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VRADA training system as a non-pharmacological dual intervention to alleviate symptoms of the pathophysiology of Mild Cognitive Impairment

Eleni E. Tzekaki [a,b,1], Georgios Katsipis [a,b,1], Eleni G. Andreadou [a,b], Christos Mouzakidis [c], Eleni N. Baldimtsi [c,d], Eleni M. Karathanasi [c], Mary Hassandra [e], Evangelos Galanis [e], Antonis Hatzigeorgiadis [e], Marios Goudas [e], Paul Zikas [f], Giannis Evangelou [f], George Papagiannakis [f,g], George Bellis [h], Christos Kokkotis [e,h], Themistoklis Tsatalas [e], Giannis Giakas [e], Yannis Theodorakis [e], Magda Tsolaki [b,c,d], Anastasia A. Pantazaki [a,b,*]

[a] Laboratory of Biochemistry, Department of Chemistry, Aristotle University of Thessaloniki, 54124, Thessaloniki, Greece
[b] Center for Interdisciplinary Research and Innovation, Laboratory of Neurodegenerative Diseases (LND), 57001, Thermi, Thessaloniki, Greece
[c] Greek Association of Alzheimer's Disease and Related Disorders (GAADRD), 54643, Thessaloniki, Greece
[d] 1st Department of Neurology, School of Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Macedonia, Greece
[e] School of Physical Education, Sport Science and Dietetics, Department of Physical Education and Sport Science, University of Thessaly, Trikala, Greece
[f] ORamaVR SA, Science and Technology Park of Crete, Heraklion, Crete, Greece
[g] Institute of Computer Science, Foundation for Research and Technology – Hellas (FORTH), University of Crete, Heraklion, Crete, Greece
[h] Biomechanical Solutions Engineering (BME), Karditsa, Greece

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ABSTRACT

In this study, a VR system called VRADA (VR Exercise App for Dementia and Alzheimer's Patients) was designed for physical and cognitive training forindividuals with Mild Cognitive Impairment (MCI). The inflammatory factors IL-1β and TNF-α, Alzheimer's disease (AD) hallmarks total tau, p181-tau, $A\beta_{42}$ and $A\beta_{40}$, the ratio of $A\beta_{42}/_{40}$ and p181-tau/$A\beta_{42}$ were assessed on the blood serum of patients diagnosed with MCI to determine the effect of VRADA training. No significant differences were verified in the levels of inflammatory markers after the end of the study, however IL-1β levels of the VRADA group were significantly lower than those of the control group, at the follow-up of the study. Also, patients following VRADA intervention presented significantly higher $A\beta_{42}/A\beta_{40}$ ratio, and lower levels of $A\beta_{42}$, of total tau, p-tau181, and of the crucial ratio p-tau181/$A\beta_{42}$, in comparison with patients of the Control group. These results are promising for the further employment of the VRADA training during early dementia, and hopefully for halting the progression to AD.

1. Introduction

Nowadays, the augmented life expectancy gave rise to malign, age-related diseases such as Mild cognitive impairment (MCI) which in many cases lead to Alzheimer's disease (AD) – a deleterious neurodegenerative disease without any effective curative reported until now. MCI patients present alterations in the abilities of memory, attention, orientation, and executive functions [1], which though are not considered an obstacle in everyday life, are not according to the established standards for their educational and age level. The cause of the disease is not yet elucidated, and the pathophysiology may differ depending on the brain region which is suffering [2–4].

AD is strongly accompanied by inflammatory dysregulation [5–7], which may attributed to commensal microbiota disruption, chronic microbial infections [8–10], or chronic astrogliosis [11–13] and microglial exacerbation [14,15], due to the presence of pathological aggregates of amyloid beta-42 peptide ($A\beta_{42}$) and hyperphosphorylated tau protein (p-tau). It is not yet fully described what is the exact contribution of inflammation in the progression of the disease [16]. The levels of several cytokines are considered as indicators of neuro-inflammation. A part of them might increase during disease progression or temporarily from the MCI stage to AD [17].

As many therapies to cure AD have proven inadequate or ineffective, the key to coping with the disease might be nested in early prevention

* Corresponding author.
E-mail address: natasa@chem.auth.gr (A.A. Pantazaki).
[1] Equally contributing authors.

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[3]. Research in the field of physical exercise (PE) shed light on the direct correlation of exercise on the down-regulation of pro-inflammatory cytokines in the brain [18]. Pro-inflammatory cytokines are found to be decreased by engagement of MCI patients in PE [19,20]. Furthermore, increased incline has been detected in anti-inflammatory cytokines [21–23]. Findings showed that PE decreases cognitive decline and dementia [24], but the effect on AD hallmarks has not yet been examined in detail [25,26].

Among the non-pharmacological therapies, combined PE with cognitive training, multicomponent intervention [27], and Virtual Reality (VR) [28] are regarded as one of the most effective ways to cease the progression of the AD [29]. PE combined with cognitive exercise has a neuroprotective effect on both non-demented individuals and patients suffering from MCI, AD, or other dementias [30–35]. VR exercise, however holds a prominent position in cognitive and physical improvement when compared to physical and cognitive exercises for normal adults and elderly [36]. VR environments are regarded beneficial for reconstruction of brain plasticity and cellular synapses [37]. This intervention aims to stimulate and "restore" cognitive function to manage the autonomy of patients [37].

VR has been employed as assistant therapy for cerebral palsy, depression, and Parkinson's disease [38]. VR technology is also highly used for neurophysiological assessment and cognitive remediation [39]. MCI and dementia patients declared that fully immersive VR cognitive training provided a safe environment, satisfaction, engagement, and lower anxiety when compared to usual pen-paper training ensuring the adherence of patients to the training [40].

VR is additionally considered a driving force for MCI patients to be capable to perform activities of everyday life such as walking, feeding, and shopping on their own [39,41]. It is noteworthy that the rehabilitation of MCI individuals performing activities of everyday life mitigates the risk of AD's manifestation [42]. Augmented cognitive and executive function found in MCI and AD patients using computer or VR technology [43].

Given this, to increase the evidence of VR in the rehabilitation of cognitive function in MCI patients, we aimed to assess the enriched environment that VR bicycle offers on MCI patients through a 3-month intervention process. In this study, a nonpharmacological intervention with dual task VR system for physical and cognitive training of people with MCI was designed [44]. A person-centered technology was adapted to provide a comfortable environment for the users which customs an immersive VR training system named VRADA (VR Exercise App for Dementia and Alzheimer's Patients). The program of physical and cognitive training was previously tested [45]. The VRADA system was designed to be fully adapted to the needs of users effectively and efficiently achieving a more accurate assessment of user desire. The efficiency of the VRADA system to assist in the amelioration of inflammation and amyloid and tau pathology, was assessed in MCI patients for 3 months. Blood serum was received from the patients prior and after the study, and cordial disease-related biomarkers, specifically Tumor Necrosis Factor α (TNF-α), Interleukin-1β (IL-1β), $A\beta_{42}$, $A\beta_{40}$, total tau, p181-tau, and the ratios $A\beta_{42}/A\beta_{40}$ and p181-tau/$A\beta_{42}$ were measured with ELISA. The results from biomarker analysis and further statistical assessment of the results, imply that VRADA training could be beneficial for stalling MCI progression to AD.

2. Material and methods

2.1. Subjects

The participants were from a Mediterranean area (Thessaloniki region, northern Greece) and were employed between 2020 and 2022. All subjects were white, community-dwelling individuals. Participants were literate and were not suffering from any debilitating diseases (e.g. cancer) as ascertained from their medical history, and physical and neurological examination tests. All participants were recruited from the Greek Association of Alzheimer's Disease and Related Disorders' Day Care centre "Saint Helen" (Alzheimer Hellas, DCCSH) and underwent neuropsychological assessments, such as the Mini-Mental State Examination (MMSE). MCI patients were randomly divided into the groups of Control before and Control after ($n = 17$) and VRADA before and after ($n = 12$). MCI patients in the Control Group followed no program of physical or mental activity.

All participants' demographic characteristics are provided in Table 1, as mean values ± standard deviations (SD).

2.2. Study design

The training system consisted of a cycle-ergometer (stationary seated bike type; Toorx, ChronoLine, BRX R 300) and a VR head-mounted display (the Oculus Go headset) with a single 3DOF controller. VRADA application was based on a platform called ORamaVR MAGES. The VR environment consisted of training in a forest that was generated as the MCI patient moved along the forest path. Mental stimulation succeeded through the animal appearance during the VR training. Patients were asked to recall them at the end of the session. Moreover, the patient was asked to perform simple math calculations.

All groups performed 2 or 3 sessions a week for 12 weeks. The duration of VR exercise for the first 5 sessions was 20 min and 20 km/h speed of cycling. The time and the speed of cycling were progressively elevated to 30 min and 30 km/h (mild to moderate intensity), respectively.

2.3. Manipulation of blood serum samples

Whole blood samples were collected from subjects in the morning hours after overnight fasting, before the onset of the study (Baseline) and after its completion (After study), 3 months later. Serum separator tubes were employed, allowing samples to clot for 30 min at room temperature. Then, blood was centrifuged for 20 min at 1,000xg, sera were collected, aliquoted, and stored at −80°C until analysis. Care has been taken to avoid multiple freeze-thaw cycles. Necessary dilutions of the sera before the analyses were performed with commercial dilution buffer provided with the kits described below, just before the analyses.

2.4. Analyses of biomarkers levels by ELISA

All studied biomarkers in the sera of the study participants were detected by commercial sandwich, HRP-conjugated ELISA kits provided by Assay Genie (Dublin, Ireland), as follows: Human IL-1 beta PharmaGenie ELISA kit (#SBRS0740), Human TNF-alpha PharmaGenie kit (#HUDC0073), Human Amyloid Beta 42 / AB 1–42 kit (#HUFI02245), Human Amyloid Beta 40 / AB 1–40 ELISA kit (#HUFI02244), Human MAPtau (Microtubule Associated Protein Tau/Tau Protein) kit (#HUES02072), and Human Phospho Tau (P181) kit (#HUFI03189). All analyses were run per the manufacturer's instructions, in duplicates. Baseline and after-study serum samples were analyzed by different researchers, who were blind to the origin of the samples, as well as to the analyses performed by each other. Quantification of biomarkers' levels in blood serum samples was performed after the construction of standard curves, employing protein standards included in the kits. Double distilled water was used in all cases when needed.

2.5. Statistical analyses

Statistical analysis and graphs were conducted with GraphPad Prism 8 (GraphPad Software Inc.). Outcome data were presented as means ± SD. Possible differences for participants' demographics were evaluated with standard unpaired t-tests, while for gender, Chi-Squared analysis with Yates' correction was used.

Statistical tests exploited in the study were chosen after performing analyses for normal (Gaussian) distribution and homogeneity of the

Table 1
Demographics data and biomarkers' levels (IL-1β, TNF-α, $A\beta_{42}$, $A\beta_{40}$, total tau protein, and p181-tau) of MCI patients assigned to VRADA intervention, or to the Control group, from whom blood serum was received.

Blood Donor Demographics				Biomarker Analysis of Study Groups				
Demographics	Control	VRADA	P values	Biomarker	Control Baseline	Control After	VRADA Baseline	VRADA After
Participants Number (N)	17	12		**IL-1β (pg/mL)**	35.13 ± 8.26	42.37 ± 9.10	34.72 ± 8.13	28.14 ± 7.33[b]
Gender (Female/Male)	15/2	9/3	0.4239	**TNF-α (pg/mL)**	17.94 ± 4.74	17.16 ± 7.09	17.33 ± 4.51	16.43 ± 4.23
Age (years)	76.1 ± 6.8	67.6 ± 10.7	0.0697	**$A\beta_{42}$ (pg/mL)**	30.63 ± 5.91	23.73 ± 4.79[a]	29.24 ± 7.18	29.73 ± 8.46[b]
Education (years)	11.2 ± 3.7	14.2 ± 1.8	0.0697	**$A\beta_{40}$ (pg/mL)**	92.42 ± 12.36	106.70 ± 12.33[a]	93.06 ± 26.88	92.48 ± 22.32[b]
Body Mass Index (kg/m^2)	27.9 ± 3.7	27.8 ± 5.7	>0.9999	**Tau total (pg/mL)**	0.18 ± 0.04	0.27 ± 0.07[a]	0.19 ± 0.04	0.18 ± 0.04[b]
				p181-tau (pg/mL)	0.57 ± 0.20	0.96 ± 0.21[a]	0.60 ± 0.15	0.60 ± 0.15[b]
				$A\beta_{42}/A\beta_{40}$	0.34 ± 0.07	0.22 ± 0.05[a]	0.33 ± 0.08	0.35 ± 0.16[b]
				p181-tau/$A\beta_{42}$	0.019 ± 0.007	0.042 ± 0.011[a]	0.021 ± 0.003	0.022 ± 0.009[b]

The levels of studied biomarkes, were determined with Sandwich, quantitative ELISA. Ratios of $A\beta_{42}/A\beta_{40}$, and p181-tau/$A\beta_{42}$ were calculated afterward. All values are provided as Means ± Standard Deviation (SD). Statistical analysis for differences in the demographic data between groups was performed with the usage of the Graph Pad Prism 8 statistical package. No statistically significant differences ($p < 0.05$) were found between the demographics of the studied groups. Paired *t*-test was applied for determining statistical differences between baseline and after-study values, for each study group. An unpaired *t*-test was performed to estimate differences between the effects after VRADA intervention and Control group after the study. Statistically significant differences ($p < 0.05$) with.

[a] Baseline.

[b] Control after study.

MCI: Mild Cognitive Impairment; VRADA: virtual reality application for the exercise of dementia and Alzheimer's patients.

data, with the tests of Anderson-Darling and Bartlett, respectively. To examine possible discrimination for biomarkers' levels between baseline and after the performance of the interventions, paired *t*-test was employed. Based on the outcomes of the tests of Anderson-Darling and Bartlett, Wilcoxon non-parametric paired *t*-test has been employed for comparing the titers of IL-1β, $A\beta_{40}$ and of the ratio p-tau181/$A\beta_{42}$ for control group, and of the ratio $A\beta_{42}/A\beta_{/40}$ for Control and VRADA groups. In all other cases, standard paired *t*-test was used. To examine possible discrimination between the control group after the study, and the group of patients assigned to VRADA intervention, a standard unpaired *t*-test was performed. To verify the power of the conducted analyses, a post-hoc power analysis was performed, with G*Power 3.1 [46].

Correlation analysis with Spearman's test has been used for determining the relationships among analyzed variables and multilinear regression with backward elimination for evaluating the best predictor for the p-tau181/$A\beta_{42}$ ratio. All the analyses were carried between the start and the end of the clinical trial for every cohort. Levels of $A\beta_{42}$ and p-tau181 have been excluded from multilinear regression analysis, due to their immediate relationship to the ratio, which would subsequently lead to artificially lower significance for the other factors.

The statistical significance was set at $p < 0.05$ in all cases.

3. Results

3.1. Statistical analysis of subjects' demographics and power analysis

Participants' baseline demographics and the p values of the statistical analyses are summarized in Table 1, where the values displayed represent mean values ± SD. Graphs containing the comparison of age, gender, education, BMI, and the number of participants are depicted in Supplementary Fig. S1. Comparing the demographics mentioned, no significant differences were observed between the two studied groups.

Post-hoc power analysis was performed for determining the power of the performed analyses and screenshots of the received results are provided in the Supplementary material. Biomarkers' analyses, except for TNF-α, were found to be of good statistical power (>0.8).

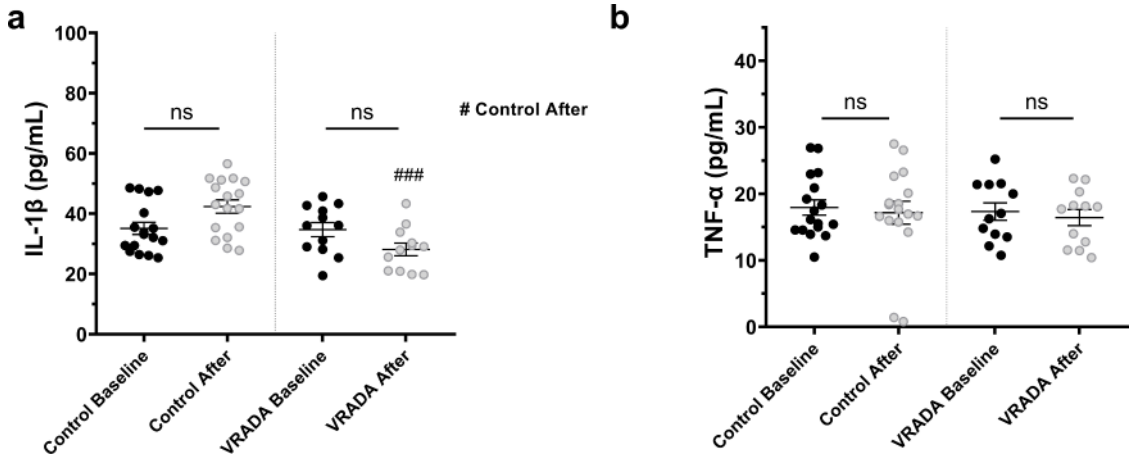

Fig. 1. Blood serum levels of (a) IL-1β and (b) TNF-α as measured from the implemented ELISA on patients with MCI who did not receive any intervened therapy (Controls) ($n = 17$) compared to MCI patients who followed VRADA intervention ($n = 12$). Blood from patients was collected at the Baseline levels (Control and VRADA baseline) and the end of the study (Control and VRADA after). Results are provided with individual values scatter plots which as mean values ± standard deviation (SD). All samples were analyzed at least in duplicates. Statistical analyses were performed with Graph Pad Prism 8.0 statistical software. Paired t-test was employed to examine differences between baseline and after biomarker titers and unpaired t-test for differences between biomarker titers of the control group and of the VRADA group after the completition of the study. #Differences between follow-up (After) levels of the Control group and the VRADA group. ns: non-significant, #: $p < 0.05$, ##: $p < 0.01$, ###: $p < 0.001$, ####: $p < 0.0001$.

3.2. Assessment of inflammatory markers in sera of MCI patients after VRADA intervention compared to the control group

By implementing ELISA, IL-1β and TNF-α levels in the blood showed a reduction thanks to VRADA engagement. The mean levels ± SD of the tested biomarkers of the two groups are provided in Table 1, and the distribution of those is depicted in Fig. 1.

IL-1β levels have an augmented tendency in the control group at the end of the study, while a reduction has been found in the VRADA group, with no statistical significance differences ($p > 0.05$, in both cases). Comparing the levels of IL-1β of the Control after and VRADA after, a significant reduction of IL-1β levels was observed in the VRADA group ($p = 0.001$). As regard to TNF-α levels, no significant changes were observed between the studied groups. The aforementioned results imply that physical and brain stimulation through VRADA technology could be able to exert an anti-inflammatory effect, as may be observed from the decline of IL-1β levels. However, the low power found as regard to TNF-α should be taken under notice, and therefore these results should be verified in the future with bigger cohorts.

3.3. The effect of VRADA technology on $A\beta$ species before and after the intervention on MCI patients

Previous studies showed that reduction of $A\beta_{42}$ and $A\beta_{40}$ plasma levels was associated with cognitive decline, indicating AD manifestation (Janelidze et al., [47]). To further investigate the effects of VRADA on the hallmarks of AD, we assessed amyloidosis in our clinical samples. All the received data as mean ± SD of the tested biomarkers are provided in Table 1 and the tendency of those is depicted in Fig. 2.

To estimate the effect of VRADA on the MCI group on the $A\beta_{42}$ and $A\beta_{40}$ levels, we determined the levels of these Aβ species in the sera of all the cohort groups before and after the treatment. Significantly lower levels of $A\beta_{42}$ ($p = 0.0023$) were found at the Control After group, indicating that amyloid homeostasis and clearance from the brain worsened in the absence of intervention. On the other hand, no significant differences were found in the VRADA group at the follow-up. After the VRADA intervention, patients presented significantly higher levels of serum $A\beta_{42}$ in comparison with the Control group ($p = 0.0219$), which may imply an ameliorative effect of VRADA technology, as higher levels of $A\beta_{42}$ in serum are regarded to correlate with higher clearance from the brain of the patients.

Serum $A\beta_{40}$ levels were found significantly elevated in the Control group at the end of the trial ($p < 0.0001$), which may be related to increased production of amyloid peptides due to gradual disease progression to the AD stage. Conversely, no significant changes in $A\beta_{40}$ levels were found for patients following the VRADA intervention. Additionally, $A\beta_{40}$ levels of the VRADA patients were significantly lower than those of the Control group ($p = 0.0364$), at the 3-month follow-up of the study. Also, in the Control group, the $A\beta_{42}/A\beta_{40}$ ratio was diminished in a significant manner ($p = 0.001$), while no changes were found in the VRADA groups. The patients employed in VRADA intervention presented significantly higher levels of $A\beta_{42}/A\beta_{40}$ ratio at the follow-up ($p = 0.0069$). Decreased plasma levels of $A\beta_{42}$ and lower $A\beta_{42}/A\beta_{40}$ ratio were previously regarded crucial for progression of MCI to dementia stage in a recent study conducted on 485 patients with MCI [48]. Also, *Fandos* et al. reported that a diminished $A\beta_{42}/A\beta_{40}$ plasma ratio is correlated with a higher risk of dementia. To summarize, an ameliorative tendency is brewed from the comparison of the VRADA baseline and VRADA group after intervention regarding general amyloidosis in MCI patients (Fandos et al., [49]).

3.4. The effect of VRADA reduces tau species

We next assessed the effect of VRADA intervention on tau levels through the measurement of total tau protein levels and phosphorylated tau species in the serum of all participants. P-tau181 protein levels in the serum (phosphorylation of threonine at 181 site) have been considered to be responsible for loss of physiological function, gain of toxicity, and its aggregation to form neurofibrillary tangles (NFTs) (Miao et al., [50]). In addition, we determined the ratio of p-tau181/$A\beta_{42}$ as a general marker of cognitive decline. The mean levels ± SD of tau species of every studied group, before and post-intervention, are summarized in Table 1, and the distribution of those is depicted in Fig. 3. Notably, elevated levels of tau-total were detected in the Control group after the intervention compared to baseline ($p < 0.0001$). On the contrary, when Control group at the follow-up was compared with the VRADA group after intervention, a significantly lower level of tau-total was measured ($p = 0.0002$).

Given that tau-total levels were changed thanks to VRADA intervention, the p-tau181 form has been next examined in the serum of MCI participants to detect the response to VRADA on hyperphosphorylation of tau. As far as the Control group is concerned, we observed an elevated tendency of p-tau181 after the end of the study on a significantly high level ($p < 0.0001$). On the other side, the comparison of the VRADA group with the Control group after intervention showed an overall amelioration of p-tau181 ($p < 0.0001$), implying that VRADA treatment may reduce toxic p-tau181 and total tau level. The same tendency was proved from the measurement of p-tau181/$A\beta_{42}$, where significantly higher levels were observed in patients who did not follow any intervention compared to the VRADA group after the end of the study ($p < 0.0001$). These results may potentially imply that VR technology as a non-pharmacological intervention can delay the progression of the

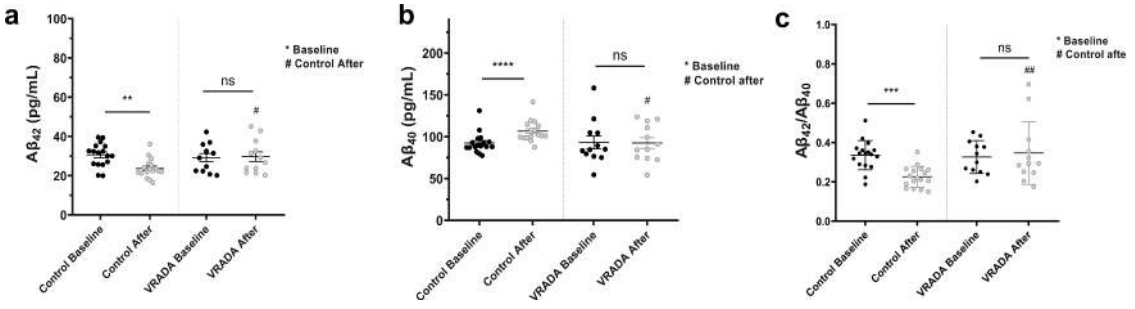

Fig. 2. Blood serum levels of **(a)** amyloid peptide 1–42 ($A\beta_{42}$), **(b)** amyloid peptide 1–40 ($A\beta_{40}$), and **(c)** of the ratio $A\beta_{42}/A\beta_{40}$ as measured from the implemented ELISA on patients with MCI who did not receive any intervened therapy (Controls) ($n = 17$) compared to MCI patients who followed VRADA intervention ($n = 12$). Blood from patients was collected at the Baseline levels (Control and VRADA baseline) and the end of the study (Control and VRADA after). Results are provided with individual values scatter plots which as mean values ± standard deviation (SD). All samples were analyzed at least in duplicates. Statistical analyses were performed with Graph Pad Prism 8.0 statistical software. Paired t-test was employed to examine differences between baseline and after biomarker titers and unpaired t-test for differences between biomarker titers of the control group and of the VRADA group after the completition of the study. *Differences between follow-up and baseline levels. #Differences between follow-up (After) levels of the Control group and the VRADA group. ns: non-significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

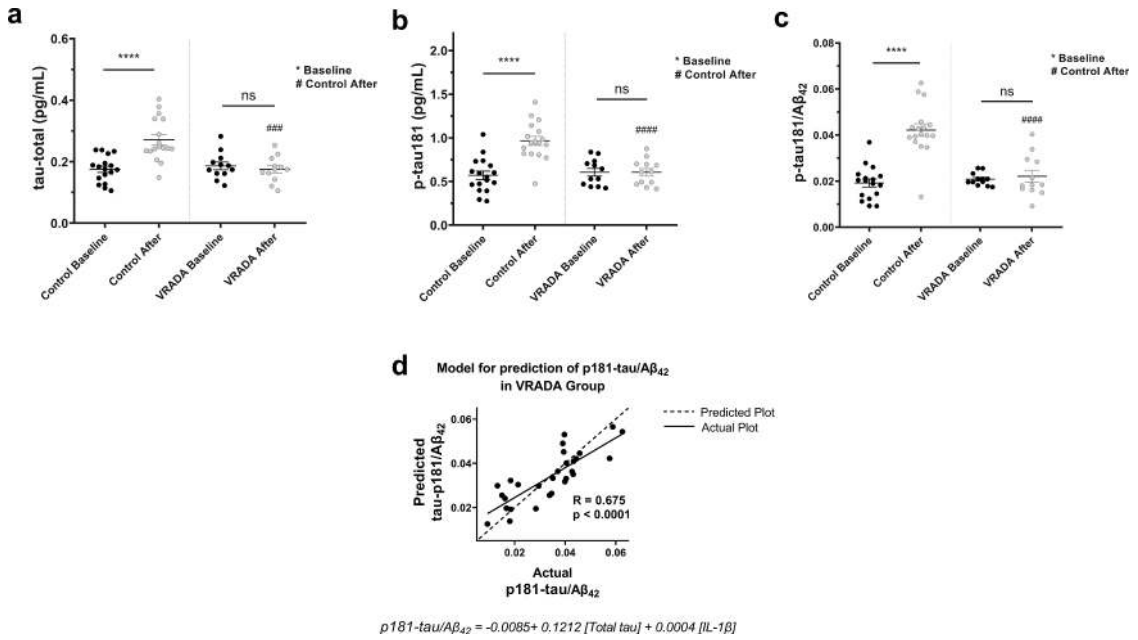

Fig. 3. Blood serum levels of **(a)** total tau protein (tau-total), **(b)** tau protein phosphorylated at Threonine-181 (p181-tau), and **(c)** of the ratio p181-tau/$A\beta_{42}$ as measured from the implemented ELISA on patients with MCI who did not receive any intervened therapy (Controls) ($n = 17$) compared to MCI patients who followed VRADA intervention ($n = 12$). Blood from patients was collected at the Baseline levels (Control and VRADA baseline) and the end of the study (Control and VRADA after). Results are provided with individual values scatter plots which as mean values ± standard deviation (SD). All samples were analyzed at least in duplicates. Statistical analyses were performed with Graph Pad Prism 8.0 statistical software. Paired t-test was employed to examine differences between baseline and after biomarker titers and unpaired t-test for differences between biomarker titers of the control group and of the VRADA group after the completition of the study. *Differences between follow-up and baseline levels. #Differences between follow-up (After) levels of the Control group and the VRADA group. ns: non-significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$. **(d)** Scatter plot of tau-p181/$A\beta_{42}$ levels against predicted tau-p181/$A\beta_{42}$ levels. Multilinear regression analysis with stepwise elimination has been performed for the evaluation of the effect of each biochemical and demographical characteristic of VRADA group. R squared values of the proposed models and p value of the corresponding equation is provided in the plot. Statistical analyses were performed with Graph Pad Prism 8.0 statistical software.

disease from MCI to AD or holistic deter the onset of deleterious effects of the disease regarding tau pathology.

3.5. Correlation analysis

The possible correlation of the levels of all studied markers between each other was also assessed. Regarding inflammatory markers results are provided in Table 2. IL-1β baseline levels were not found to correlate with any studied biomarker, while at the follow-up of the study, significant correlations were demonstrated with AD hallmarks, $A\beta_{42}$, total tau, p-tau181, and the ratios $A\beta_{42}/A\beta_{40}$, and p-tau181/$A\beta_{42}$. IL-1β also correlated positively with tau species and the ratio p-tau181/$A\beta_{42}$, while a negative correlation was demonstrated for $A\beta_{42}$ and the ratio $A\beta_{42}/A\beta_{40}$. In addition, TNF-α levels were found to positively correlate with total tau and p-tau181 levels at both the baseline and the follow-up of the study. Significant correlations were also demonstrated only at the follow-up of some biomarkers, namely a negative correlation with $A\beta_{42}$ levels and $A\beta_{42}/A\beta_{40}$ ratio, and positive correlations with $A\beta_{40}$ and p-tau181/$A\beta_{42}$. Finally, a positive correlation between baseline TNF-α levels and follow-up levels was verified. In, Supplementary Figure S2, the correlations of the ratios $A\beta_{42}/A\beta_{40}$ and p181-tau/$A\beta_{42}$ with the inflammatory markers are also provided. These correlations underline the implication of inflammation on amyloid and tau hallmarks of AD.

Table 2
Correlation analysis of serum cytokines IL-1β and TNF-α with the analyzed biomarkers and demographics of the study.

Biomarker	Correlation analysis of study groups			
	IL-1β		TNFα	
	Baseline	After	Baseline	After
IL-1β		−0.099	0.310	0.340
TNF-α	0.310	0.340		0.516**
$A\beta_{42}$	0.140	−0.399*	0.313	−0.335****
$A\beta_{40}$	−0.007	0.246	0.343	0.450*
Total tau	0.131	0.463*	0.392*	0.531**
p181-tau	0.066	0.490**	0.523**	0.475**
$A\beta_{42}/A\beta_{40}$	0.061	−0.446*	−0.042	−0.442*
p181-Tau/$A\beta_{42}$	−0.077	0.591**	0.262	0.562**
Age	−0.239	0.301	0.001	0.051
Education	−0.281	−0.325	−0.066	0.054
BMI	0.248	−0.060	0.033	0.155

Correlation analysis has been performed with Graph Pad Prism 8.0 statistical package and rank correlation coefficients (r) and their corresponding p values were evaluated using Spearman's test.
* : $p < 0.05$.
** : $p < 0.01$
*** : $p < 0.001$.
**** : $p < 0.0001$.

As far as AD hallmarks are concerned, Aβ species correlations are given in Table 3, while regarding tau species, correlation results can be found in Table 4. $A\beta_{42}$ levels were found to correlate positively with p181-tau levels only at the baseline. A strong positive correlation with $A\beta_{42}/A\beta_{40}$ ratio at both baseline and follow-up as well as a strong negative correlation between $A\beta_{42}$ level only at follow-up and p181-tau/$A\beta_{42}$ ratio, was also demonstrated. $A\beta_{40}$ levels were found to correlate with total tau and p181-tau levels at both baseline and follow-up. Also, positive correlations were found to be significant between $A\beta_{40}$ and the ratio p181-tau/Aβ42 ratio, and more strongly between baseline and follow-up levels of this amyloid species. Total tau levels positively correlate with p181-tau at baseline, but more strongly at the follow-up. Also, positive correlations, only at the follow-up, were found between total tau and the ratio p181-tau/$A\beta_{42}$, while a positive, significant correlation was also found between follow-up total tau and the age of the participants. Positive significant correlations regarding age were also

Table 3
Correlation analysis of serum amyloid ($A\beta_{42}$, $A\beta_{40}$, and the ratio $A\beta_{42}/A\beta_{40}$) with the analyzed biomarkers and demographics of the study.

Biomarker	Correlation Analysis of Study Groups					
	$A\beta_{42}$		$A\beta_{40}$		$A\beta_{42}/A\beta_{40}$	
	Baseline	After	Baseline	After	Baseline	After
$A\beta_{42}$		−0.216	0.352	−0.176	0.626***	0.863****
$A\beta_{40}$	0.352	−0.176		0.725****	−0.367	−0.556**
Total tau	0.352	−0.284	0.515**	0.410*	−0.080	−0.421*
p181-tau	0.428*	−0.319	0.373*	0.418**	0.032	−0.498**
$A\beta_{42}/A\beta_{40}$	0.626***	0.863****	−0.367	−0.556		0.018
p181-Tau/$A\beta_{42}$	−0.304	−0.702****	0.228	0.428*	−0.577**	−0.796****
Age	0.074	−0.109	0.136	0.166	−0.034	−0.205
Education	−0.049	0.350	0.148	0.113	−0.092	0.282
BMI	0.263	−0.283	−0.045	0.102	0.260	−0.337

Correlation analysis has been performed with Graph Pad Prism 8.0 statistical package and rank correlation coefficients (r) and their corresponding p values were evaluated using Spearman's test.
*: $p < 0.05$.
**: $p < 0.01$.
***: $p < 0.001$.
****: $p < 0.0001$.

Table 4
Correlation analysis of serum tau (T-tau, p181-tau, and the ratio p181-tau/$A\beta_{42}$) with the analyzed biomarkers and demographics of the study.

Biomarker	Correlation Analysis of Study Groups					
	Total tau		p181-tau		p181-Tau/$A\beta_{42}$	
	Baseline	After	Baseline	After	Baseline	After
Total tau		0.280	0.417*	0.871****	0.295	0.771****
p181-tau	0.417*	0.871****		0.484**	0.646****	0.843****
p181-Tau/$A\beta_{42}$	0.295	0.771****	0.646***	0.843****		0.122
Age	0.063	0.404*	0.194	0.507**	0.148	0.394*
Education	0.171	−0.226	0.271	−0.257	0.232	−0.337
BMI	0.083	0.033	−0.139	0.051	−0.320	0.148

Correlation analysis has been performed with Graph Pad Prism 8.0 statistical package and rank correlation coefficients (r) and their corresponding p values were evaluated using Spearman's test.
*: $p < 0.05$.
**: $p < 0.01$.
***: $p < 0.001$.
****: $p < 0.0001$.

verified for p-tau181 levels and the ratio p181-tau/$A\beta_{42}$, implying a significant implication of the age of the participants, for VRADA intervention to present better effects.

3.6. Multilinear regression analysis for determinants of ratio p181-tau/$A\beta_{42}$

To establish how the studied biomarkers are implicated in the general AD pathology for both Control and VRADA groups, a multilinear regression analysis was performed for best predictors of p181-tau/$A\beta_{42}$ levels - a crucial ratio reflecting the status of AD hallmarks. The results are presented in Table 5. At baseline, the multilinear model derived after backward elimination could not be verified as statistically significant, with no studied biomarkers presenting any significant effect on the ratio. To be noted, $A\beta_{42}$ and p-tau, which would present significant implications on the ratio, were omitted from this study, to verify the effect of other determinants.

A very different result was found after the competition of the VRADA intervention. A strong, significant positive effect of total tau levels was verified, while also IL-1β was found to be a significant, though less strong, determinant of the ratio levels. The multilinear model (which is provided in Fig. 3d), was found to be significant. Thus, this analysis proves that VRADA intervention would possibly lead to a diminished p181-tau/$A\beta_{42}$ ratio, possibly through a pathway including a decrease of soluble total tau levels. This effect could reflect a decreased rate of axon degeneration, through a simultaneous alleviation of inflammatory pathways, as is found by the reduced IL-1β levels proven in this study.

Table 5
Independent predictors of ratio p181-tau/$A\beta_{42}$ from a multilinear regression model using backward elimination.

Predictor marker	Multilinear regression analysis of ratio p-tau181/$A\beta_{42}$			
	Baseline		Follow-up	
	β	p	β	p
IL-1β	< 0.0001	ns	0.0004	*
TNF-α	0.0003	ns	0.0001	ns
$A\beta_{40}$	< 0.0001	ns	0.0001	ns
Total Tau	0.0073	ns	0.1212	****
Age	< 0.0001	ns	0.0002	ns
Gender	0.0036	ns	0.0019	ns
Education	0.0006	ns	−0.0002	ns
BMI	−0.0003	ns	0.0005	ns
Intercept	0.0124	**	−0.0085	ns
R Squared	0.1270	ns	0.6750	****

The biomarkers IL-1β, TNF-α, $A\beta_{40,}$ and tau-total that were analyzed in blood sera of MCI patients before and at the follow-up of the VRADA intervention, and demographic parameters (age, education, BMI) were included in the multivariable analysis (Graph Pad Prism 8) and were eliminated step to step, based on the significance of the contribution to the p181-tau/$A\beta_{42}$. β: standardized regression coefficient; MCI: Mild Cognitive Impairment; BMI: Body Mass Index ns: non-significant.
*: $p < 0.05$.
**: $p < 0.01$
***: $p < 0.001$.
****: $p < 0.0001$.

4. Discussion

Dementia is considered a multifactorial disease, with the mechanisms involved are not yet fully understood. A critical role is attributed to the interplay between inflammatory and environmental factors. Currently, the unearthing of non-toxic or non-pharmacological interventions targeting AD has attracted considerable attention in AD research. As the elixir to cope with the deleterious effects of the disease still not come to light, disease prevention appears to be a potential possibility to manage the progression to AD.

VRADA training model was designed by a group of experts according to the previous guidelines and methodology for artificial therapeutic and health apps [51,52]. The VR system was formed to have a dual performance of physical and cognitive training for the elderly suffering from MCI. The pleasure, engagement, tolerance, acceptance, and easy-to-use VRADA system were reflected in the measured scores which were proved to be above the mean and succeeded in higher scores compared to a group of students [44]. This kind of technology system, which is continuously tested, based on human needs proved to be user-friendly [44]. Patients unfamiliar with the application had short-term training for their encouragement to use VR. While past studies support that the use of immersive VR by older adults creates symptoms such as instability and sensory conflict [53], no side effects such as nausea, dizziness, and anxiety were declared from patients who suffered from MCI and participated in VRADA training.

Here, we aimed to assess and quantify the possible amelioration at biomarker-level in older adults with MCI following VRADA, compared to patients receiving no intervention. More specifically, we focused on the inflammatory cascade to examine possible mitigation by 3-month engagement on VRADA, reflecting in the overall amelioration of AD biomarkers. For this reason, having in mind that there were no reported studies that have correlated the improvement thanks to VR technology, this is the first research that embodied inflammatory and AD biomarkers to provide data in detail for the possible potential of this new technology. Moreover, our aim was to investigate if a non-pharmacological intervention may halt or mitigate the progression of MCI to AD state.

A multi-tasking device, except for the attainment of a more active lifestyle, mostly affects cognitive function in the elderly community-dwelling population with MCI. This VR stationary bike system, called VRADA, provides physical and cognitive training performance creating a safe, comfortable, and engaged environment to evaluate the possible potential of a non-pharmacological intervention to ameliorate the deleterious pathophysiology of dementia patients or to overcome the progression of the disease. The dual stimulation both physically and mentally adopted in the present protocol was sufficient and able to achieve a preliminary mitigated effect reflecting an alleviation of patients who suffered from MCI. To determine the effect of VRADA intervention on blood serum biomarkers of patients diagnosed with MCI, this study accompanied cognitive stimulation by assessment of inflammatory factors (IL-1β, TNF-α), the biomarkers hallmarks of AD (total tau, p181-tau, Aβ42, and Aβ40, as well as the ratios of Aβ42/40 and p181-tau/Aβ42).

First, concerning inflammation, a significant decrease in IL-1β levels was measured after the VRADA intervention, with a significant difference between the Control group and VRADA group at the follow-up. The decrease in IL-1β may imply that patients suffering from MCI may profit from a healthier status after the VRADA sessions. These results come in agreement with those of *Kohanpour* et al. where patients were engaged in aerobic exercise with simultaneous consumption of *Glycyrrhiza glabra* extract. Moreover, augmented levels of IL-1 are considered to be pathological evidence of AD due to their implication in memory disturbance [54]. In a transgenic animal model, the chronic silence of the IL-1 receptor resulted in the amelioration of neuroinflammation and neuronal pathology [55]. Secondly, TNF-α levels were found to decrease, despite the lack of significant group differences possibly because of the short period of intervention [56]. Previous studies also presented the ameliorative effect of PE in inflammation. Pro-inflammatory cytokine IL-6 increases in response to abrupt decrement in the post hoc period triggering the augmentation of anti-inflammatory response through IL-1ra and IL-10 [57,58]. The activation of IL-6 via physical activity seems to have an anti-inflammatory role which counteracting as an obstacle to TNF-α activation in healthy individuals [59].

As for amyloid peptides, $A\beta_{42}$ had an increased tendency in the post-VRADA group with a significant difference compared to the post-Control group reinforcing the evidence that supports PE for Aβ clearance. The ratio $A\beta_{42}/A\beta_{40}$ had an improvement in the VRADA-after group despite the lack of significance between the post-VRADA and post-Control groups. All studied biomarkers were assessed in serum as an alternative and easy-to-obtain source that reflects the pathology of the disease [60,61]. More promising results were revealed from the measurement of tau forms in the sera of the MCI VRADA patients group compared to the control group at baseline level and at the end of the study. We found a decisive amelioration of total tau and p-tau181 after the intervention, with a significant difference compared to the Control group after the intervention. The same improvement turns out to have the ratio of p-tau181/$A\beta_{42}$. This could indicate that at the start of the study, participants with higher $A\beta_{42}$ levels tended to have higher p-tau181 levels. However, as the study progressed and follow-up measurements were taken, this correlation might have altered or diminished altogether. The initial positive correlation might reflect an early stage of the disease, but as it progresses, other factors could become more influential, leading to a weakening or disappearance of the correlation. The study involved VRADA interventions which could have an impact on the biomarker's level, thus affecting the correlation observed at the baseline.

The aforementioned results are strongly supported by statistical analysis through Multilinear Regression. The biomarker which is actively contributed to the amelioration of p-tau181/$A\beta_{42}$ observed in the VRADA group is owed to tau mitigation and IL-1β levels.

An interplay between AD and inflammation is well-proven [62]. It has not been elucidated if inflammation is the driving force for Aβ deposition or Aβ toxicity provokes inflammatory response [63]. On Amyloid Precursor Protein (APP) transgenic mice, physical exercise was sufficient to activate the neprilysin, the Aβ-degrading enzyme, alleviating Aβ pathology, and memory [64]. In addition, PE augments adulthood neurogenesis in mice, as proved by morphological studies and attenuated cytokine-level [65].

The main element of amyloid plaques is found to be $A\beta_{42}$ which forms aggregations in the brain of AD patients. Both Positron emission tomography (PET) results and CSF-blood amyloid levels confirmed the aggregation of cerebral amyloid [66,67]. To the best of our knowledge, till now, the literature reported data have not been correlated to a specific tendency of Aβ levels of MCI patients [68]. Consequently, $A\beta_{40}$ is not counted as a predictor of dementia opposing to the ratio of $A\beta_{42/40}$ [69]. Studies suggested that decreased plasma levels of $A\beta_{42/}A\beta_{40}$ [70] are strongly associated with the onset of AD and future cognitive decline ([71,72], Fandos et al., [49]).

Fibrillary form of Aβ extracellularly conglomerates and form senile plaques which in turn stimulate tau hyperphosphorylation generating a neurotoxic environment [73]. However, it is not yet fully understood whether the neurotoxicity of Aβ is dependent on tau [73]. A significant increase of p181-tau depleted $A\beta_{42}$, and increased tau-total evidence the amyloid deposition and faster cognitive decline [74] which are associated with CSF biomarkers at the clinical stage of AD [75–78]. P181-tau deleterious augmentation is prodromal of AD stage in MCI patients [79]. In a recent longitudinal study plasma p181-tau levels were greater, at baseline of MCI and AD dementia, and augmented over time at the preclinical, prodromal, and dementia stage of AD [74]. It is underwritten that the ratio of p181-tau/$A\beta_{42}$ constitutes a useful tool for discrimination of the disease stage [80]. As high as the ratio is the risk of AD onset is enhanced [80]. In addition, the plasma p181-tau/$A\beta_{42}$ ratio is considered a double-sided mirror to depict tau deposition in diffuse brain regions [81].

To the best of our knowledge, two studies for older adults with MCI have detected the amelioration owing to VR-PE on general cognitive function [36,82], executive function [83,84,36], working memory capacity [83], short-term memory [85] and verbal episodic memory [86] compared to the control groups. A significant improvement was found in the MCI group compared to healthy elderly in executive functions [87]. As for the dual role of intervention, VR and physical exercise have been reported to overcome the inhibition performance in older adults with and without mild cognitive decline [83,37]. VR-PE was effective in shifting performance measured by trial making [88] or color trials [83] in healthy older adults. Two studies have measured dual-task performance [89,36], and one study the abstract thinking [84].

Even though this research has recruited a small number of participants, the physical performance in conjunction with cognitive tasks in a virtual reality environment was able to verify decreased inflammatory cytokines, IL-1β, TNF-α and increased ratios of $A\beta_{42/40}$, and p181-tau/$A\beta_{42}$ by implemented ELISA in serum of all patients with MCI and comparing to control group. The reason that may plausibly justify this evidence is summarized in the concept that exercise activates the body's recovery system resulting in the production of trophic, anti-oxidant, and anti-inflammatory factors which in turn improve brain plasticity and function [90–93]. As a result, the adopted training protocol resulted in the amelioration of MMSE and the mitigation of the disease [44]. The subjects in the VR intervention group strongly adhered to the training and the dropout number was low.

In addition, patients' declarations revealed engagement with the training performance of VRADA, a pleasured environment, and a broad acceptance as a multi-useful tool for conducting cognitive tasks combined with delight retrieved from the PE. The training program was well put in and accepted by all participants who declared in most cases thrilled for the use of VR system, which represents a key tool when coping with the elderly [94].

The adaptation of a lifestyle in nourishing [95,96] combined with physical activity on a moderate level day-to-day is considered as the main element to alleviate or overcome cognitive decline [30–35]. Therefore, it is out of emergency the adoption of preventive measures of everyday life to confront the disease. VR constitutes a new trend that floods our society, but it stood to preliminary applications as a therapy tool. In the domain of prevention and treatment of MCI, VR continues to require more clinical research. As this method is expanded to patients with different dementias levels, it is expected to gain more therapeutic strategies having in its center the patients' needs with a view to qualified. The future of VR in MCI therapeutic strategies is strongly correlated with new technological data, that aim to create an ever more engaging and safer artificial interactive environment.

5. Limitations

Even though VRADA technology presented advantages such as acceptability, usability, and tolerability, however, its effectiveness has some limitations indeed. These limitations are concerned with its pilot study characteristics (i.e., small sample and a short period of treatment). The monitoring of the physical activity in the experimental sessions and the social activity program in the control group strengthen the clinical study. A backbone study with a long-term follow-up is indispensable to evaluate the rehabilitation efficacy in detail. Despite the small number of patients, the strength of this study includes the supervised exercise program, a high rate of adherence to the intervention, and the use of validated outcome measures.

Declaration of Competing Interest

All the authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.dscb.2023.100090.

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