCs, Peripheral Blood Mononuclear Cells; LOAD, Late-onset Alzheimer's disease.

0.506 were obtained. Independent t-test showed that there was a statistically significant difference in the Age and MMSE score variables between two groups ( $p < 0.001$ ,  $p < 0.0001$ , respectively).

### 3.2. Comparisons of 5-HTR2A, 5-HTR3A, and MAO-A mRNA expression between the two groups

Independent t-test showed that the relative level of 5-HTR2A, 5-HTR3A, and MAO-A mRNA expression in PBMCs significantly decreased in the LOAD group compared to the control group ( $p = 0.0008$ ,  $p = 0.0046$ ,  $p < 0.0001$ , respectively).

The relative levels of 5-HTR2A, 5-HTR3A, and MAO-A mRNA expression based on the log<sub>2</sub>-transformed value are shown in Figure 1.

### 3.3. Correlation analysis of 5HTR2A, 5HTR3A and MAO-A mRNA expression with demographic characteristics

In the control group, the Pearson correlation test was performed between each of the variables age and MMSE total score with relative expression of 5-HTR2A, 5-HTR3A and MAO-A genes, respectively. According to the obtained results, no statistically significant relationship was observed between the mentioned variables.

## 4. Discussion

The results of this study showed a significant decrease in 5-HTR2A, 5-HTR3A and MAO mRNA levels in PBMCs of patients with LOAD. We suspect that a decrease in the expression of 5-HTR2A and 5-HTR3A mRNA in PBMCs of patients with LOAD may reflect a decrease in their brain levels. Since CNS tissues are generally not available for genetic studies, the idea of replacing more accessible tissues such as peripheral blood lymphocytes has been proposed. It has been shown that careful and deliberate use of peripheral gene expression may be a useful alternative to gene expression in the CNS, and the peripheral gene expression pattern establishes a reasonable relationship with CNS gene expression, especially when identifying the respective genes in the target tissues [11].

5-HT receptors (5-HTRs) include seven classes from 5-HTR1 to 5-HTR7, some of which have a variety of subclasses. The 5-HTR2A class, along with the other 5 classes, are all G-protein-coupled receptors, while the 5-HTR3A receptor is a ligand-gated ion channel receptor that regulates permeability to sodium, potassium and calcium ions in the CNS and peripheral nervous system [28, 29]. In the brain, 5-HT serves as a neurotransmitter while in the periphery it serves as a hormone regulating a range of processes. Because 5-HT does not transit the Blood-Brain Barrier (BBB), peripheral and CNS 5-HT has no cross over, and functionally can be considered to two distinct molecules [30].

5-HT is a component of the monoaminergic system that modulates cognitive functions and some symptoms of AD are attributed to disorders of the serotonergic system [31, 32]. Serotonergic neurons are composed of a vast and complex network that is present in almost all brain structures [1] and as expected, extensive serotonergic denervation has long been reported in the brain of patients with AD [33, 34, 35]. Aligned with these findings, 5-HT depletion, as well as its metabolites, have been observed in post-mortem AD brain tissue [36, 37, 38]. 5-HT receptors are selectively affected in AD and the 5-HT<sub>2</sub> receptor family is generally defective [39, 40]. In support of this finding, Studies using various methods such as immunohistochemistry, post-mortem, and imaging have all shown a reduction in brain 5-HTR2A density in patients with AD [41, 42, 43, 44, 45, 46]. Moreover, reduction of 5-HTR2A in patients with amnesic mild cognitive impairment is widespread and occurs early in the course of the disease [47]. Based on the peripheral marker hypothesis that suggests the expression level of neurotransmitter receptors in peripheral blood lymphocytes is parallel to the expression level in the brain [48], our results also showed a decrease in 5-HTR2A gene expression in PBMCs of patients with LOAD, which is consistent with the results of previous similar studies on Alzheimer's brains. While the 5-HTR3

location in brain areas is related to the phenomenon of cognition, so far few studies have been conducted on the involvement of 5-HT<sub>3</sub> receptors in cognitive functions, and most studies are limited to the effects of receptor antagonists on improving memory [49, 50, 51, 52].

In human peripheral blood, the immune cells express 5-HT receptors of 5-HTR1, 5-HTR2, 5-HTR3, 5-HTR4, and 5-HTR7 classes and MAO. 5-HTR2A and 5-HTR3A are expressed by monocytes, macrophages, and T cells. Besides, 5-HTR2A is expressed by eosinophils, B cells, and platelets. MAO is expressed by monocytes, macrophages, T cells, and platelets [53]. One of the strongest evidence that immune cells respond to 5-HT is that they essentially express the molecular machinery that makes up the serotonergic system [54]. As mentioned earlier, some researchers have identified AD as a systemic disease that can also affect peripheral tissues outside the central nervous system [23, 55], and that peripheral and CNS 5-HT are not physiologically able to cross BBB [30], they somehow complicate the role of 5-HT in the pathogenesis of AD. The presence of 5-HT receptors in both PBMCs and the nervous system indicates a possible role for 5-HT in the relationship between the CNS and the immune system [56]. One possible intermediate system in the human body that can interact with both 5-HT and AD is the immune system. Our possible assumption is that 5-HT affects the immune system, and the altered immune system, in turn, keys the mechanisms for brain damage and AD progression. This assumption is reinforced by recent studies showing that 5-HT regulates a wide range of leukocyte functions, from activating the immune response to the formation of immune memory cells. Interestingly, 5-HT modulates cytokine secretion in monocytes/macrophages, or it can suppress Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1  $\beta$  (IL-1  $\beta$ ) by activating 5-HTR2A in PBMCs [54]. Other researchers have shown that 5-HT in human monocytes modulates the release of various cytokines and chemokines, mainly through 5-HTR3, 5-HTR4 and 5-HTR7 [57].

Growing studies have shown that in AD, the presence of persistent and unresolved inflammation in neurons (Neuroinflammation) may lead to the destruction of neurons and a decrease in functional and cognitive abilities [58, 59, 60, 61, 62]. This inability to relieve inflammation in the brains of AD patients, which results in the activation of microglial cells and astrocytes, leads to the use of peripheral immune cells and the overproduction of pro-inflammatory mediators [63, 64]. In the CNS, activated microglial cells have been shown to release pro-inflammatory cytokines, including IL-1  $\beta$ , IL-6, and TNF- $\alpha$ , thereby leading to the dendritic spine loss [65, 66]. As mentioned earlier, an increase in the release of IL-1  $\beta$  and TNF- $\alpha$  outside the CNS has also been observed in response to the inactivation of 5-HTR2A in PBMCs [54]. Therefore, such similar events could indicate possible links between the hormonal role of 5-HT in the peripheral and the neurotransmitter role of 5-HT in the CNS, which requires further studies. Furthermore, changes in BBB in AD may be an early and important step in pathogenesis [67]. Microglial priming could sensitize the BBB to disruptive changes [68]. The cellular infiltration during systemic inflammation in AD is also associated with several BBB changes without disturbance, which may increase amyloid deposition and contribute to disease progression [69]. Because both acute and chronic systemic inflammation can accelerate the progression of AD [70], peripheral 5-HT may also be able to exert its regulatory effects on the brain, either through primary BBB changes or through systemic inflammation that causes secondary BBB changes. However, more research is needed to clarify these issues.

In a review of the literature, no similar studies of 5-HTR2A, 5-HTR3A and MAO-A gene expression in PBMC of patients with LOAD were found, but so far the peripheral expression profiles of each of these genes (alone or in combination) have been investigated in other human disorders such as depression, multiple sclerosis, breast cancer, and allergic asthma [56, 71, 72, 73, 74]. Although only one study using [3H]LSD as a radio-ligand showed that platelet density of 5-HT<sub>2</sub> receptors in AD patients was not significantly different from that of healthy controls [75], no similar studies were found to investigate the 5-HT receptors in other peripheral blood cells of AD patients. Despite documented involvement of the



serotonergic system in AD, we currently lack a comprehensive understanding of the molecular downstream mechanisms that explain the effects of each component of this system on AD [76].

MAO-A, which preferentially oxidizes 5-HT, is responsible for the presynaptic degradation of 5-HT [77, 78]. Some studies have reported both increased MAO-A activity and gene expression in different brain regions of patients with AD [21, 79, 80]. Increased MAO activity will be associated with a decrease in CNS monoamine levels such as 5-HT. A change in the serotonergic system is observed in the aging brain, as well as in depressed and aggressive patients with AD, and these changes may, in turn, play an important role in memory decline and other cognitive functions [81, 82, 83, 84, 85]. In the present study, a decreased MAO-A gene expression was observed in PBMCs of patients with LOAD, which is in contrast to previous findings on the activity of this enzyme in the brain of patients with AD. This contradiction may be due to an unknown compensatory mechanism in the brain versus the periphery and warrants further studies.

As the decrease in the 5-HTR2A receptor has been reported in the early stages of mild cognitive impairment [47], our argument for a decrease in the expression of 5-HTR2A, 5-HTR3A and MAO mRNA in PBMCs of patients with LOAD is that these changes may precede the onset of AD. These genetic changes in the blood cells of patients may coincide with the progression of AD in the CNS. These changes may occur early and before pathological changes in the brain. Therefore, such changes may be used as a clinical tool in the early diagnosis of AD before overt mental deterioration.

The present study had several limitations. This study was limited to mRNA analysis and did not include the evaluation of 5-HT receptor subunit proteins. MAO activity was not measured. Larger sample sizes with more diverse participants are also needed for generalizable results. Research on peripheral blood gene expression is very promising, as it would be economically and technically difficult to detect such changes directly in vivo in the CNS. Conducting similar sets of studies in post mortem tissue in which both peripheral and CNS samples are available simultaneously, would be useful to further investigate the association between 5-HTR2A, 5-HTR3A and MAO mRNA levels in the blood and brain of AD patients.

## 5. Conclusion

The present study found that patients with LOAD have lower mRNA expression levels of 5-HTR2A, 5-HTR3A, and MAO-A comparing with their healthy counterparts. These genetic changes in the blood cells of patients with LOAD can act as an affected tissue and coincide with the progression of AD in the CNS, or even these changes occur early and before any pathological changes in the brain tissue of these patients. Our findings may help further clarify the link between serotonergic system and the immune system that have been shown to be effective in the pathogenesis of AD. However, if these molecular changes are influenced by the tissue's response to AD-related pathologies, it possibly will provide a new pathway for studies focused on blood-based markers that looking for diagnostic or prognostic biomarkers in LOAD.

## Declarations

### Author contribution statement

Masoud Neshan: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Arezoo Campbell: Conceived and designed the experiments; Wrote the paper.

Seyed Kazem Malakouti: Conceived and designed the experiments.

Mahsa Zareii: Performed the experiments.

Ghasem Ahangari: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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### Competing interest statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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