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Evaluating the Clinical Significance of Diazepam Binding Inhibitor in Alzheimer's Disease: A Comparison with Inflammatory, Oxidative, and Neurodegenerative Biomarkers

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Keywords
Diazepam binding inhibitor · Alzheimer's disease · Ageing · Biomarker · Inflammation · Oxidative stress

Abstract
Introduction: Alzheimer's disease (AD) is one of the pathologies that the scientific world is still desperate for. The aim of this study was the investigation of diazepam binding inhibitor (DBI) as a prognostic factor for AD prognosis. ***Methods:*** A total of 120 participants were divided into 3 groups. Forty new diagnosed Alzheimer patients (NDG) who have been diagnosed but have not started AD treatment, 40 patients who diagnosed 5 years ago (D5YG), and 40 healthy control groups (CG) were included in the study. Levels of DBI, oxidative stress, inflammatory, and neurodegenerative biomarkers were compared between 3 groups. ***Results:*** Plasma levels of DBI, oligomeric Aβ, total tau, glial fibrillary acidic protein, α-synuclein, interleukin (IL) 1β, IL6, tumor necrosis factor α, oxidative stress index, high-sensitive C-reactive protein, and DNA damage were found higher in D5YG and NDG as compared to CG ($p < 0.001$). On the contrary, plasma levels of total thiol, native thiol, vitamin D and vitamin B12 were lower in D5YG and NDG as compared to CG ($p < 0.001$). ***Discussion:*** DBI may be a potential plasma biomarker and promising drug target for AD. It could help physicians make a comprehensive evaluation with cognitive and neurodegenerative tests.

Introduction

Alzheimer's disease (AD) is a prevalent neurodegenerative disorder that is rapidly spreading worldwide, particularly in low- and middle-income countries. It is estimated that approximately 65 million people were affected by the disease in 2021 [1]. AD is characterized by pathological features, including neuron loss, synaptic dysfunction, senile

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plaques composed of oligomeric amyloid-beta (Aβ), and neurofibrillary tangles (NFTs) consisting of hyperphosphorylated tau and aggregates of Aβ plaques [2]. While medication is available to treat some of the symptoms of AD type dementia, no medication is currently available to prevent or slow the progression of the disease [1].

Oxidative stress is believed to occur in AD and play a role in molecular interactions. OAβ and tau may cause an increase in reactive oxygen species (ROS) and lead to mitochondrial dysfunction [2]. Additionally, elevated levels of oxidatively damaged proteins, nucleic acids, and lipids are thought to be crucial in the development of the disease [2]. DNA damage is also a significant contributor to these factors. Both tau and Aβ contribute to genomic instability, which is a characteristic of the AD pathology of NFT and senile plaques [1, 2].

Neuroinflammation is also believed to contribute to the pathology of AD. Recent studies have demonstrated that Aβ toxicity and NFTs can trigger an immune response, consisting of the activation of microglia and astrocytes, the brain immune cells [3]. It is also thought that the activation of these cells plays a critical role in the development of NFTs, leading to neuronal loss and dysfunction [4]. Toxic substances such as Aβ can activate microglial cells and astrocytes, leading to the release of various inflammatory mediators such as high-sensitive C-reactive protein (hsCRP), as well as elevated levels of proinflammatory cytokines including interleukin 6 (IL6), tumor necrosis factor α (TNF-α), and IL1β, which are observed in all stages of AD [5]. One of these mediators is diazepam binding inhibitor (DBI), known for its ability to displace benzodiazepine (BDZ) from the binding sites of the $GABA_A$ receptor [6]. DBI levels have been found to be increased in epilepsy patients [7]. It binds to the BDZ peripheral receptor, the activated microglia, and neuroinflammation imaging marker (TSPO) [8], and initiates neurosteroid biosynthesis and promotes monocyte chemotaxis [9]. In addition to its peripheral role, DBI acts centrally as a $GABA_A$ receptor allosteric modulator on the BDZ binding site, differentially modulating $GABA_A$Rs in various brain regions, including the cerebellum, amygdala, hippocampus, hypothalamus, and substantia nigra [10]. Therefore, changes in DBI levels may be linked to altered learning and memory [11, 12]. In addition, via other intracellular actions, including effects on lipid metabolism, DBI may modulate neuronal and/or glial function, and the gene responsible for its expression has omnipresent housekeeping functions [13, 14]. Hence, DBI is capable of performing various biological activities that may affect neuronal and synaptic physiology, thus modulating complex behaviors [6]. Investigating DBI plasma levels in AD patients and evaluating its role with neuroinflammation and oxidative stress parameters could aid in searching for a new biomarker.

Materials and Methods

This cross-sectional study was conducted at a university hospital in Istanbul between January 2020 and February 2021. The study was approved by the Local Ethics Committee of Clinical Research at Istanbul Medipol University Hospital with decision number 10/13.

Study Design and Participants

A total of 120 participants were included and divided into three groups: new diagnosed Alzheimer patients who have not started AD treatment (NDG), patients who were diagnosed 5 years ago (D5YG), and a healthy control group (CG), with each group consisting of 40 participants. All participants were between the ages of 66 and 91 and had similar demographics, as shown in Table 1. The inclusion criteria for the study were a diagnosis of AD and being over the age of 55, as well as having similar sociodemographic factors. The diagnosis of dementia was confirmed using DSM V and ADRA criteria. Participants with comorbid conditions such as diabetes mellitus, chronic kidney failure, collagen tissue disease, or any endocrinological and rheumatological disease, as well as other neurological diseases (e.g., mixed dementia, epilepsy) and a history of stroke were excluded from the study. All participants provided informed consent. During the hospital visit, a neurologist conducted the examination and calculated the mini-mental score and clinical dementia scores of all volunteers.

Blood Sample Collection

In all volunteers, blood samples were taken in EDTA-treated vials, and plasma samples were separated with centrifugation at 1,107 rcf for 12 min. The separated plasmas were kept at –80°C for further analysis. Among routine tests, plasma B12, vitamin D, HbA1c were measured in biochemistry-hormone auto-analyzers (Abbott Architect c16000).

Measurement of hsCRP, IL1β, IL6, TNF-α, GFAB, Total Tau, αSYN, DBI, and oAβ

Concentrations of hsCRP, IL1β, IL6, TNF-α, GFAB, total tau, αSYN, DBI, and oAβ the plasma were measured by specific commercial ELISA kit according to the manufacturer's instructions (IL1β: E-EL-H5134; IL6: E-EL-H0149; TNF-α: E-EL-H0109; GFAB: E-EL-H6093; αSYN: E-EL-H0983 – Elabscience, USA; total tau: KHB0041 – Thermo Fisher Scientific, USA; DBI: MBS063410 – MyBioSource, USA; oAβ: DEIA6190 – Creative Diagnostics, USA). Concentrations were determined with a spectrophotometric microtiter plate reader (Varioskan Flash Multimode Reader, Thermo, Waltham, USA) at 450 nm optical density.

Analysis of Oxidative Stress

Plasma total antioxidant status (TAS) was determined with a fully automatic method developed by Erel [15]. Total oxidant status (TOS) was determined with a fully automatic method

Table 1. Clinical and demographic characteristics of the groups

Parameter	Control	NDG	D5YG	*p* value[a]*
Age, years	75.07±6.04	76.77±6.17	77.37±5.61	*ns*
Gender (m/f)	19/21	19/21	19/21	*ns*
Blood pressure, systolic/diastolic, mm Hg	109.5/70.25	105.87/68.12	107.87/70.12	*ns*
BMI, kg m^{-1}	28.22±1.73	27.00±1.64	27.35±1.96	0.010
HbA1c, %	5.44±0.48	6.22±0.50	6.22±0.46	<0.001
MMSE score	28.02±1.42	24.55±2.09	22.2±1.78	<0.001
Clinical dementia rating score	0±0	1.07±0.30	2±0.10	<0.001
Oligomeric Aβ, pmol/L	37.52±16.30	495.63±53.20	750.52±86.60	<0.001
Total tau, pg/mL	57.56±15.85	463.25±158.09	625.10±175.65	<0.001
α-Synuclein, pg/mL	165.44±77.44	395.86±83.79	782.04±97.86	<0.001
GFAP, pg/mL	115.30±22.64	352.04±87.90	522.05±99.01	<0.001
DBI, ng/mL	2.02±1.54	8.10±2.30	14.48±2.27	<0.001
Vitamin D, ng/mL	46.92±12.50	37.17±11.29	25.91±5.53	<0.001
Vitamin $B_{12,}$ pg/mL	601.07±146.56	365.62±96.59	271.30±98.09	<0.001
Oxidative parameters				
TOS, μmol H_2O_2 Eq./L	10.66±0.94	14.42±1.89	17.35±1.70	<0.001
TAS, mmol Trolox Eq./L	1.17±0.06	0.79±0.09	0.52±0.07	<0.001
OSI (arbitrary unit)	9.99±0.99	18.43±3.70	33.65±6.33	<0.001
TT, mmol/L	0.57±0.04	51±0.02	0.47±0.02	<0.001
NT (SH), mmol/L	0.40±0.02	0.29±0.02	0.19±0.02	<0.001
DIS (SS), mmol/L	0.08±0.02	0.11±0.01	0.13±0.01	<0.001
Inflammatory parameters				
hsCRP, pg/L	45.77±23.27	276.99±83.41	620.02±103.38	<0.001
IL1β, pg/L	40.49±7.55	130.70±40.71	201.40±60.90	<0.001
IL6, ng/L	76.63±27.11	171.52±42.86	387.37±55.54	<0.001
TNF-α, pg/L	109.48±10.59	222.26±56.37	393.40±97.37	<0.001
DNA damage (%tail intensity)	2.56±1.81	35.17±7.24	52.71±10.80	<0.001

BMI, body mass index; MMSE, Mini-Mental State Examination; GFAP, glial fibrillary acidic protein; DBI, diazepam binding inhibitor; hsCRP, high-sensitive C-reactive protein; TOS, total oxidant status; TAS, total antioxidant status; OSI, oxidative stress index; TT, total thiol; NT, native thiol; DIS, disulfide; IL1β, interleukin 1β; IL6, interleukin 6; TNF-α, tumor necrosis factor α. *$p < 0.05$ was considered statistically significant. [a]Kruskal-Wallis test.

developed by Erel [16]. Oxidative stress index (OSI) is an indicator of the degree of oxidative stress [16]. Plasma thiol disulfide homeostasis tests were measured using an automated clinical chemistry analyzer (Abbott Architect ci16200 device, New Jersey, USA) using a recently defined method [17].

DNA Damage

Blood taken from volunteers into heparinized tubes was separated into mononuclear leukocytes using density gradient centrifugation method. DNA damage was measured using the comet assay method, which was detailed in our previous studies [18].

Statistical Analysis

Biostatistical analyses have been made with SPSS® (version 26.0) and Jamovi® statistical software. Kruskal-Wallis and Dwass-Steel-Critchlow-Fligner tests are used for groups and subgroups analysis. Results were expressed as mean ± standard deviation. The level of significance was identified at $p < 0.05$.

Results

The demographic information of the participants is given in Table 1. There was not any statistically significant difference between age, gender, and blood pressure between the groups ($p > 0.05$). The neuropsychological tests such as Mini-Mental State Examination (MMSE) and Clinical Dementia Rating Score (CDS) showed significant differences between groups ($p < 0.001$), as given in Table 1. The levels of oligomeric Aβ, total tau, α-synuclein, glial fibrillary acidic protein (GFAP), DBI were found highest in D5YG, and a statistical difference was seen between groups ($p < 0.001$).

Quantified levels of different vitamins were found to be statistically different between groups ($p < 0.001$). According to our measurements, levels of vitamin D and B12 showed a decreasing manner toward D5Y

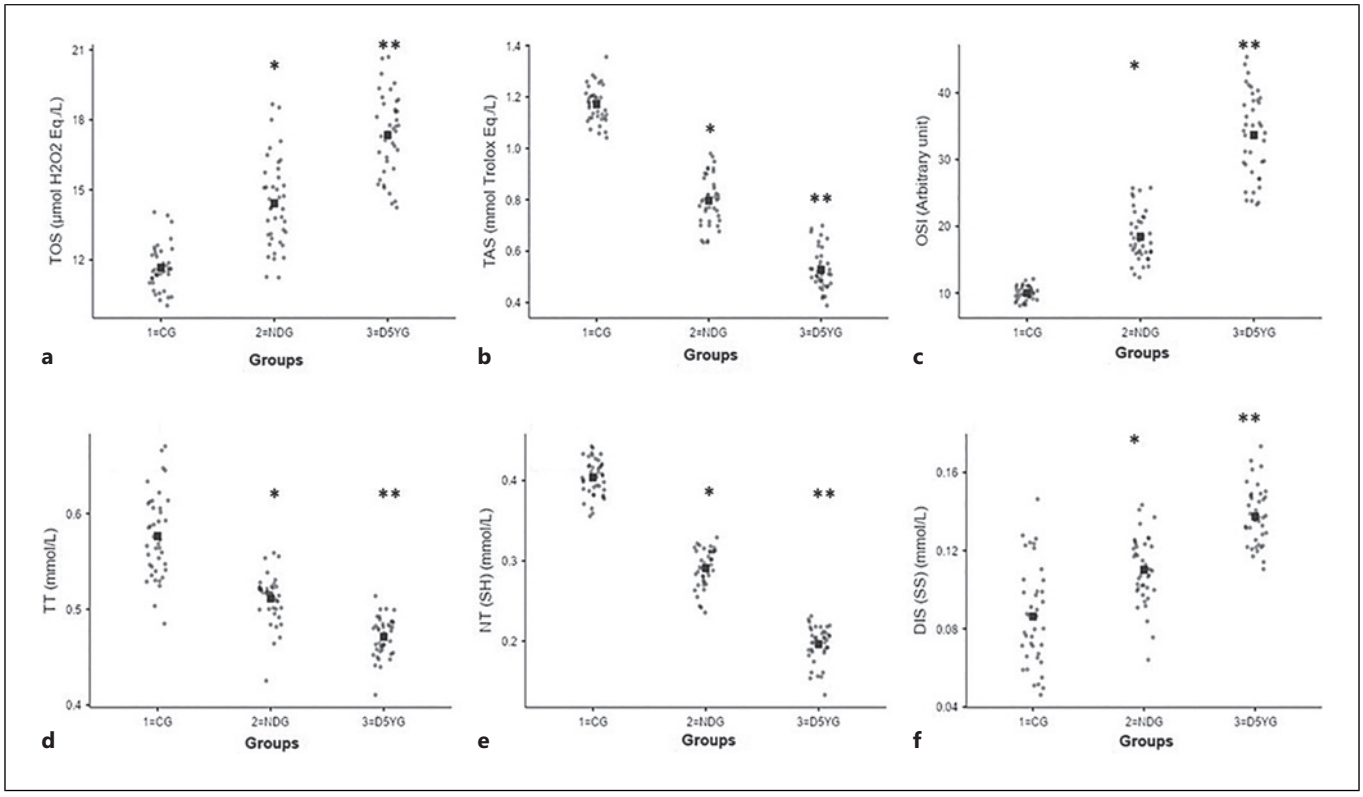

Fig. 1. Plasma oxidative stress biomarkers. **a** TOS (µmol H2O2 Eq./L). **b** TAS (mmol Trolox Eq./L). **c** OSI (arbitrary unit). **d** TT (mmol/L). **e** NT (SH) (mmol/L). **f** DIS (SS) (mmol/L). Kruskal-Wallis and Dwass-Steel-Critchlow-Fligner tests are used for groups and subgroups analysis. *$p < 0.05$ and **$p < 0.001$ were considered significant. TOS, total oxidant status; TAS, total antioxidant status; OSI, oxidative stress index; TT, total thiol; NT, native thiol; DIS, disulfide.

group (Table 1). Another important metabolic marker quantified were HbA1c, which were statistically different between CG and NDG, 5YDG ($p < 0.001$).

The mean results of oxidative stress marker levels obtained are given in Table 1 and Figure 1. The findings of analysis carried out among different groups based on their TOS, TAS, OSI, total thiol, native thiol, and disulfide values. According to the results, there is a significant difference among the groups concerning TOS assays, with a p value of less than 0.001. After examining the TAS values, it was discovered that there is a statistically significant difference among the CG, NDG, and D5YG groups, with a p value of less than 0.001. Additionally, the calculated OSI values suggest a significant difference among the groups, respectively, with a p value of less than 0.001. Regarding the results obtained from total thiol, native thiol, and disulfide, a significant difference was observed among the groups, with a p value of less than 0.001.

The results of the % tail intensity values used to calculate the DNA damage are given in Table 1. The results obtained from the DNA damage assay conducted on CG, NDG, and D5YG groups revealed a significant difference with regards to % tail intensity ($p < 0.001$).

The results of the analysis of inflammatory markers are presented in Table 1 and Figure 2. The quantification of hsCRP revealed a statistically significant difference among the CG, NDG, and D5YG groups, respectively, with a p value of less than 0.001. The investigation of IL1β results also indicated a significant difference, with a p value of less than 0.001. Similarly, the measurement of IL6 levels demonstrated a notable difference among the groups ($p < 0.001$). The assessment of TNF-α results revealed a statistical difference among CG, NDG, and D5YG groups, with a p value of less than 0.001. In addition, a statistically significant difference has been observed in serum levels of DBI among different groups with a p value of less than 0.001 (Table 1; Fig. 3).

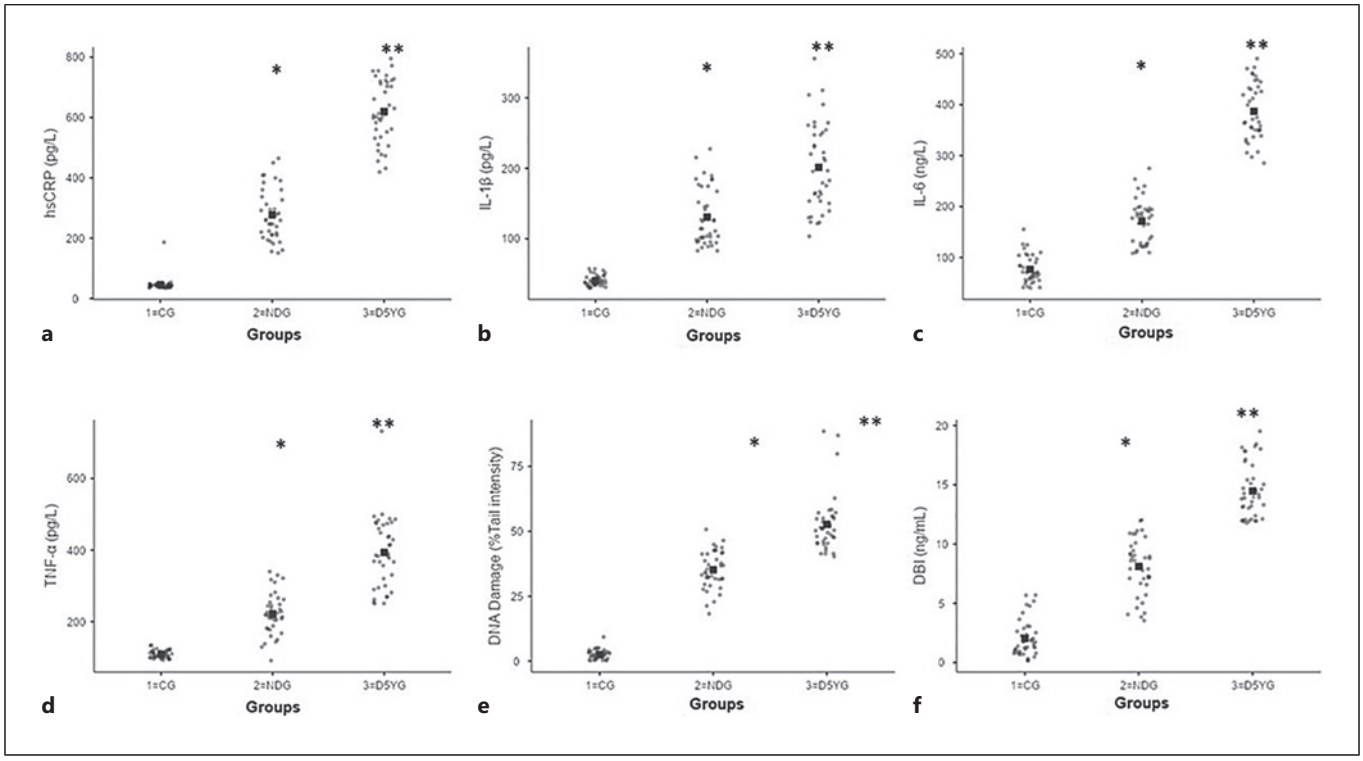

Fig. 2. Plasma inflammatory biomarkers. **a** Plasma level of hsCRP (pg/L). **b** IL1β (pg/L). **c** IL6 (ng/L). **d** TNF-α (pg/L). **e** DNA damage (%tail intensity). **f** Diazepam binding inhibitor levels (ng/mL). Kruskal-Wallis and Dwass-Steel-Critchlow-Fligner tests are used for groups and subgroups analysis. *$p < 0.05$ and **$p < 0.001$ were considered significant. hsCRP, high-sensitive C-reactive protein; IL1β, interleukin 1β; IL6, interleukin 6; TNF-α, tumor necrosis factor α.

Discussion

Currently, the diagnosis of AD is typically based on a combination of clinical assessments, imaging tests, and cognitive tests. However, these methods can be subjective and may not always accurately reflect the underlying pathology of the disease. Biomarkers, on the other hand, have the potential to provide a more objective measure of the disease process, which could improve the accuracy of diagnosis and the effectiveness of treatment [19]. One potential biomarker for AD is the presence of Aβ plaques in the brain. Aβ plaques are abnormal protein deposits that accumulate in the brains of individuals with AD, and they are thought to contribute to the deterioration of neurons and the loss of cognitive function. Other potential biomarkers for AD include tau protein tangles, inflammation, and oxidative stress [20].

Different studies showed patients with severe anxiety had increased DBI levels. However, DBI level in vascular dementia and Parkinson-type dementia was not significantly changed [21, 22]. On the other hand, the effects of DBI are unclear; in an animal study, it is proposed that over expressed DBI were decreasing hippocampal learning and does not affect anxiety and conflict behavior [23]. Whereas, in another animal study, DBI knockout murine were failed to learn and spatial navigation memory was not affected [3]. As seen in the literature, the concept of DBI is not a brand new idea for neuroscientists.

However, the evaluation of DBI in AD is a rather new concept. There are few studies in the literature where DBI levels are measured on plasma in AD patients. According to Conti et al. [4], DBI levels were dramatically increased due to inflammation compared to CG. Also, studies have shown that levels of DBI are altered in the brains of individuals with AD, and that these changes may be related to the progression of the AD. These studies, including our results, demonstrate the undeniable role of DBI in both clinical and preclinical studies. Whether the increase in DBI is related to disease progression, inflammation, or any other reason, evidence shows that there is a strong relationship between DBI and AD progression. There is a demonstration of the emergence of inflammation with progression in AD patients. As we

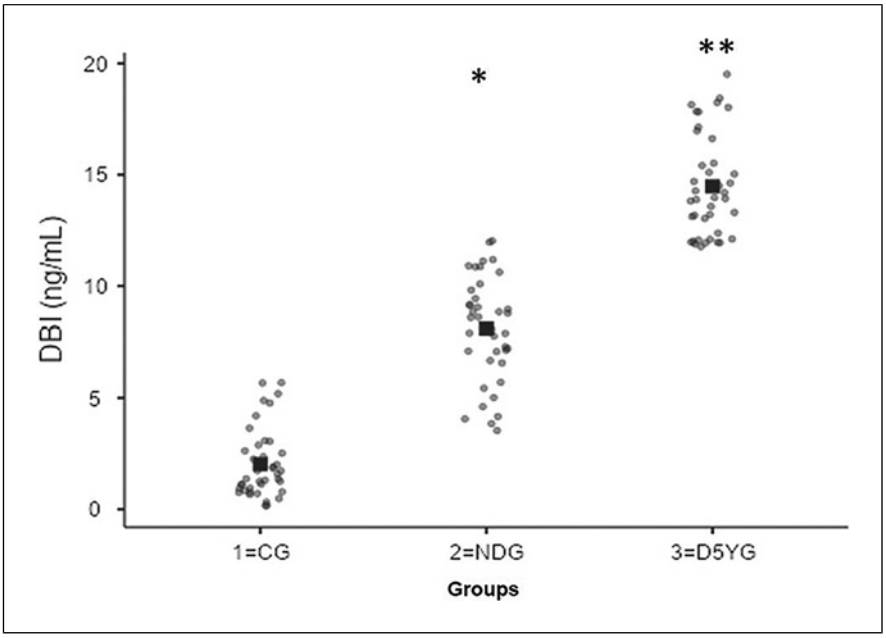

Fig. 3. Plasma level of diazepam binding inhibitor, DBI (ng/mL); $p < 0.001$. Kruskal-Wallis and Dwass-Steel-Critchlow-Fligner tests are used for groups and subgroups analysis. *$p < 0.05$ and *$p < 0.001$ were considered significant.

have shown in our study, the DBI intervals suggest that late-stage AD patients are characterized by a higher level of inflammation with a predominance of constructive features compared to early AD patients [4]. Another study pointed out that accumulation of Aβ is related with increased DBI expression [24].

In addition, cohort studies have determined that in AD patients, the plasma Aβ concentration is increased compared with mild cognitive impairment (MCI) or healthy controls [8]. Longitudinal analysis in the Alzheimer's Disease Neuroimaging Initiative showed that plasma tau level was associated with worsening of cognition, increased atrophy, and hypometabolism, thus AD-type dementia [13]. Individuals with MCI had higher plasma tau, a decrease in cortical thickness, and a decrease in memory performance were found compared to the healthy CG [11]. As a result, it has been reported that plasma tau level is highly associated with neurodegeneration [14]. Depending on the progression, an increase in these proteins was observed in late-stage AD patients compared to early-stage AD patients. Similar results to the literature, increased amount of tau was observed in the D5YG group in comparison to the control and NDG ($p < 0.001$). Similar to the literature, our results showed increased amount of Aβ and plasma tau levels in the late-stage AD group compared to early-stage and healthy CGs (Table 1). Additionally, our results indicated a strong positive correlation between DBI, Aβ levels, and plasma tau levels, respectively ($r = 0.917$, $r = 0.766$, $p < 0.001$). Therefore, as Aβ and plasma tau levels, DBI also could be interpreted as a promising biomarker in AD progression.

In the literature, α-synuclein levels were found to be higher in CSF levels in AD patients compared to healthy subjects [25, 26]. A recent study has also shown that α-synuclein plays an important role in AD pathophysiology. In particular, an increase in α-synuclein concentration was found in individuals with APoE4 alleles [27]. However, there is no AD study investigating α-synuclein levels through blood data. Therefore, as seen in our study, the dramatic increase in plasma levels of α-synuclein in late-stage AD patients is consistent with disease progression, as is the increase in CSF.

GFAP is a protein that increases in all neurodegenerative diseases. In a study where early-onset and late-onset Alzheimer's patients were compared with healthy controls, GFAP values of both AD groups were found to be higher than those of the CG. In our study, in accordance with the literature, GFAP protein was significantly increased in AD patients [28].

According to the literature, vitamin D deficiency increases aging and senile diseases [29, 30] and is an important risk factor for dementia and Alzheimer's development [31]. Studies have suggested that low levels of vitamin D may be associated with an increased risk of AD, and that vitamin D supplementation may have protective effects against the development of the disease [32, 33]. In our study, vitamin D level continued to decrease significantly compared to the progression of AD 5 years later.

Vitamin B12 decreases in neurodegenerative diseases, especially AD [34–36]. Similarly, relative to healthy controls, plasma B12 levels were found to be significantly lower

in individuals with MCI and AD patients [30]. According to results of Seshadri et al. [37], vitamin B12 supplementation was associated with a reduced risk of cognitive decline in older individuals with high levels of homocysteine, a protein that has been linked to an increased risk of AD. Perhaps by increasing vitamin D and vitamin B12 levels, AD can be improved or its progression slowed down.

Studies on C-reactive protein (CRP) have produced controversial results. In a study in which plasma CRP levels were measured, no significant difference was found between the MCI, AD, and CGs [38]. In addition, it has been stated in a study that CRP plasma levels are lower in individuals with AD compared to MCI and normal elderly individuals [39]. In a study in which plasma CRP was evaluated, they found that high CRP created 3 times more dementia and Alzheimer's risk [40]. The drastic rise in plasma CRP seen in early and late-stage AD patients relative to the CG is therefore not entirely consistent with the literature in our research. There is no direct relationship between CRP and DBI. However, inflammation has been linked to changes in the metabolism of some medications, including BDZ [38, 41].

Elevated levels of proinflammatory cytokines have been linked to an increased risk of several chronic diseases, including AD [42–44]. The fact that these metabolites are in an increasing trend in our late-stage AD patients is also directly proportional to the progression of the disease. There is also evidence to suggest that proinflammatory cytokines may have an effect on DBI levels in the brain and serum, whether in the literature and our results [4].

According to the literature, DNA damage was increased in the brains of individuals with AD compared to controls, and this was correlated with the severity of the AD [45]. It was also showed that DNA repair pathways were disrupted in neurons from individuals with AD, and that this disruption was associated with increased DNA damage and decreased brain function [46–48].

It has been revealed by many studies that oxidative stress in AD, as in many diseases, impairs the ability of cells to repair damaged DNA and leads to further damage accumulation [2, 49–52]. According to the results of Tadokoro et al. [53], a statistically significant improvement in MMSE scores was obtained when food supplement with known antioxidant properties was compared with placebo. Similar to the literature, our results showed that increased ROS was correlated with lower MMSE scores ($r = -0.685$, $p < 0.001$). Not surprisingly, most studies have found a general decrease in the amount and activity of antioxidants in the blood of patients in the early stages of AD, suggesting that the physiological balance between ROS/RNS production and antioxidants is altered, and therefore the amount of available antioxidants is greatly compromised [54, 55].

There were several limitations identified in our study. First, the study was conducted at a single center, which raises concerns about the generalizability of the findings to the wider population. Second, the investigation of the effects of DBI requires further investigation to confirm the routine use of DBI as a biomarker for AD. Third, as with the increase in Aβ levels, it is difficult to say that the high levels of DBI, oxidative stress, and tau are the cause of AD. Additionally, confounding factors such as medical conditions, comorbidities, diet, sleep patterns, and exercise status, which could impact DBI levels, inflammation, and oxidative stress levels, were not controlled for in our analysis. On the other hand, the lack of consideration of cerebrospinal fluid levels of DBI and the direct effects of DBI on AD limits our understanding of the potential of DBI as a biomarker for AD. Finally, the small sample size evaluated in our study is another limitation, and a larger sample size study may have yielded more reliable comparisons and more remarkable results.

In conclusion, the level of DBI were consistent with the commonly used biomarkers for diagnosis and follow-up of AD such as oligomeric Aβ, total tau, α-synuclein, GFAP, vitamin B12, vitamin D, oxidative stress, and inflammatory cytokines. According to the literature, DBI could be produced by peripheral organs [56]. The increased oxidative stress and inflammatory response may be the reason for the increased DBI levels. This may further compromise our theory however whether in the literature and our observations the increased DBI levels were related with the neuroinflammation due to pathology of the AD and recruited peripheral monocytes [4].

Although this study has yielded promising results, more research is required to confirm the utility of these compounds as a biomarker and to determine the optimal methods for measuring DBI levels. Further research is needed to fully understand the potential of DBI as a biomarker for AD.

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Statement of Ethics

The study has been approved by the Local Ethics Committee of Clinical Research with the decision number of 10/13. Written informed consent was obtained from all individual participants

included in the study. All procedures performed in the study were in accordance with the ethical standards of the University of Siena and with the 1964 Helsinki Declaration and its later amendments.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Mustafa Gokce: conceptualization, data curation, formal analysis, investigation, methodology, validation, and writing – original draft. Halil Aziz Velioglu: investigation, conceptualization, methodology, resources, supervision, validation, and writing – review and editing. Muhammed Yunus Bektay: conceptualization, resources, visualization, and writing – review and editing. Eray Metin Guler: methodology, funding acquisition, supervision, validation, and writing – review and editing.

Data Availability Statement

Data are not publicly available due to ethical reasons. Further inquiries can be directed to the corresponding author.

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