The Association between TNF-alpha, IL-1 alpha and IL-10 with Alzheimer's Disease

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Source / Izvornik: **Current Alzheimer Research, 2021, 17, 972 - 984**

Journal article, Accepted version
Rad u časopisu, Završna verzija rukopisa prihvaćena za objavljivanje (postprint)

https://doi.org/10.2174/1567205017666201130092427

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:105:201249

RESEARCH ARTICLE

1. INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, primarily affecting the hippocampal regions and cerebral cortex [1,2]. Patients with AD develop changes in memory, thinking, coping with space, as well as alternations in their behavior and functioning [3]. Observed degenerative changes in AD brain include appearance of senile plaques and neurofibrillary tangles, atrophy of the cerebral cortex, and loss of neurons leading to the reduction of the total brain mass, which are accompanied by a decrease in the psychic and physical abilities [2]. Mild Cognitive Impairment (MCI) has been characterized by a slight but visible and measurable decline in cognitive ability, including

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1567-2050/19 $58.00+.00

problems with memory, language, thinking, and judgment [4,5]. Patients with MCI, due to their increased risk of developing AD or other types of dementia [6], represent a group of significant interest for investigating the pathophysiology of MCI transition to AD. Specifically, it has been demonstrated that 10-15% of MCI patients annually develop AD and that 80% of patients with MCI are subsequently diagnosed with AD [7]. Therefore, current research aims at early diagnosis of transition phases between normal aging, MCI and dementia using potential biomarkers [8].

The chronic inflammatory processes are shown to accompany AD [9], and inflammation has been proposed to play a key role in the development of neurodegeneration in AD [10]. However, it remains unclear whether inflammation might be a protective response to AD pathology or it can contribute to the onset or progression of the disease [11,12]. Cytokines are products of specific cells of immune system [13,14] and they affect a wide range of biological functions, including immunity, hematopoiesis, inflammation and repair, as well as cell signaling [15]. In the brain, cytokines are produced by and act on both neurons and glial cells [16]. Although most cytokines are expressed at very low levels in a healthy brain, the neuroinflammation associated with the disease can be detected years before neuronal apoptosis [17]. Whereas proinflammatory cytokines represent immunoregulatory molecules, which are secreted from immune cells that promote inflammation [18,19], the anti-inflammatory cytokines control the proinflammatory cytokine response [20]. The inflammation is characterized by an interplay between pro- and anti-inflammatory cytokines [21], and an imbalance between pro-inflammatory and anti-inflammatory cytokines may be an important factor in AD.

Tumor necrosis factor α (TNF-α) plays a central role in the cytokine cascade during an inflammatory response and it is the most studied proinflammatory cytokine in the pathophysiology of AD. In spite of many contradictory findings, TNF-α seems to increase slightly but steadily over the time during the course of AD, in both blood as well as in the cerebrospinal fluid (CSF) [22]. Its concentrations are elevated in blood [23] and central nervous system (CNS) [24] of AD patients, while many clinical and animal studies indicated association between increased concentrations of TNF-α in the brain and AD [25]. It has been proposed that in patients with AD, activated microglia cells, especially those associated with amyloid deposits, generate high concentrations of TNF-α. Elevated TNF-α concentrations appear to correlate with disease progression [26]. High TNF-α concentrations are present in the serum of patients with severe AD relative to individuals with mild to moderate disease [27, 28]. In addition, there is a significant correlation between AD progression and increased TNF-α concentration in the CSF [29]. It has been shown that TNF-α increases the Aβ production through upregulation of β-secretase expression [30] and γ-secretase activity [31] and inhibits the microglial clearance of Aβ [32]. Chronic neuronal TNF-α expression causes synaptic dysfunction [33] and extensive neuronal cell death [34], leading to the progression of the AD and cognitive decline [35]. Moreover, genetic studies have found that particular polymorphisms in the *TNF* gene are associated with AD [36]. The *TNF* G308A (rs1800629) single nucleotide polymorphism (SNP) affects the TNF-α activity, with the A allele associated with the higher transcription activity than the G allele, resulting in higher TNF-α concentrations [37]. It was shown that A allele carriers are at greater risk for autoimmune diseases and inflammatory disorders [38], as well as for AD [39]. However, there are also studies indicating opposite results [40, 41].

Interleukin-1 (IL-1) family is a complex network of 11 proinflammatory cytokines that play a major role in the regulation of acute and chronic inflammatory responses [42-44]. Studies suggested the possible role of IL-1 in triggering adaptive innate immune processes during the course of chronic neurodegenerative disease, such as AD [45]. IL-1α and IL-1β were the first members of the IL-1 family to be described, and both signal via the same receptor, IL-1R [46]. IL-1α is expressed by both hematopoietic and non-hematopoietic cells, and might be rapidly upregulated by a diverse array of inflammatory stimuli [47]. IL-1α possesses many biological functions, such as physiological, and hematopoietic activities, as well as immunological and inflammatory functions [48]. It has been reported that IL-1 is overexpressed in the brain of patients with AD [49]. Observed overexpression of IL-1 in the brain has been correlated with formation of Aβ-plaques and neurofibrillary tangles, as well as with DNA damage in neurons [50]. IL-1 induces the deposition of Aβ fragments in plaques [51] as well as the release of secreted beta-amyloid precursor protein (APP) in neurons [52]. The IL-1α was also increased in the serum of subjects with AD [53]. The gene encoding IL1 alpha (IL1A) has a common polymorphism in its 5' regulatory region (rs1800587) with possible functional effects. Despite the contrary results, most studies demonstrated that *IL1A* T/T genotype has been associated with higher risk for AD [54-56].

Interleukin-10 (IL-10) is a cytokine with strong anti-inflammatory properties, suppressing the expression of inflammatory cytokines such as TNF-α, IL-6 and IL-1 [57]. It appears to be a suppressor of both immunoproliferative and inflammatory responses in the brain, by reducing the synthesis of pro-inflammatory cytokines, suppressing the cytokine receptor expression, as well as by the inhibiting their receptor activation [58,59]. It has been suggested that IL-10 may have neuroprotective effects, as it ameliorates neuroinflammation, cognitive dysfunction or neurodegeneration, however, the mechanisms involved are not completely clear [60]. IL-10 may be a potential therapy for AD since this cytokine can act to reduce amyloid, inducing the production of anti-inflammatory molecules and inhibiting pro-inflammatory cytokines [61]. Patients with AD showed a significant upregulation of IL-10 concentrations in serum and their significant inverse correlation with CFS levels of Aβ42 and the Aβ42/p-tau ratio [62]. In addition, high concentrations of IL-10 in the blood were significantly associated with amyloid deposition, suggesting that it might be used as biomarker to detect individuals at risk for AD [63]. However, some studies did not detect abnormal levels of IL-10 in either CSF or serum of AD patients [64]. Moreover, it has been hypothesized that polymorphisms of *IL10* gene might affect the risk of developing AD; however, there are conflicting results suggesting a

possible association between -1082 A > G polymorphism (rs1800896) and AD [65, 66]. The haplotype analysis also showed the association of this and other *IL10* polymorphisms, not only with AD, but possibly with other dementias [67].

As the results of numerous studies investigating the role of both pro- and anti-inflammatory cytokines in AD are contradictory, the aim of the present study was to assess possible association of *TNF* (rs1800629), *IL1A* (rs1800587) and *IL10* (rs1800896) gene polymorphisms with AD, as well as to determine serum TNF-α, IL-1α and IL-10 concentrations in AD patients and in subjects with MCI.

2. MATERIALS AND METHOD

Study design follows the recommendations given by STrengthening the REporting of Genetic Association studies (STREGA) initiative which is an extension of the Strengthening the Reporting of OBservational Studies in Epidemiology (STROBE) Statement [68].

While reporting our results and methods we also tried to follow the guiding principles (22 items) suggested by STREGA initiative [68].

2.1. Participants

This study included 645 participants: 395 subjects with AD and 250 subjects with MCI. All subjects were recruited from the University Psychiatric Hospital Vrapce, Zagreb, Croatia, in the period from 2016 till 2019. The diagnosis of AD [69] or MCI [7, 70] was based on the criteria listed in the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5) and the criteria of the National Institute of Neurological and Communication Disorders and Stroke, which is part of the American National Institute of Health (NINCDS-ADRDA; National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association). For the participation in this study, all included subjects signed informed consent form. The consent forms were explained in details to the patients with AD or subjects with MCI and their caregivers. All procedures were approved by the Ethical Committee of the University Hospital Vrapce Zagreb, and were carried out in line with the Helsinki Declaration [71]. Patients were also evaluated using Mini-Mental State Examination (MMSE). The study was approved by the Ethics Committee of University Psychiatric Hospital Vrapce, Zagreb, Croatia and carried out in accordance with the Helsinki Declaration (1964).

2.2. Genetic and biochemical analyses

During regular check-ups, venous blood samples were collected from all subjects.

For genetic analyses, 645 participants: 395 subjects with AD and 250 subjects with MCI were evaluated. Their venous blood samples were collected in vacutainer tubes containing ACD anticoagulant. Genomic DNA was isolated from peripheral blood leukocytes by a salting out method [72] and stored at -20ºC at Rudjer Boskovic Institute (RBI). Genotyping of the *IL1A* (rs1800587), *TNF* (rs1800629) and *IL10* (rs1800896) polymorphisms were determined using commercially available TaqMan® primer and assay mixtures (Applied Biosystems®, USA) purchased from Applied Biosystems as TaqMan® SNP Genotyping Assay using real-time PCR. Samples were determined on ABI Prism 7300 Real Time PCR System apparatus (Applied Biosystems, Foster city, CA, USA) at RBI. The 10 μL reaction volume contained around 20 ng of DNA. Assay IDs were C___9546481_30, C___7514879_10, and C___1747360_10. Around 10% of randomly selected samples were genotyped again as a quality control for genotyping assays.

For the determination of serum cytokines (IL-1 α, IL-10 and TNF-α) concentration, out of 645 participants, 174 subjects were evaluated: 74 patients with AD and 100 subjects with MCI. Venous blood samples were collected in VACUETTE® TUBE 6 ml Serum Clot Activator, in the morning after overnight fasting and immediately centrifuged at a speed of 4000/rpm for 10 minutes to obtain serum. All samples of the participants were stored at −80 ° C immediately after separation from the peripheral blood prior to the analysis. Serum IL-1 α, IL-10, TNF-α concentrations were determined using solid-phase sandwich enzyme-linked immunosorbent assay ELISA kits (IL-1 α, IL-10, TNF-α: AviBion, Orgenium Laboratories, Finland; IL-18: Invitrogen, Carlsbad, CA, USA; resistin: R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions [73]. The absorbance of the samples was measured at 450 nm using an Organon Teknika 530 Microplate Reader (Anthos Labtec Instruments GmbH, Salzburg, Austria). The IL-1α, IL-10, and TNF-α concentrations were determined by interpolation on the standard curve created by the absorbance of the standards. Serum IL-1α, IL-10, and TNF-α concentrations were expressed in pg / ml.

2.3. Statistical analysis

Statistical processing of the results was made using the statistical program R, version 3.6.2. and using the following software packages: rcompanion, FSA, expss, readxl, xlsx, foreign, car, corrplot, ggcorrplot, data.table, table1, effects, nlme, multcomp, RColorBrewer, knitr, kableExtra, tidyverse, dplyr, ggpubr, genetics, Hardy-Weinberg. The Shapiro-Wilk test was used to determine the presumption of normal distribution for all data groups and subsets [74]. For variables (IL-1 α, IL-10, TNF-α concentrations) that were not normally distributed, non-parametric tests were applied and the results were expressed as median and minimum-maximum ranges. Mann-Whitney test was used to compare the two groups, while Kruskal-Wallis ANOVA by ranks was used to compare three or more groups. After significant Kruskal-Wallis ANOVA, Dunn's multiple comparison test was used for post-hoc comparisons. For variables that followed normal distribution (age, MMSE scores) Student-t test was used

and results are presented as means ± SD. Multiple linear regression tested the demographic variables as predictors of cytokine concentrations. Prior to interpreting the linear regression model, assumptions were tested: linear correlation of predictors and outcomes, residual normality, homoskedasticity of variance, absence of multicollinearity, and absence of extreme values that could have a negative effect on the model. Age, MMSE scores TNF-α, IL1-α and IL-10 concentrations were correlated using Spearman's correlation coefficient. Prior to genotype frequency testing, a $\chi 2$ test was performed to identify potential deviation from the Hardy-Weinberg equilibrium (HWE). The frequencies of demographics, genotypes and alleles were compared using the $\chi 2$-test. The significance level was set at <0.05. The Benjamini-Hochberg correction (false discovery rate) was used to correct the p-value due to multiple testing. G∗Power 3 Software [75] was used to calculate the needed sample size and statistical power. With expected small effect size = 0.15, and statistical power set to 0.800, for the $\chi 2$ test (i.e. for the genotyping analyses), the required sample size was N=429. As this part of the study for the genotyping included 645 participants, it had adequate sample size and statistical power to detect significant differences among the groups, if they existed. For the biochemical analyses (Student t-test), with expected moderate effect size = 0.50, and statistical power set to 0.800, needed sample size was 128 (64 per group); for ANOVA with 3 groups; moderate effect size = 0.25, and statistical power set to 0.800, needed sample size was 159. Therefore, this part of the study with 174 subjects had adequate sample size to detect significant differences if they existed.

3. RESULTS

There was no significant difference in the frequency of male and female patients between two groups of patients (Table 1). In both groups (patients with AD and patients diagnosed with MCI), there was a higher frequency of female subjects (Table 1). As shown in Table 1, there were significant differences between patients with AD and MCI in age and the number of MMSE scores. As expected, patients with AD were significantly older and had had significantly lower MMSE scores than subjects with MCI (Table 1).

Table 1. The demographic and clinical characteristics of patients with Alzheimer's disease (AD) and subjects with mild cognitive impairment (MCI)

	AD (n=395)	MCI (n=250)	Statistical tests
Gender			
Male (%)	142 (35.9%)	95 (38.0%)	χ^2=0.28; p=0.599
Female (%)	253 (64.1%)	155 (62.0%)	
Age Mean ± SD	78.83 ± 2.21	71.95 ± 4.88	Student t-test; p<0.0001
MMSE scores Mean ± SD	13.92 ± 3.51	26.15 ± 1.40	Student t-test; p<0.0001

MMSE= Mini-Mental State Examination.

To evaluate possible sex related differences in the frequency of the *TNF* rs1800629, *IL1A* rs1800587 and *IL10* rs1800896 genotypes between subjects with AD and MCI, all subjects were subdivided according to sex. $\chi 2$ revealed that sex did not affect significantly the frequency of the *TNF* rs1800629 ($\chi 2$=1.63; p=0.443), *IL1A* rs1800587 ($\chi 2$=0.88; p=0.645) and *IL10* rs1800896 ($\chi 2$=1.13; p=0.569) genotypes in male and female patients with AD. Similarly, there were no significant differences in the distribution of the *TNF* rs1800629 ($\chi 2$=1.66.; p=0.432), *IL1A* rs1800587($\chi 2$=073; p=0.694) and *IL10* rs1800896 ($\chi 2$=3.97; p=0.137) genotypes between male and female subjects with MCI. Therefore, in the further genotype analyses male and female subjects were merged together.

Table 2. Frequencies of *TNF* rs1800629, *IL1A* rs1800587 and *IL10* rs1800896 genotypes and alleles in patients with Alzheimer's disease (AD) and subjects with mild cognitive impairment (MCI)

	Genotype n (%)			Statistics	Allele n		Statistics
***TNF* rs1800629**	**AA**	**GA**	**GG**	**df =1**	**A**	**G**	**df =1**
AD n=395	6 (1.5%)	153 (38.6%)	237 (59.9%)	χ^2=0.318; df=2; p=0.853	165 (20.8%)	627 (79.2%)	χ^2=0.001: df=2; p = 0.976
MCI n=250	5 (2.0%)	93 (37.2%)	152 (60.8%)		103 (20.6%)	397 (79.4%)	
	Genotype n (%)			**Statistics**	**Allele n**		**Statistics**
***IL1A* rs1800587**	**CC**	**TC**	**TT**	**df =2**	**T**	**C**	**df =1**
AD n=395	212 (53.5%)	158 (39.9%)	26 (6.6%)	χ^2=1.824; df=2; p=0.402	582 (73.5%)	210 (26.5%)	χ^2=1.110; df=2; p = 0.292
MCI n=250	124 (50.6%)	98 (40.0%)	23 (9.4%)		346 (70.6%)	144 (29.4%)	
	Genotype n (%)			**Statistics**	**Allele n**		**Statistics**
***IL10* rs1800896**	**AA**	**GA**	**GG**	**df =2**	**A**	**G**	**df =1**
AD n=395	135 (34.2%)	188 (47.6%)	72 (18.2%)	χ^2=3.414; df=2; p=0.181	458 (56.5%)	352 (43.5%)	χ^2=0.314; df=2; p = 0.575
MCI n=250	84 (33.6%)	106 (42.4%)	60 (24.0%)		274 (54.8%)	226 (45.2%)	

TNF=tumor necrosis factor α; *IL1A*=interleukin-1α; *IL10*=interleukin 10.

Table 2 shows the lack of significant difference in the genotype/allele distribution of the *TNF* rs1800629, *IL1A* rs1800587 and *IL10* rs1800896 between subjects with AD and MCI. No significant differences were found in the distribution of the *TNF* rs1800629 AA, AG and GG genotypes, or carriers of the A and G allele between subjects with AD and MCI (Table 2). There were no significant differences in the frequencies of the IL1-α rs1800587 TC, CC, and TT genotypes, or T and C allele between patients with AD and subjects with MCI. Frequency of the *IL10* rs1800896 AA, GG and GA genotypes, or G and A allele did not differ significantly between subjects with AD and MCI (Table 2). Our results revealed that *TNF* rs1800629, *IL1A* rs1800587 and *IL10* rs1800896 polymorphisms were not associated with AD.

Multiple linear regression analyses revealed that age and sex were not good predictors of the TNF-α (corrected R2=-0.027; F=0.043; p=0.95), IL-1α (corrected R2=-0.013; F=0.528; p=0.59) and IL-10 (corrected R2=-0.032; F=0.651; p=0.52) concentrations in subjects with AD. In addition, age and sex were not good predictors of the TNF-α (corrected R2=-0.023;

F=1.158; p=0.32), IL-1α (corrected R2=-0.004; F=0.79; p=0.46) and IL-10 (corrected R2=-0.01; F=0.52; p=0.60) concentrations in subjects with MCI, shown by multiple linear regression analyses. Therefore, although patients with AD were significantly older than subjects with MCI, this difference in age did not affect significantly cytokine concentrations. Consequently, in the further analyses, patients with AD and subjects with MCI were not subdivided according to the sex, when determining serum IL-1 α, IL-10, TNF-α concentrations.

As shown in Table 3, there were significant differences between patients with AD and MCI in serum concentrations of the TNF-α, IL1-α and IL-10. Patients with AD had significantly decreased serum concentration of IL-1α and IL-10, and significantly increased TNF-α concentration compared to subjects with MCI (Table 3).

Table 3. IL-1α, TNF-α and IL-10 concentrations (pg/ml) in patients with Alzheimer's disease (AD) and subjects with mild cognitive impairment (MCI)

	AD (n=74)	MCI (n=100)	Statistical tests
TNF-α concentration Median (min, max)	18.6 (3.13; 41.4)	6.49 (1.96; 48.59)	U=966.5; p<0.0001
IL1-α concentration Median (min, max)	0.36 (0.08; 2.15)	1.30 (0.17; 3.90)	U=464; p<0.0001
IL-10 concentration Median (min, max)	3.90 (0.17; 21.92)	3.96 (0.52; 21.61)	U=1873; p<0.0001

TNF-α=tumor necrosis factor α; IL1-α=interleukin-1α; IL-10=interleukin 10.

To further evaluate the association between TNF-α, IL-1α and IL-10 concentrations and their respective polymorphisms, we subdivided separately patients with AD and subjects with MCI according to their *TNF* rs1800629, *IL1A* rs1800587 and *IL10* rs1800896 genotypes and analyzed the possible differences of the IL-1 α, IL-10, TNF-α concentration between these groups. There were significant (p<0.001; Kruskal Wallis ANOVA) differences in the concentration TNF-α, IL-1 α and IL-10 between patients with AD and subjects with MCI, when they were subdivided into carriers of the specific *TNF* rs1800629, *IL1A* rs1800587 and *IL10* rs1800896 genotypes. However, Dunn's test revealed that TNF-α, IL-1α and IL-10 concentrations did not differ in patients with AD and subjects with MCI subdivided according to their *TNF* rs1800629, *IL1A* rs1800587 and *IL10* rs1800896 genotypes. Therefore, TNF-α, IL-1α and IL-10 concentrations in serum were not significantly associated with *TNF* rs1800629, *IL1A* rs1800587 and *IL10* rs1800896 genotypes. The significant differences (Kruskal-Wallis ANOVA and Dunn's multiple test) were detected only in the cytokine concentrations between patients with AD compared to subjects with MCI, confirming our previous cytokine concentration data. Namely, TNF-α concentration differed significantly (H=72.09; p<0.001) between patients with AD and MCI subdivided into A vs GG carriers of the *TNF* rs1800629, since GG carriers with AD had significantly (p<0.001) higher TNF-α concentration that GG carriers with MCI, and A carriers with AD had significantly (p<0.01) higher TNF-α concentration that A carriers with MCI. Carriers of the *IL1A* rs1800587 TC genotype with MCI had significantly higher (p<0.0001) IL1-α concentration than TC carriers in patients with AD; and CC carriers with MCI had significantly higher (p<0.0001) IL1-α concentration than CC carriers with AD (H=97.5; p<0.001). Significant differences in the IL-10 concentration (H=24.5; p<0.001) were found since AA (p<0.0001), GG (p<0.001) and GA (p<0.01) carriers with MCI had significantly higher IL1-10 concentration than the corresponding AA, GG and GAG carriers with AD, respectively. These results revealed that serum TNF-α, IL-1α and IL-10 concentrations were not significantly associated with their *TNF* rs1800629, *IL1A* rs1800587 and *IL10* rs1800896 genotypes, either in patients with AD or in subjects with MCI.

Table 4 shows that there are no significant correlations between age and Mini-Mental State Examination (MMSE) with TNF-α, IL-1α and IL-10 concentrations in patients with AD and subjects with MCI. Therefore, age or MMSE scores did not affect significantly TNF-α, IL-1α and IL-10 concentrations in patients with AD and subjects with MCI.

Table 4. Correlation of age and Mini-Mental State Examination (MMSE) with TNF-α, IL-1α and IL-10 concentrations (pg/ml) in patients with Alzheimer's disease (AD) and subjects with mild cognitive impairment (MCI)

	AD (n=74)		MCI (n=100)	
	Age	MMSE	Age	MMSE
TNF-α concentration	r=-0.028; p=0.086	r=-0.170; p=0.158	r=-0.020; p=0.554	r=0.030; p=0.639
IL1-α concentration	r=0.010; p=0.798	r=0.100; p=0.978	r=-0.210; p=0.158	r=-0.350; p=0.063
IL-10 concentration	r=0.270; p=0.418	r=0.250; p=0.443	r=-0.170; p=0.172	r=-0.200; p=0.133

TNF-α=tumor necrosis factor α; IL1-α=interleukin-1α; IL-10=interleukin 10; MMSE= Mini-Mental State Examination; r= Spearman's coefficient of correlation.

4. DISCUSSION

The results of the present study showed that 1) patients with AD and subjects with MCI had similar distribution of the *IL1A* rs1800587, *TNF* rs1800629 or *IL10* rs1800896 genotypes or alleles, suggesting that specific *TNF* rs1800629, *IL1A* rs1800587 and *IL10* rs1800896 risk genotypes are not associated with vulnerability to develop AD; 2) patients with AD and subjects with MCI had significantly different TNF-α, IL-1α and IL-10 concentrations, controlled for the effect of age, sex and ethnicity;

patients with AD had significantly lower IL-1α and IL-10 concentrations than subjects with MCI; and significantly higher TNF-α concentration than subjects with MCI; 3) IL-1α, TNF-α and IL-10 concentrations were similar in *IL1A* rs1800587, *TNF* rs1800629 or *IL10* rs1800896 genotype carriers in patients with AD and subjects with MCI, revealing that serum IL-1α, TNF-α and IL-10 concentrations were not significantly associated with *IL1A* rs1800587, TNF rs1800629 or *IL10* rs1800896 polymorphisms.

It is a widely accepted fact that neuroinflammatory processes are a key feature of AD, which is supported by increased production of pro-inflammatory cytokines. Alterations of specific cytokine levels in CNS and periphery reflect the immune system disturbances in AD, however, relying on the evidence we have so far, we cannot yet say with certainty whether inflammation is the cause or an outcome of dementia. Various case-control studies investigated the association between the risks of developing AD, specific inflammatory cytokines and related genetic polymorphisms, including ones associated with IL-1α, TNF-α and IL-10 [76]. However, the results are not straightforward.

4.1. Interleukin-1α

This study reported a lack of significant association between AD and *IL1A* rs1800587. IL-1 was found to be overexpressed in the CNS of subjects with AD, and associated with β-amyloid plaque and neurofibrillary tangle formation in human brain [49, 77]. Numerous studies investigated the association between *IL1A* rs1800587 polymorphism, situated in 5' regulatory region, and the risk of developing AD. *IL1A* rs1800587 polymorphism was found to have an effect on *IL1A* gene transcription and protein synthesis [78, 79], with T allele being associated with increased IL-1α gene transcription which leads to overexpression of pro-inflammatory cytokines and neuroinflammation. Some of the studies indicated a significant association of *IL1A* rs1800587 polymorphism with AD development [55, 80-83]. Reported studies suggested that *IL1A* rs1800587 T allele could be a risk factor for developing AD [80, 81], which was also confirmed by meta-analyses [55, 82], showing that the risk for developing AD was the highest in subjects with TT genotype. Hayes and colleagues [84] demonstrated greater activation of microglia in AD patients that were T allele carriers, especially in those who were also carriers of apolipoprotein E (*APOE*) ε4 allele. This evidence suggests that the *IL1A* could be a candidate gene for AD susceptibility in Caucasian subjects, but this association was not confirmed by other research [83-86], or by our results, obtained on a large number of subjects with AD and MCI. In Chinese subjects, carriers of the CT genotype, or the T allele carriers, had significantly higher risk of AD than carriers of the CC genotype [83]. These inconsistencies could be a result of an inadequate statistical power for some of the studies, or the consequence of the race/ethnicity influence and inadequate corrections for multiple hypothesis testing.

Even though we did not find a significant association between *IL1A* rs1800587 polymorphism and vulnerability to AD, we detected significantly lower circulating levels of IL-1α in patients diagnosed with AD, compared to subjects with MCI. IL-1α concentration was not affected by the MMSE scores or by sex or age of the subjects. Cytokines IL-1α and IL-1β are difficult to detect in human circulation because they are most often closely related to the specific tissue in which inflammation occurs [89]. In contrast to our findings, a small, but significant, increase in IL-1α and IL-1β concentration was detected in serum samples from AD patients [52]. In addition, increased concentration of the soluble form of the ligand-binding signaling receptor (sIL-1R1) was observed in patients diagnosed with AD, compared to normal healthy subjects, subject diagnosed with MCI and patients with subjective memory complaints [52]. These findings suggest that AD is characterized by an excessive IL-1 activity, which is opposite to our findings. Most of the studies focused more on the role of IL-1β and not of the IL-1α, in AD. A recent meta-analysis [90] suggested significantly higher peripheral concentration of IL-1β in elderly subjects with AD, but this significance was lost after Bonferroni adjustment. This finding is not surprising because it is consistent with the proposed role of this cytokine in the deposition of β-amyloid plaques [91] and in tau phosphorylation [92]. Similar results were observed for serum IL-1α concentration in AD patients. Study including a sample from Baghdad and surrounding governorates, detected significantly higher serum concentration of IL-1α in AD patients, compared to healthy control subjects [93]. Results from a study following the effects of long-term inflammation in Korean community on AD development [94], suggested that dementia could be a cause of inflammation, rather than a consequence of elevated levels of pro-inflammatory cytokines, such as TNF-α, IL1-α, and IL-1β. Contrary to the inflammatory hypothesis, we found lower serum concentration of IL-1α in AD patients than in subjects with MCI. However, most of the above-mentioned studies compared AD subjects to healthy controls, while in our study we focused on a difference between AD and MCI, as a potential transition state from normal aging to dementia. In our study we eliminated the effect of age and sex on IL-α concentration between subjects with MCI and AD patients, but this still leaves the possibility of confounding bias, due to other covariates that were not taken into account. In the future, more studies will be needed to demonstrate the true relationships between IL-1α and dementia.

We failed to detect a significant association between IL1-α concentration and *IL1A* rs1800587 polymorphism. Namely, IL1-α concentration did not differ in carriers of the TT, TC and CC genotypes either in patients with AD or in subjects with MCI. Only significant differences were found, as conformation, between subjects with AD and MCI. To the best of our knowledge, there are no data in the literature comparing IL1-α concentration in patients with AD and MCI subdivided into carriers of the *IL1A* rs1800587 genotypes. In vitro findings (in lipopolysaccharide-stimulated mononuclear cells and in human astrocyte cell line) revealed that *IL1A* rs1800587 T allele was associated with higher *IL1A* gene transcription and increased production of the protein [78, 79], while Kawaguchi et al. [95] reported no significant effects of *IL1A* rs1800587 on the transcriptional activity and processing of the precursor IL-1α in skin fibroblasts. In disagreement with some of the in vitro data, in our study IL1-α

concentration was not affected by the MMSE scores, sex and age of the subjects and was not associated with *IL1A* rs1800587 either in patients with AD or in subjects with MCI.

4.2. Tumor necrosis factor alpha

TNF-α is reported to be associated with increased β-amyloid and tau pathology in animal models of AD [34], however, there is also evidence suggesting neuroprotective effect of TNF-α [96]. A small pilot study [97] demonstrated that the use of TNF-α inhibitor may improve the cognition in AD patients. This all leads to the conclusion that TNF-α has a multifunctional role in AD pathology.

We failed to detect a significant difference in the frequency of the *TNF* rs1800629 genotypes between patients with AD and subjects with MCI. Several polymorphisms in the gene coding for TNF-α were investigated regarding their possible relationship with AD development. Few studies investigated *TNF* rs1800629, including our study. No association between *TNF* rs1800629 and AD is in line with the most studies reporting a lack of association of *TNF* rs1800629 with AD risk [40, 41, 98-100]. Negative results were also confirmed by our results and meta-analysis performed by Di Bona and colleagues [36]. Our recent study [84] investigated the association of specific CSF AD biomarkers with *TNF* rs1800629 polymorphism and the results showed pathological levels of examined biomarkers: higher p-tau and VILIP-1 CSF levels in patients with the GG *TNF* rs1800629 genotype compared to GG homozygotes diagnosed with AD. In line with our data, this previous study, that included only part of the subjects evaluated here (N=168), also failed to find significant association between *TNF* rs1800629 and AD [85]. However, in Chinese subjects, the A allele frequency was higher in AD than in control subjects [101]. *TNF* rs1800629 A allele was shown to have higher transcriptional activity when compared to G allele [102], but, so far, most of the studies [100] support our results and demonstrate no significant influence of *TNF* rs1800629 polymorphism on AD susceptibility. Conflicting results were also reported [103], showing evidence of a significant association between earlier onset of AD symptoms and *TNF* rs1800629 polymorphism in Italian population. It has also been suggested that an interaction of *TNF* rs1800629 polymorphism with other polymorphisms could potentiate the risk for developing AD. For example, a significant association between *TNF* rs1800629/*IL10* rs1800896 AA haplotype and AD risk was reported [104]. The same study revealed that *TNF* rs1800629/*IL6* rs1800795 AC haplotype increases the risk for developing AD [104]. Meta-analysis [105] indicated a significant association of *TNF* rs1800629 A allele with the development of AD, but only in East Asian subjects. All of the above points to inconsistency in the results, which is most likely a consequence of the heterogeneity between the studied populations, ethnic differences, small sample sizes and low statistical power.

Our results suggested a significant association between peripheral concentration of TNF-α and AD. TNF-α concentration was not affected by sex, age or MMSE scores. We detected significantly higher TNF-α concentration in patients diagnosed with AD, when compared to subjects with MCI. Our findings are in line with data from the Chinese subjects, where higher TNF-a concentration was detected in serum of AD group compared to values in control group [101]. Although results are still inconclusive, we could say that most of the evidence so far favors elevated pro-inflammatory cytokines, including TNF-α, in the CSF and peripheral circulation of individuals diagnosed with AD [22]. TNF-α is one of the most frequently investigated cytokines regarding its association with AD development. The evidence from the literature is contradictory. Some studies suggested upregulation of peripheral TNF-α levels in AD patients [23, 28, 106-109], but there is also evidence for downregulation of TNF-α in circulation [110-112]. Other studies found no association between peripheral TNF-α concentration and AD diagnosis [113, 114]. Most of the above-mentioned studies focused on differences between subjects with AD and healthy control subjects. When comparing AD patients to subject with MCI, some studies indicated no differences in peripheral TNF-α concentration between these two groups of subjects [115], however, some of them support our results [116]. Results presented by King and colleagues [116] suggest that higher concentrations of plasma TNF-α are associated with more severe cognitive impairment, since they demonstrated decreased TNF-α concentration in patients with MCI, compared to subjects diagnosed with AD. Other cytokines they investigated, IL-10, IL-1β, IL-4 and IL-2, were increased in MCI subjects compared to individuals with AD, suggesting that increased peripheral inflammation is characteristic for the early MCI stage, while it decreases with disease severity [116]. This supports our results regarding the peripheral concentration of pro-inflammatory cytokine TNF-α, and points out to the importance of early anti-inflammatory treatment in dementia.

In the present study TNF-α concentration was not different either in patients with AD or in subjects with MCI when they were subdivided into carriers of the AA, GA or GG genotypes of the *TNF* rs1800629. Only significant differences were those related to AD and MCI diagnoses. As far as we know, other studies did not evaluate TNF-α concentrations in subjects with AD or MCI and its association with *TNF* rs1800629. This lack of relationship is partly in line with the results obtained in Caucasian population with myocardial infarction, who also show increased TNF-α concentrations compared to controls, where *TNF* rs1800629 was not associated with differences in TNF-α concentration [117]. In vitro data also support these findings, since both the highest and the lowest TNF production in the whole blood cultures upon stimulation with LPS from patients with multiple sclerosis was not influenced by the *TNF* rs1800629 polymorphism [118]. In Iranian patients with other diagnosis (i.e. chronic hepatitis B virus infection), their decreased amount of TNF-α was related to G allele of the *TNF* rs1800629 [119]. As nicely summarized [100], *TNF* gene and *TNF* polymorphisms influence susceptibility or resilience to different human diseases.

4.3. Interleukin-10

Unlike IL-α and TNF-α, IL-10 is an anti-inflammatory cytokine with a very important neuroprotective role in CNS [120]. There are conflicting results regarding the effect of IL-10 in animal models of AD. Some studies suggest a positive effect on neurogenesis and cognition [121], while others implicate the role of this interleukin in AD pathology [122].

One of the most investigated *IL10* gene polymorphisms is rs1800896. This polymorphism was associated with altered IL-10 gene transcription and IL-10 plasma concentration [122, 123]. In our study patients with AD and subjects with MCI had similar distribution of the *IL10* rs1800896 genotypes or alleles, suggesting that *IL10* rs1800896 is not associated with vulnerability to develop AD. There are conflicting results in the literature, suggesting either a significant association with the risk of developing AD [66, 99, 104, 125, 126] or, similarly to results of our study, no association with AD [123, 127, 128]. Some studies suggest a possible role of sex-specific effects in the relationship between AD risk and *IL10* rs1800896 polymorphism [129], but this was controlled in our study and sex did not affects significantly frequency of the *IL10* rs1800896 genotypes. Meta-analyses by two different groups of authors [66, 126] support the idea of a significant association between *IL10* rs1800896 allele G and reduced risk of developing AD in subjects with European origin. This finding might be in line with the results of our recent study [85] reporting increased p-tau CSF levels in patients with AD, carriers of the AA genotype of the *IL10* rs1800896. However, these results are opposite to the results obtained on a large sample of subject from Brazil, which showed that *IL10* rs1800896 AA homozygotes have almost 40% lower chance for developing AD [130]. Our previous smaller [85] and a present study, including a fairly large sample, failed to replicate these results, that is, we did not confirm a significant association between *IL10* rs1800896 and diagnosis of AD. Differences between studies might be assigned to ethnic differences which play a large role in genetic studies.

In this study IL-10 concentration, controlled for the effect of age, sex, MMSE scores and ethnicity was significantly lower in patients with AD than in subjects with MCI. Defective IL-10 signaling has been implicated in neurodegeneration because of its important role in regulating the immune crosstalk. IL-10 is expected to reduce inflammation in AD, however, results from other studies [93, 131], suggest higher concentration of IL-10 in demented patients. Same trend was reported for IL-10 concentration in the brain tissue of AD subjects and subjects with different neurological diseases [120, 132]. However, study by Kim and colleagues [111] detected no difference in IL-10 plasma concentration of AD patients and controls. It is possible that the observed elevated peripheral concentration of IL-10 represents compensation for chronic inflammation accompanying AD. This is consistent with the role of IL-10 in suppressing the secretion of proinflammatory cytokines from microglia by triggering M2c polarization associated with microglia deactivation. Results of our study are also supported by the evidence gathered in studies with animal models [133]. These studies demonstrate elevated plasma IL-10 concentration in mice immunized with full length β-amyloid [135] and also in mice expressing mutant amyloid precursor protein and human presenilin 1 that were immunized with an adenovirus vector encoding 11 tandem repeats of Abeta1-6 fragment [135]. Upregulation of IL-10 in animal model of AD was also associated with impaired microglial phagocytosis of amyloid-β which resulted in cognitive impairment [122, 132]. This leads us to conclude that future studies of dementia treatment should focus and pay more attention on exploring novel cytokine inhibitors that would be able to restore the balance of pro- and anti- inflammatory activity in AD.

We failed to detect any significant difference in IL-10 concentration between subjects with AD or MCI subdivided into *IL10* rs1800896 genotypes. Their serum IL-10 concentration differed only between diagnoses. No data are available for the possible association between IL-10 concentration and *IL10* rs1800896 in AD or MCI. In contrast to the lack of effect of IL-10 rs1800896 on serum in IL-10 concentration in subjects with ad AD and MCI, in vitro studies revealed that G allele of the *IL10* rs1800896 was associated with the over expression of IL-10 in vitro [136], while IL-10 production was decreased in AA+AG vs GG in stimulated peripheral blood mononuclear cells isolated from healthy controls [137].

Although the sample size was adequate (N=645), the possible limitation of the study might be in small subsample for cytokine concentrations. However, this part of study had adequate sample size and enough statistical power to detect significant differences i.e. when calculated sample size in advance using G* power. Genotyping part of the study also had adequate sample size and statistical power. Therefore, a lack of significant association between selected *IL1A* rs1800587, *TNF* rs1800629 or *IL10* rs1800896 polymorphism and AD could not be explained by the small sample size or the lack of statistical power. Mixed results from the literature and non-replication of the genotyping data are the rule, rather than exception, due to the small effect size of each individual SNP, different effect of the same SNP on the risk for development of AD, population and ethnic differences, but also diet, exercise, infection, other comorbid diagnoses [76]. Strengths of the present study are in three SNPs evaluation (*IL1A* rs1800587, *TNF* rs1800629 or *IL10* rs1800896), evaluation of the three cytokine concentration (IL1-α, TNF-α and IL-10), corrected for age, sex and MMSE scores, inclusion of ethnically homogenous Caucasian patients with AD or MCI, from the same center. Therefore, further studies that wish to confirm or discard the association between *IL1A*, *TNF* and *IL10* polymorphism and the risk of AD should take into account the influence of race/ethnicity, have an adequate control group and be aware of the need to adjust the results for multiple testing.

CONCLUSION

Our study aimed to associate the risk of AD with blood serum concentrations and particular gene polymorphisms that are related to the transcriptional activity of three cytokines, IL-1α, TNF-α and IL-10. Results showed that specific *TNF* rs1800629, *IL1A* rs1800587 and *IL10* rs1800896 risk genotypes are not associated with vulnerability to develop AD. However, the study confirmed the important role of the immune system in AD, demonstrating decreased levels of IL-1α and IL-10 and elevated levels of TNF-α in the blood serum of AD patients, compared to subject with MCI. Our results partly supported evidence of

dysregulation in the pro- and anti-inflammatory response in AD [138], either as the cause or the consequence of the neurodegenerative process. Though, the mechanism behind the effect of these three cytokines in promoting These inconsistenciesneuroinflammation and neurodegeneration is still not completely resolved. These results support future work and additional studies, needed to clarify the role of Il-1α, TNF-α and IL-10 during AD development and their potential as peripheral AD biomarkers.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Ethics Committee of University Psychiatric Hospital Vrapce, Zagreb, Croatia and carried out in accordance with the Helsinki Declaration (1964).

HUMAN AND ANIMAL RIGHTS

No Animals were used for this study.

CONFLICT OF INTEREST

The authors declare no conflict of interest, with none of the authors having financial or other relationships that could lead to it. This study was partly supported by the Croatian Science Foundation, project no. IP-2019-04-6100.

ACKNOWLEDGEMENTS

Nela Pivac developed the original idea. Marija Culjak determined cytokine concentrations in serum; Matea Nikolac Perkovic, Dubravka Svob Strac, Gordana Nedic Erjavec, Mirjana Babic Leko, Lucija Tudor and Marcela Konjevod managed the experimental work, collected blood samples, isolated DNA and did the genotyping. Matea Nikolac Perkovic did the statistical evaluation of the data; Marija Culjak, Matea Nikolac Perkovic, Dubravka Svob Strac, Gordana Nedic Erjavec and Nela Pivac did the data analysis and interpretation, performed literature search and wrote the first draft of the article. Suzana Uzun, Goran Simic, Oliver Kozumplik and Ninoslav Mimica explained the research goals and described protocol in details to the patients; explained the inclusion/exclusion criteria, insured participant adherence for the participation in the study, motivated, selected, diagnosed, evaluated and sampled patients with AD and MCI. Nela Pivac wrote the final draft of the article. All authors have read and approved the final version and have contributed substantially to the design, performance, analysis, and reporting of this study.

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