

EXPRESSION OF SELECTED miRNAs IN CIRCULATING BLOOD OF EARLY AND LATE-ONSET ALZHEIMER DISEASE PATIENTS

ERKEN VE GEÇ BAŞLANGIÇLI ALZHEIMER HASTALARININ PERİFERİK KANLARINDA SEÇİLMİŞ miRNA'LARIN ANLATIM DÜZEYİ

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Cite this article as: Guven G, Lohmann E, Gulec C, Bilgic B, Ozer E, Hanagasi H, et al. Expression of selected miRNAs in circulating blood of early and late-onset Alzheimer disease patients. J Ist Faculty Med 2021;84(2):165-74. doi: 10.26650/IUITFD.2020.0061

ABSTRACT

Objective: Inflammation and associated microRNAs (miRNA) both play essential roles in the pathogenesis of Alzheimer's disease (AD). The expression profile of miRNA's in an AD brain, also reflected in the peripheral blood mononuclear cells, may give support to the inflammatory changes seen in the course of AD. We aimed to investigate the expression levels of specific inflammatory miRNAs (mir-146a, mir-144, mir-34a) in both blood leukocytes and plasma of AD patients and to evaluate their potential usability as biomarkers in AD diagnosis and to also demonstrate whether the expression of these miRNAs differ between early and late-onset AD patients.

Methods: We investigated the expression levels of miRNAs in 16 early-onset and 26 late-onset AD patients and in their respective controls by using qRT-PCR.

Results: Plasma mir-144 levels were significantly different between EOAD and LOAD patients ($p=0.015$). In addition, levels of leukocyte mir-34a were significantly down-regulated in EOAD compared to LOAD patients ($p=0.027$). Our results also showed significant positive correlations between age and plasma mir-144 and leukocyte mir-34a expressions.

Conclusion: The differential expression of plasma mir-144 and leukocyte mir-34a between EOAD and LOAD patients might ref-

ÖZET

Amaç: Enflamasyon ve ilişkili mikroRNA'lar (miRNA), Alzheimer hastalığının (AH) patogeneğinde önemli rol oynamaktadırlar. AH beyininde görülen miRNA'ların anlatım profilindeki değişikliklerin periferik kan mononükleer hücrelerine de yansımış olması, AH sırasında görülen enflamatuvar değişikliklerin bir göstergesi olabilir. Bu çalışmada, Alzheimer hastalarının hem periferik lökositlerinde hem de plazmalarında belirli enflamatuvar miRNA'ların (mir-146a, mir-144, mir-34a) anlatım düzeylerindeki değişiklikleri araştırmayı ve ve AH tanısında biyobelirteç olarak kullanılabilirliklerinin değerlendirilmesini amaçladık. Ayrıca, bu miRNA'ların anlatımlarının erken ve geç başlangıçlı AH arasında farklılık gösterip göstermediğini belirlemeyi amaçladık.

Gereç ve Yöntem: Gerçek zamanlı polimeraz zincir reaksiyonu yöntemini kullanarak 16 erken başlangıçlı ve 26 geç başlangıçlı AH hastası ve yaş-uyumlu kontrollerinde miRNA'ların anlatım düzeylerini araştırdık.

Bulgular: Plazma mir-144 anlatım düzeyinin erken ve geç başlangıçlı AH hastaları arasında anlamlı farklılık gösterdiğini saptadık ($p=0,015$). Ek olarak, lökosit mir-34a anlatım düzeyi erken başlangıçlı AH'de geç başlangıçlı AH hastalarına göre anlamlı derecede düşük bulundu ($p=0,027$). Sonuçlarımız ayrıca, yaş ve plazma mir-144 ($r=0,319$, $p=0,02$) ile lökosit mir-34a

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Submitted/Başvuru: 15.05.2020 • **Revision Requested/Revizyon Talebi:** 20.07.2020 •

Last Revision Received/Son Revizyon: 27.08.2020 • **Accepted/Kabul:** 07.09.2020 • **Published Online/Online Yayın:** 09.03.2021



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lect age-dependent changes in miRNA expression and might be independent of the disease status.

Keywords: Alzheimer disease, miRNA, miRNA expression

($r=0,414$, $p=0,001$) anlatım düzeyleri arasında anlamlı pozitif korelasyon olduğunu gösterdi.

Sonuç: Bulgularımız erken ve geç başlangıçlı AH hastaları arasında plazma mir-144 ve lökosit mir-34a anlatımlarında görülen farklılığın miRNA ekspresyonundaki yaşa bağlı değişiklikleri yansıttığını ve hastalık durumundan bağımsız olabileceğini düşündürmektedir.

Anahtar Kelimeler: Alzheimer hastalığı, miRNA, miRNA anlatımı

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease leading to cognitive decline, interfering with the activities of daily living. The main pathological hallmarks of the disease are extracellular protein deposits called amyloid plaques (A β) and intracellular deposits called neurofibrillary tangles, consisting of phosphorylated tau protein. Alzheimer's disease is divided into two groups according to the age of onset; early-onset (<65 years old) and late-onset (> 65 years old). Early-onset AD (EOAD) is mostly familial and caused by rare mutations in one of three responsible genes: *Amyloid precursor protein (APP)*, *Presenilin 1 (PSEN1)* and *Presenilin 2 (PSEN2)*. Late-onset AD (LOAD), a more common type of AD, is mostly sporadic. Numerous genetic and environmental factors pose risks for the development of AD, affecting the age at onset and the progression of the disease. Inflammation is one of the core factors in the pathogenesis of AD. The mechanisms underlying the process from A β production to neurodegeneration partly depend on fluctuation in the expression of genes involved in inflammation, which suggests the transcriptional and/or post-transcriptional regulation of these genes. One of the regulatory factors that may play a role in this process is the behaviour of small non-coding RNA molecules called micro RNAs (miRNAs) that regulate gene expression in the post-transcriptional stage (1). miRNAs have fine-tuning roles in inflammatory processes, and their expression profiles are known to change in various diseases (2).

Studies have shown that the expression levels of several miRNAs have altered in AD and moreover, these changes have been reported not only in disease-related brain regions (3) but also in peripheral body fluids such as blood, cerebrospinal fluid (CSF), plasma and serum (4). Determination of these changes in peripheral samples enables these miRNAs to be used as non-invasive biomarkers for the early diagnosis of AD. Accordingly, studies have found significant differences in the expression levels of miRNAs, which involved a number of important pathways including inflammation, in peripheral mononuclear cells of Alzheimer's patients (5).

Therefore, in our study we first aimed to investigate whether the expression levels of specific inflammatory miRNAs could be identified in both peripheral blood

cells (leukocytes) and cell-free plasma of Alzheimer patients and to evaluate their potential usability as a biomarker in AD diagnosis in the Turkish cohort of AD patients. Secondly, our aim was to determine whether the expression of the selected miRNAs differed between early and late-onset AD.

In the selection of miRNAs included in our study, we considered that they showed altered expression profile in peripheral body fluids of AD patients as well as the fact that they were not previously studied in the Turkish population. Based on this, three candidate miRNAs (mir-146a, mir-144 and mir-34a) known to be involved in inflammatory processes and whose target genes are associated with A β metabolism were selected for the study (Table 1). Mir-146a plays a key role in the negative feedback regulation of the inflammatory pathway activated by nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) by suppressing the expression of its target gene; Interleukin-1 receptor-associated (*IRAK1*) (6). mir-144 is a negative regulator of ADAM10, a major α -secretase responsible for cleaving APP, and up-regulation of mir-144 decreases expression of ADAM10 protein (7). Mir-34a is a NF κ B regulated miRNA that is involved in inflammatory processes and through down-regulation of its target gene triggering receptor expressed in myeloid cells 2 (*TREM2*), it may be involved in AD pathogenesis (8).

MATERIALS AND METHODS

Patients and controls

The study population was comprised of 42 Turkish AD patients and 20 Turkish control subjects with no history of any major neurologic and psychiatric disease. Patients were separated into two groups according to age at onset; EOAD (n=16) and LOAD (n=26) and also controls were divided into two groups as young (age <65 yrs, n=8) and elderly (age $\geq$ 65yrs, n=12). Participants were recruited in the Behavioural Neurology and Movement disorders Unit of the Neurology Department. All participants underwent detailed clinical and neuropsychological examination with neuroimaging. The diagnosis of dementia was based on the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's disease (9). A Mini Mental State Examination test (MMSE) was used to evaluate the global cognitive status in the

Table 1: Selected circulating miRNAs specifying their regulation status, source analysed, target genes and detection method

miRNA	Target gene	Source	miRNA detection method	Status	Reference
mir-146	<i>TRAF6, IRAK1, TSPAN12</i>	CSF	Microarray	increased	(14)
			qRT-PCR	increased	(25)
			qRT-PCR	decreased	(16)
			Microarray	increased	(15)
			Microarray/qRT-PCR	decreased	(17)
			qRT-PCR	decreased	(26)
mir-144	<i>ADAM10</i>	blood	Small RNA seq	decreased	(26)
			qRT-PCR	decreased	(29)
mir-34a	<i>TREM2, BECLIN1</i>	CSF	qRT-PCR	decreased	(27)
			Microarray	increased	(19)
			qRT-PCR	decreased	(28)
		plasma	qRT-PCR	decreased	(20)
			qRT-PCR	increased	(30)
			qRT-PCR	increased	(18)
		blood	Microarray	increased	(26)

CSF=Cerebrospinal fluid, PBMC=Peripheral blood mononuclear cell, qRT-PCR=quantitative real-time polymerase chain reaction

participants. The study was approved by the Ethics Committee of the University (Date:12/08/2014, No:1221). The procedures used in this study adhere to the tenets of the Declaration of Helsinki. Informed consent was obtained from the individual participants or the legal guardians of the subjects unable to consent.

Total RNA isolation from blood leukocytes and plasma

20 ml peripheral blood was collected into EDTA containing tubes to isolate leukocytes and plasma. Blood samples were centrifuged at 1400xg for 10 min and separated plasma samples were stored at -80°C for further use. Total RNA was isolated from peripheral blood leukocytes by using a miRNeasy Mini Kit (Qiagen) and from 600 µl of plasma by using a mirVana PARIS Isolation Kit (Applied Biosystems) according to the manufacturer's instructions. During the first step of the plasma RNA isolation protocol, 0.30 fmols synthetic miRNA-39 from *Caenorhabditis elegans* cel-miR-39 (Qiagen) was added to each sample to normalise and monitor the extraction efficiency in RNA isolation.

Quantitative real-time PCR

MicroRNAs were transcribed by using the Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems), with specific miRNA stem-loop primers according to the manufacturer's instructions. 15 µl reverse transcription (RT) reaction mixture consists of 0.15 µl of 100 mM dNTP, 1 µl of MultiScribe reverse transcriptase (50 U/µL), 1.5 µl of 10× RT

buffer, 0.19 µl of RNase inhibitor (20 U/µl), 4.16 µl of RNase-free water, 3 µL of 5×miRNA-specific stem-loop RT primer and 5 µl of the RNA sample (1-10ng total RNA). RT reaction for each specific miRNA was performed at 16°C for 30 min, at 42°C for 30 min, and at 85°C for 5 min.

Quantitative real-time polymerase chain reaction (RT-qPCR) was performed to determine the expression levels of miRNAs using the TaqMan MicroRNA Assays (Applied Biosystems). A total of 10 µl qPCR reaction mix was composed of 1 µl TaqMan Small RNA Assay (Applied Biosystems), 5 µl LightCycler 480 Probes Master (Roche), 2 µl RNase-free water and 2 µl RT reaction product. The qRT-PCR was carried out on a LightCycler 480 system (Roche) under the following conditions: 95°C 10 min, 45 cycles of 95°C 15 s, and 60°C 1 min. All qRT-PCR reactions were run in triplicate. The relative expression level of each miRNA was calculated using the $2^{-\Delta\Delta C_t}$ method. RNU48 was used as an endogenous control in $2^{-\Delta\Delta C_t}$ calculations for the leukocyte samples. The Ct values of plasma samples were first normalised against cel-miR-39 and then against RNU6, as described previously (10).

Statistical analysis

Relative mRNA levels between groups were compared using a two-tailed Mann-Whitney U test. Correlations of miRNA levels with age and MMSE in all subjects and correlations of miRNA levels with age at onset in AD patients

were analysed using Spearman's correlation test. Descriptive statistical analysis of the groups was performed with an χ^2 test for the categorical variables, and a Student's t test for the continuous variables. We used receiver operating characteristic (ROC) curve analysis and calculated the area under the curve (AUC) of the miRNAs to evaluate their sensitivity and specificity for distinguishing AD from controls or EOAD from LOAD. The criterion for significance was set at $p < 0.05$. The stability of the candidate reference gene expression in groups was evaluated by the NormFinder (11) and BestKeeper (12) programs. All statistical analyses were performed with SPSS 21.0 software (IBM Corp, Armonk, NY, USA).

RESULTS

Characteristics of the study group

Demographic and clinical characteristics of the subjects are summarised in Table 2. As shown in table 2, mean MMSE scores were similar between EOAD and LOAD groups but as expected, lower than their age-matched controls.

miRNA expression levels

mir-146a levels

The plasma and leukocyte expression levels of mir-146a did not show a significant difference between any of the AD patients and controls. Figure 1 shows plasma and leukocyte mir-146 expression in EOAD, LOAD groups and

Table 2: Descriptive characteristics of patient groups and controls

	EOAD (n=16)	Young controls (n=8)	p-value	LOAD (n=26)	Elderly controls (n=12)	p-value
Age at recruitment, years	58.3±9.6	54.25±8.44	0.33	76.5±4.4	73.7±5.3	0.09
Age of onset, years	53±8.8			71.6±4.5		
Gender, n (%)						
Male	4 (25)	4 (50)	0.22	9 (34.6)	5 (41.7)	0.67
Female	12 (75)	4 (50)		17 (65.4)	7 (58.3)	
MMSE score	16.4±7.7 (n=12)	28.25±1.2 (n=4)	0.009	17.7±6.0 (n=25)	28.45±1.5 (n=11)	<0.001
APOE ϵ 4 status, n (%)						
ϵ 4 carrier	6 (40)	2 (25)	0.47	12 (46.2)	2 (16.7)	0.08
ϵ 4 non-carrier	9 (60)	6 (75)		14 (53.8)	10 (83.3)	

Continuous variables are presented as mean±SD and dichotomous variables as percentage. A t-test was used for comparison of means and χ^2 test for percentages. Abbreviations: AD=Alzheimer's disease, MMSE=Mini mental state examination, n=number of individuals

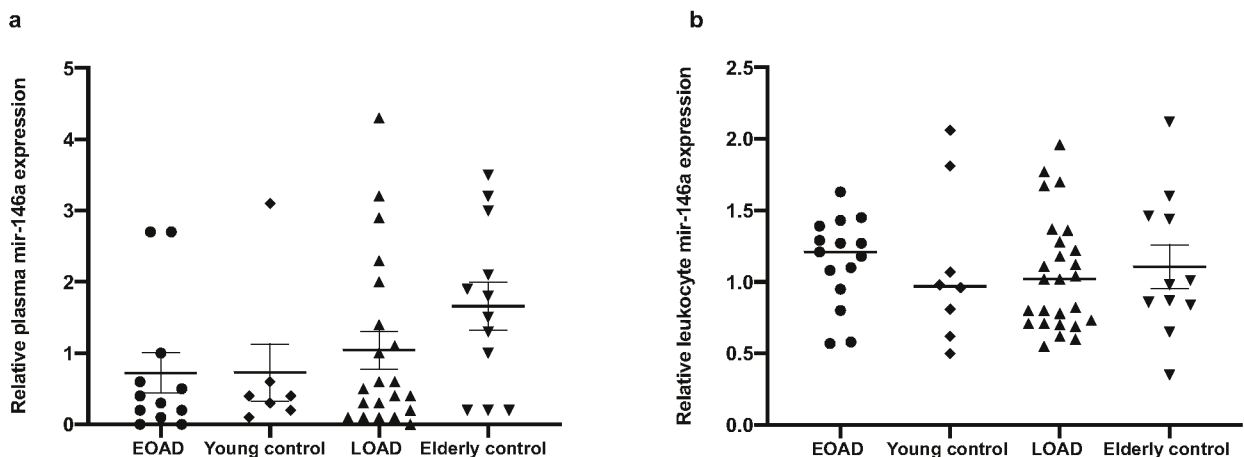


Figure 1: Plasma (a) and leukocyte (b) mir-146a levels in EOAD, LOAD, young and elderly controls. Data are presented as a scatter plot with mean±SEM. EOAD=Early-onset Alzheimer Disease, LOAD=Late-onset Alzheimer Disease. plasma mir-146a: EOAD vs young control $p=0.766$, LOAD vs elderly control $p=0.111$, EOAD vs LOAD $p=0.388$, young control vs elderly control $p=0.116$; leukocyte mir-146a: EOAD vs young control $p=0.401$, LOAD vs elderly control $p=0.690$, EOAD vs LOAD $p=0.273$, young control vs elderly control $p=0.836$

their respective controls. Plasma mir-146a expression in EOAD group (mean±SEM; 0.725 ± 0.28) was similar to young controls (mean±SEM; 0.729 ± 0.39 , $p=0.766$) but lower levels of mir-146a were observed in LOAD group (mean±SEM; 1.04 ± 0.26) than elderly controls (mean±SEM; 1.66 ± 0.33), without any significance ($p=0.111$; Figure 1a). Also, there was no significant difference between EOAD and LOAD patients, in terms of plasma mir-146a expression levels ($p=0.388$). As shown in Figure 1b, leukocyte mir-146a expression was similar between EOAD (mean±SEM; 1.15 ± 0.08) and young controls (mean±SEM; 1.10 ± 0.19 , $p=0.401$), also similar between LOAD (mean±SEM; 1.05 ± 0.08) and elderly controls (mean±SEM; 1.10 ± 0.15 , $p=0.690$). In addition, there was no significant difference between the EOAD and LOAD groups ($p=0.273$).

mir-144 levels

The plasma and leukocyte expression levels of mir-144 did not show any significant difference between AD patients and controls. Figure 2 shows plasma and leukocyte mir-144 expression in EOAD, LOAD groups and their respective controls. Plasma mir-144 levels did not differ significantly in EOAD (mean±SEM; 0.54 ± 0.19) compared to young controls (mean±SEM; 0.82 ± 0.32 , $p=0.366$) and in LOAD (mean±SEM; 2.94 ± 0.74) compared to elderly controls (mean±SEM; 3.14 ± 1.17 , $p=0.946$) but there was a significant decrease in EOAD compared to LOAD ($p=0.015$; Figure 2a). Also, plasma mir-144 expression seems to be decreased in young controls compared with elderly, but this was not statistically significant ($p=0.178$). As shown in Figure 2b, leukocyte mir-144 levels in EOAD (mean±SEM; 0.99 ± 0.22) and young controls (mean±SEM; 0.89 ± 0.17) were similar ($p=0.746$). Also, no significant

difference between the LOAD (mean±SEM; 1.46 ± 0.27) and elderly controls (mean±SEM; 1.77 ± 0.45) were found ($p=0.637$). Although the leukocyte mir-144 level seems to decrease in EOAD patients compared to LOAD patients, no significant difference was found ($p=0.787$). In addition, a lower leukocyte mir-144 level was observed in the young controls than in the elderly controls without any significance ($p=0.537$).

mir-34a levels

The plasma and leukocyte expression levels of mir-34a did not show any significant difference between the AD patients and the controls. Figure 3 shows plasma and leukocyte mir-34a expression in EOAD, LOAD groups and their respective controls. Plasma mir-34a expression did not show a significant difference between EOAD (mean±SEM; 1.20 ± 0.32) and young controls (mean±SEM; 0.94 ± 0.42 , $p=0.623$) but mir-34a expression seems to decrease in LOAD (mean±SEM; 1.57 ± 0.48) compared to elderly controls (mean±SEM; 2.35 ± 0.76) without any significance ($p=0.293$; Figure 3a). Besides, plasma mir-34a expression was similar between the EOAD and LOAD groups ($p=1.000$). As shown in Figure 3b Leukocyte mir-34a levels did not differ significantly in EOAD (mean±SEM; 0.98 ± 0.17) compared to young controls (mean±SEM; 1.04 ± 0.18 , $p=0.481$) and in LOAD (mean±SEM; 1.17 ± 0.07) compared to elderly controls (mean±SEM; 1.39 ± 0.2 ; $p=0.420$) but it was significantly decreased in the EOAD patients compared with the LOAD patients ($p=0.027$). In addition, leukocyte mir-34a expression seems to decrease in the young controls compared with the elderly, but this was not statistically significant ($p=0.342$).

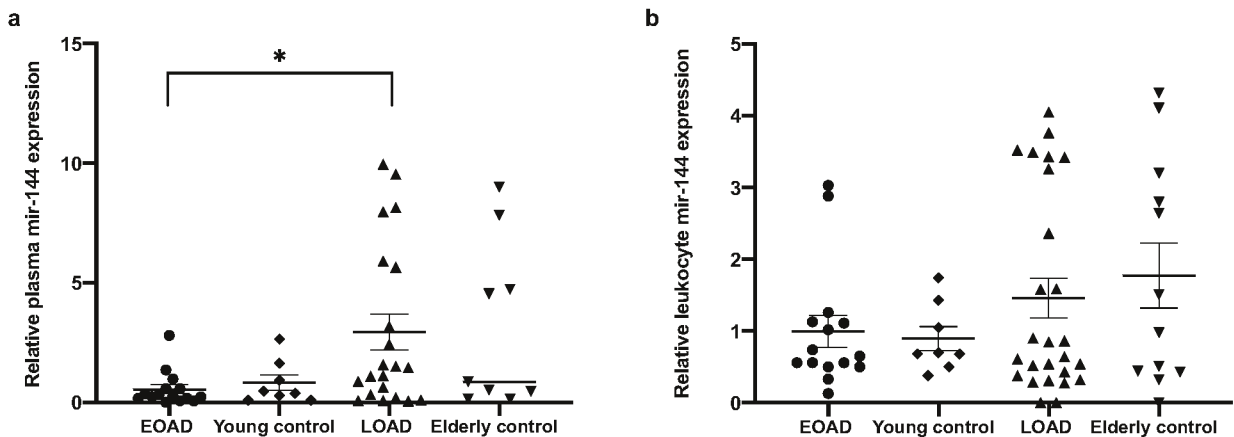


Figure 2: Plasma (a) and leukocyte (b) mir-144 levels in EOAD, LOAD, young and elderly controls. Data are presented as a scatter plot with mean±SEM. * $p < 0.05$, EOAD=Early-onset Alzheimer Disease, LOAD=Late-onset Alzheimer Disease. plasma mir-144: EOAD vs young control $p=0.366$, LOAD vs elderly control $p=0.946$, EOAD vs LOAD $p=0.015$, young control vs elderly control $p=0.178$; leukocyte mir-144: EOAD vs young control $p=0.746$, LOAD vs elderly control $p=0.637$, EOAD vs LOAD $p=0.787$, young control vs elderly control $p=0.537$

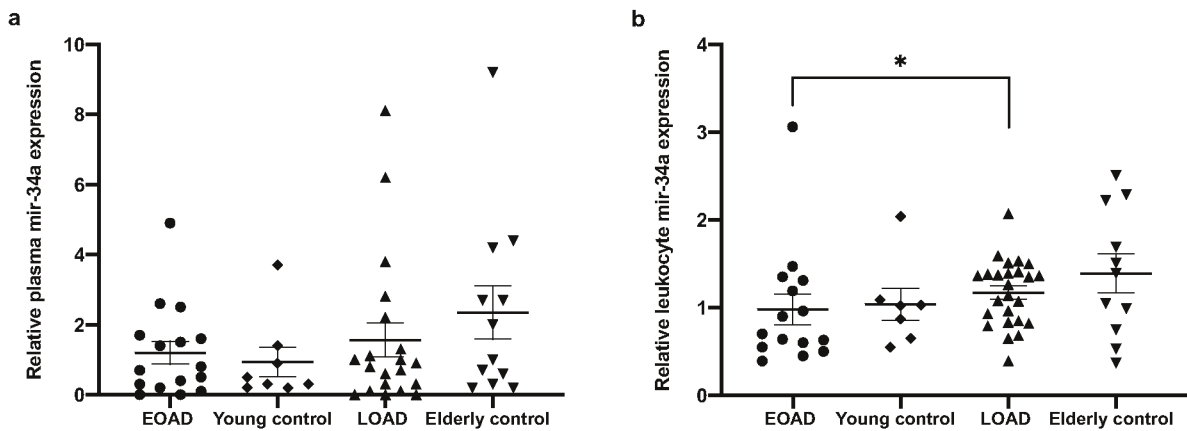


Figure 3: Plasma (a) and leukocyte (b) mir-34a levels in EOAD, LOAD, young and elderly controls. Data are presented as a scatter plot with mean±SEM. * $p<0.05$, EOAD=Early-onset Alzheimer Disease, LOAD=Late-onset Alzheimer Disease. plasma mir-34a: EOAD vs young control $p=0.623$, LOAD vs elderly control $p=0.293$, EOAD vs LOAD $p=1.000$, young control vs elderly control $p=0.163$; leukocyte mir-34a: EOAD vs young control $p=0.481$, LOAD vs elderly control $p=0.420$, EOAD vs LOAD $p=0.027$, young control vs elderly control $p=0.342$

ROC curve analysis

To evaluate the sensitivity and specificity of the significantly altered miRNAs, a receiver operating characteristic (ROC) curve analysis was used. The ROC curve analysis and calculated area under the curve (AUC) of significant miRNAs are presented in Table 3. Both plasma mir-144 and leukocyte mir-34a were sensitive to distinguish LOAD from EOAD (Figure 4a-b). The AUC for plasma mir-144 was 0.741 (95% CI, 0.577-0.905), with 71.4% sensitivity and 80% specificity. Similarly, the AUC for leukocyte mir-34a was 0.711 (95% CI, 0.533-0.888), with 44% sensitivity and 86.7% specificity. However, the diagnostic power values of plasma mir-144 and leukocyte mir-34a in differentiation of EOAD or LOAD patients from controls as measured

by AUC were lower than 0.70, indicating poor diagnostic value for distinguishing the patients from the controls (Table 3). As we performed ROC analysis for both miRNAs in all young and elderly individuals regardless of the disease status, both miRNAs were sensitive to distinguish young individuals from elderly, the AUC for plasma mir-144 was 0.711 (95% CI, 0.572-0.850), with 56.7% sensitivity and 82.6% specificity and the AUC for leukocyte mir-34a was 0.684 (95% CI, 0.540-0.829), with 63.9% sensitivity and 68.2% specificity.

Correlation analysis

Since the expression levels of the miRNAs may vary depending on age and the MMSE score, the relationship

Table 3: The receiver operating characteristic (ROC) curve analysis of significant miRNAs

	Cutoff value	AUC	Sensitivity	Specificity	95%CI	p-value	Distinguish between
plasma mir-144	≤ 0.58	0.741	0.714	0.800	0.577-0.905	0.015	EOAD from LOAD
	< 0.38	0.617	0.625	0.667	0.371-0.862	0.366	EOAD from young control
	< 4.55	0.492	0.444	0.714	0.265-0.719	0.946	LOAD from elderly control
	< 0.53	0.694	0.667	0.625	0.438-0.951	0.178	young control from elderly control
	< 1.09	0.711	0.567	0.826	0.572-0.850	0.009	young subjects from elderly subjects
leukocyte mir-34a	< 1.36	0.711	0.440	0.867	0.533-0.888	0.027	EOAD from LOAD
	< 1.02	0.595	0.571	0.667	0.353-0.837	0.481	EOAD from young control
	≤ 1.39	0.585	0.545	0.720	0.342-0.829	0.420	LOAD from elderly control
	< 1.05	0.636	0.636	0.714	0.375-0.898	0.342	young control from elderly control
	< 1.05	0.684	0.639	0.682	0.540-0.829	0.019	young subjects from elderly subjects

AUC=Area under curve, CI=Confidence interval

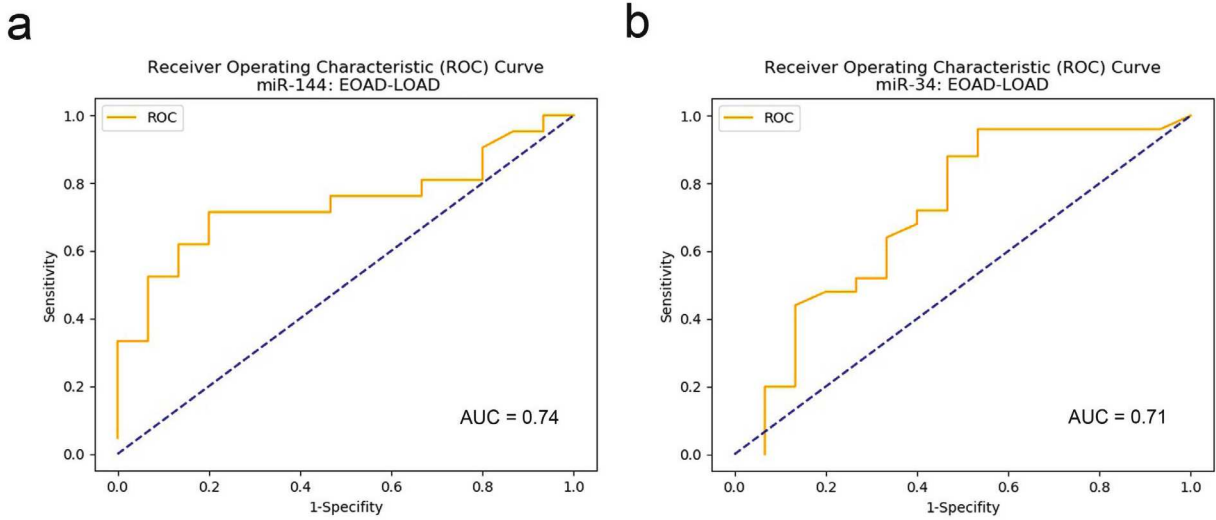


Figure 4: The receiver operating characteristic (ROC) curve plots of plasma mir-144 (a) and leukocyte mir-34a (b) for discriminative ability between EOAD and LOAD. AUC=Area under curve

between age, age of onset, MMSE and the expression levels of the miRNAs were examined by correlation analysis. The correlation analyses pointed out that plasma mir-144 levels were positively correlated with age and age of onset ($r=0.319$, $p=0.02$; $r=0.343$, $p=0.041$; respectively; Figure 5a-b). Also, leukocyte mir-34a expression lev-

els were positively correlated with age and age of onset ($r=0.414$, $p=0.001$; $r=0.399$, $p=0.011$; respectively; Figure 5c-d). There was no significant correlation between age, age of onset and levels of other miRNAs. No significant correlation was found between the MMSE score and levels of miRNAs.

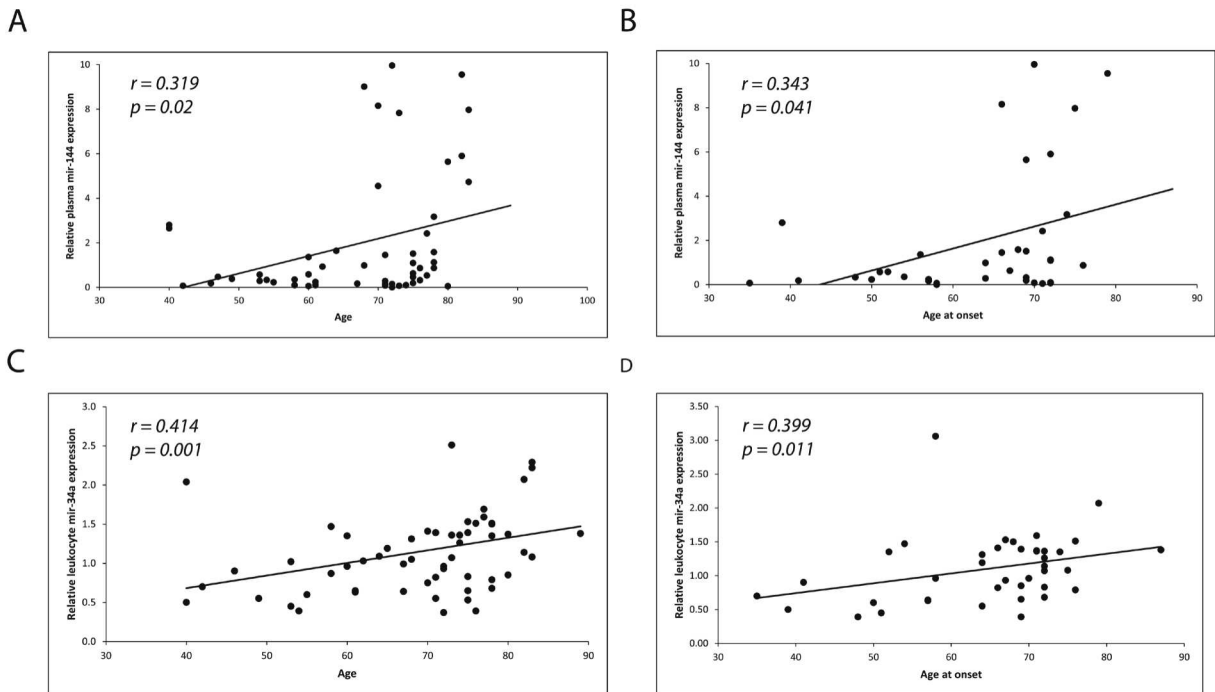


Figure 5: Correlations of plasma mir-144 and leukocyte mir-34a expression levels with age (a-c) and age at onset (b-d). age at onset, r :Spearman's correlation coefficient

DISCUSSION

The present work is a preliminary study with a small number of patients and miRNAs. We aimed to perform a comparative analysis of the selected miRNAs, the expression of which are known to change in AD patients. Therefore, we focused on three circulating miRNA (mir-146a, mir-144 and mir-34a) which were previously identified in microarray or RNA sequencing studies. The first aim of this study was to test whether the expression of specific miRNAs could be identified in both the cellular component (leukocyte) and cell-free plasma, and could serve as a peripheral biomarker in AD diagnosis. Then secondly, our next aim was to determine the expression levels of the selected miRNAs in AD patients and whether they differed in EOAD and LOAD. Distinct from previous studies where these miRNAs were investigated, our study is the first comparative study dealing with the cellular origin of the selected miRNAs. Since the cellular origin of a biomarker miRNA is important to explain the mechanism of the relation between the biomarker-miRNA and the disease, we used both cellular (leukocyte) and free (plasma) miRNA. Furthermore, the expression levels of some miRNAs are known to differ among populations (13) and to the best of our knowledge, the selected miRNAs have not been investigated within the Turkish population. Therefore, our study is the first in literature to investigate the mentioned miRNAs in Turkish AD patients.

AD-related alterations in the expression of miRNAs have been described by several studies and these alterations have been reported not only in disease-related brain regions but also in peripheral body fluids such as CSF (14-17), blood (18), plasma (19) and serum (20). Compared to brain tissue, peripheral samples, including blood, plasma, and serum are much easier to collect and determination of miRNA expression profiles in peripheral components enables these miRNAs to be used as a source of non-invasive biomarkers in AD diagnosis. So far, there has only been one study in literature to investigate the expression levels of miRNAs in Turkish AD patients (21). In their study Yılmaz et al. examined seven miRNAs (mir-9-5p, mir-29a-3p, mir-106a-5p, mir-106b-5p, mir-107, mir-125a-3p, and mir-125b-5p) whose target genes were associated with the A β pathway, in blood samples of AD patients. They found that the expression levels of mir-9-5p, 106a-5p, mir-106b-5p and mir-107 were down-regulated in AD patients and indicated that these miRNAs conferred a protection against AD.

Mir-146a expression levels were reported to increase in AD mouse models and human AD brain (22, 23). Also, it has been shown that the expression of mir-146a was higher in the hippocampus and neocortex regions of a human AD brain, and the increase in the mir-146 level was associated with the severity of AD pathology (24). Higher levels of mir-

146a were also reported in CSF (14, 15, 25), plasma (19) and serum (20) samples of AD patients; on the contrary, lower levels of mir-146a were observed both in CSF (16, 17, 26), plasma (26, 27) and serum (28). A study by Kiko et al. showed decreased levels of plasma mir-146a in elderly AD patients (mean age:80.7 yrs) (15). However, that study included a small sample size, and there was a significant difference in age between patients and controls. A recent study by Maffioletti et al. showed no significant association in plasma mir-146a levels comparing AD patients and age-matched controls (29). Strikingly, in their study, Maffioletti et al. observed significantly higher mir-146a levels when AD patients were compared with age-unmatched controls (29). Furthermore, they found a positive correlation between mir-146a levels and age, which pointed out the influence of ageing on peripheral mir-146a levels. In these two studies, the mean ages of AD patients (80.7 and 74.6 years) were similar to the mean age of our LOAD patients (76.5 years). Unlike the study by Kiko et al. but in line with Maffioletti et al., we did not find a significant difference in plasma levels of mir-146a between LOAD patients and elderly controls (26, 29). Although there have been a few studies analysing plasma mir-146 expression in AD patients, such a study in blood leukocytes has not been performed so far. Therefore, our study is the first to examine leukocytes mir-146 expression in AD patients and we have found that the expression of leukocyte mir-146a does not significantly differ between each group, but is very similar. Overall, we can suggest that the differential expression of mir-146a detected in a human AD brain may not be present in leukocytes or plasma. However, it should be taken into consideration that our study and other studies investigating the peripheral mir-146a levels in AD patients were performed in small study cohorts.

Previously, mir-144 expression was found to be down-regulated in blood samples of AD patients (18, 30). Unlike Satoh et al., we found that blood mir-144 levels did not differ in early and late-onset patients compared to their age-matched controls. However, our study was conducted on a relatively small sample size compared with that of Satoh et al., thus, further research in larger cohorts will be needed to validate the reported results (30). Plasma mir-144 levels in AD patients have not been examined so far. Our results showed no significant difference in early and late-onset patients compared to their age-matched controls but we observed significantly increased plasma mir-144 levels in LOAD patients when compared with EOAD, and this increase was also detected in leukocytes although statistically non-significant. Moreover, we found that plasma mir-144 levels were positively correlated with age. Up-regulation of miR-144 was reported in the ageing primate cerebellum and cortex and also in the cortex of human AD brain (31). The increase of miR-144 levels suggested that miR-144 may play a regulatory role in the post-transcriptional regulation of certain genes in the

ageing brain (32). In line with this finding, both in plasma and leukocyte samples we observed lower levels of mir-144 in young controls than in elderly controls but due to the low number of individuals in each group, this decrease did not reach a statistical significance. Therefore, we can suggest that the differential expression of mir-144 between EOAD and LOAD groups might be independent of the disease status and results from an age-dependent increase in mir-144 expression.

mir-34a is up-regulated in the hippocampus of an AD brain (33) and the increased expression of mir-34a in AD brains correlated with the severity of AD pathology (34). Alterations in the expression of mir-34a were described in plasma samples of AD patients, although with contradictory results (10, 26, 27). Two studies (26, 27) showed lower levels of plasma mir-34a in AD patients while in one study, higher levels were observed (10). Unlike these studies, we did not find a significant difference in plasma mir-34a levels in early and late-onset patients compared to their age-matched controls. So far, two studies have investigated mir-34a expression in blood cells of AD patients (5, 10). In both studies, analyses were performed in elderly AD patients and higher mir-34a levels were observed. However, in our study blood leukocyte levels of mir-34a were not different in neither the EOAD nor the LOAD group compared to their respective controls. Strikingly, leukocyte mir-34a expression in EOAD patients was significantly lower than the expression in LOAD patients. Furthermore, young controls seem to have lower levels of leukocyte mir-34a expression than elderly controls. Thus, we can suggest that this differential expression of mir-34a between EOAD and LOAD might be due to the age-dependent changes in expression of mir-34a (35). In addition, the significant positive correlation of mir-34a expression with age was another finding of our study, in line with this evidence. Since studies examining peripheral mir-34a expression showed contradictory results, further studies are needed to clarify the role of mir-34a in AD.

To be used as a biomarker, a miRNA level is expected to have sufficient levels of specificity and sensitivity. Though our data demonstrated that relative expression levels of plasma mir-144 and leukocyte mir-34a seemed to distinguish EOAD from LOAD with significant specificity and sensitivity, these findings were due to dependence of both miRNAs on the age, consistent with the results of the correlation analyses.

In conclusion, our results could suggest that the differential expression of miRNAs between EOAD and LOAD groups might be independent of the AD disease status and result from age-dependent changes. Also, altered expression of miRNAs in the brain may not be correlated in plasma or blood cells. The relatively small sample size is the main limitation of our study. Future studies

with larger cohorts are needed to validate results reported here, and reveal whether miRNA changes specific to EOAD and LOAD could be used as a biomarker.

Ethics Committee Approval: This study was approved by the Ethical Committee of the Istanbul University, Istanbul Faculty of Medicine (Date:12.08.2014, No:1221).

Informed Consent: Written consent was obtained from the participants.

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- G.G., Ç.G.; Data Acquisition- G.G., E.Ö., E.L., B.B., H.G., H.H.; Data Analysis/Interpretation- G.G., Ç.G., N.Ü.; Drafting Manuscript- G.G., B.B., E.L., Ç.G.; Critical Revision of Manuscript- H.H., H.G., E.Ö., N.Ü.; Final Approval and Accountability- G.G., E.L., Ç.G., B.B., E.Ö., H.H., H.G., N.Ü.

Conflict of Interest: Authors declared no conflict of interest.

Financial Disclosure: This study was supported by Istanbul University Scientific Research Projects (Project No:48601)

Etik Komite Onayı: Bu çalışma için etik komite onayı İstanbul Üniversitesi, İstanbul Tıp Fakültesi Etik Kurulu'ndan alınmıştır (Tarih:12.08.2014, Sayı:1221).

Bilgilendirilmiş Onam: Katılımcılardan bilgilendirilmiş onam alınmıştır.

Hakem Değerlendirmesi: Dış bağımsız.

Yazar Katkıları: Çalışma Konsepti/Tasarım- G.G., Ç.G.; Veri Toplama- G.G., E.Ö., E.L., B.B., H.G., H.H.; Veri Analizi/Yorumlama- G.G., Ç.G., N.Ü.; Yazı Taslağı- G.G., B.B., E.L., Ç.G.; İçerğin Eleştirel İncelemesi- H.H., H.G., E.Ö., N.Ü.; Son Onay ve Sorumluluk- G.G., E.L., Ç.G., B.B., E.Ö., H.H., H.G., N.Ü.

Çıkar Çatışması: Yazarlar çıkar çatışması beyan etmemişlerdir.

Finansal Destek: Bu çalışma İstanbul Üniversitesi Bilimsel Araştırma Projeleri kapsamında desteklenmiştir (Proje No:48601).

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