CLINICAL STUDY RESULTS DOCUMENT

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Effects of Aerobic Exercise on Clinical Progression Outcomes and Neurovascular Unit–related Biomarkers in Individuals with Multiple Sclerosis: A Randomized Controlled Trial

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Study Design

This study was designed as a randomized controlled trial to comprehensively investigate the effects of aerobic exercise on NVU biomarkers in individuals with MS. The study was conducted between February and June 2025 at the MS outpatient clinic of the Department of Neurology, Ondokuz Mayıs University Faculty of Medicine. Participants were randomly assigned to either a control group or an exercise group. Assessments were performed at baseline (pre-intervention) and eight weeks after the intervention (post-intervention). The study procedure is summarized in the CONSORT flow diagram (Figure 1). No modifications or deviations from the original study protocol were made after the initiation of the trial.

Participants

Individuals with a definitive diagnosis of MS according to the McDonald criteria who presented to the Neurology Clinic of Ondokuz Mayıs University were invited to participate in the study. A total of 38 patients who met the inclusion criteria and provided written informed consent were allocated into two groups the exercise group (n = 19) and the control group (n = 19) using a simple randomization method. Randomization was performed by the physician responsible for patient referral using a computerized random allocation sequence. Importantly, the referring physician was blinded to the specific interventions applied in each study group. Outcome assessors and laboratory personnel responsible for biochemical analyses were also blinded to group allocation throughout the study. Group allocation was concealed until the completion of baseline assessments. Due to the nature of the exercise intervention, participant and therapist blinding was not feasible, and therefore neither participants nor therapists were blinded to group allocation.

A total of 38 participants were randomized into the study. During the follow-up period, four participants in the control group did not complete the study (one due to an MS relapse, one due to non–MS-related health problems, and two due to non-attendance at

the final assessment). In the exercise group, four participants did not complete the study (two due to non-completion of the exercise program and two due to changes in pharmacological treatment). Thirty participants completed the intervention and post-intervention assessments (control group: n = 15; exercise group: n = 15) (Figure 1).

Inclusion criteria were as follows: a definitive diagnosis of MS according to the McDonald criteria; age between 20 and 50 years; a Standardized Mini-Mental Test (SMMT) score of ≥24; an EDSS score between 0 and 4.0; absence of any other serious medical condition that would preclude exercise participation; and no changes in pharmacological treatment within the previous six months.

Exclusion criteria included: experiencing an MS relapse within the past six months; presence of orthopedic or systemic conditions that would limit participation in exercise; having a known neuromuscular disorder other than MS; receipt of immunomodulatory treatment within the previous six months; presence of cardiopulmonary conditions that could contraindicate exercise; and pregnancy or breastfeeding.

Withdrawal criteria were defined as: failure to attend three consecutive exercise sessions; changes in or interruption of ongoing pharmacological treatment; occurrence of an MS relapse; or withdrawal from the study due to health-related or personal reasons.

Measures

Participants were assessed before group allocation (baseline assessment) and at the eighth week following completion of the aerobic exercise program (post-intervention assessment). All participants with a definitive diagnosis of MS underwent the Standardized Mini-Mental Test (SMMT), and their EDSS scores were determined. Patients who scored ≥24 on the SMMT, had an EDSS score between 0 and 4.0, and met the inclusion criteria underwent baseline assessments to determine clinical progression. These assessments included a demographic data form, the 6MWT, the 9HPT, and the TUG. In addition, blood samples were collected to evaluate NVU related biomarkers.

Following completion of the aerobic exercise program, the 6MWT, 9HPT, and TUG were re-administered by the researchers, post-intervention blood samples were obtained, and final assessments were completed. All clinical evaluations were conducted by expert physiotherapists with experience in neurological rehabilitation. To ensure objectivity of the measurements, the assessors were blinded to the results of the biochemical analyses.

Demographic Data Form:

A demographic data form was developed for this study in accordance with the literature to collect information on participants' demographic characteristics and MS-related symptoms (Knowles et al., 2024). The form included data on age, sex, use of assistive devices, MS type, date of MS diagnosis, number and timing of relapses, time since the most recent relapse, current medications, comorbid chronic diseases, history of surgical interventions, presence of fatigue, and exercise habits.

Expanded Disability Status Scale (EDSS):

The EDSS is a practical and reliable scale frequently used in clinical settings to evaluate disease progression and treatment response in individuals with MS. It was originally developed by neurologist John Kurtzke as a 10-step Disability Status Scale. The EDSS is widely used by clinicians worldwide to assess functional systems of the central nervous system in individuals with MS. Scoring is based on the extent of impairment in MS-affected body functions, and the total score constitutes the EDSS value, ranging from 0 to 10 (Demir, 2022).

Standardized Mini-Mental Test (SMMT):

The SMMT consists of 11 items grouped into five main domains: orientation, registration memory, attention and calculation, recall, and language. Scores between 0–9 indicate severe cognitive impairment, 10–19 moderate cognitive impairment, 20–23 mild cognitive impairment, and 24–30 normal cognitive function. The validity and reliability of the SMMT for the Turkish population were established by Güngen et al. (Güngen et al., 2002).

6-Minute Walk Test (6MWT):

The 6MWT is a clinical exercise test used for the objective assessment of functional

exercise capacity and provides an estimate of disease-related effects of MS on walking endurance. The test measures the distance walked by the participant over six minutes on a flat 30-meter corridor. In healthy individuals, the expected walking distance ranges between 400 and 700 meters. Blood pressure, oxygen saturation, heart rate, and perceived levels of dyspnea and fatigue using the Borg scale are assessed before and after the test. If the participant stops to rest during the test, the stopwatch is not paused. The test is terminated in the presence of chest pain, intolerable dyspnea, leg cramps, unsteadiness, diaphoresis, cyanosis or pallor, or if oxygen saturation falls below 85% (Savci et al., 2005).

9-Hole Peg Test (9HPT):

The 9HPT is used to assess upper extremity function and fine motor skills in clinical populations. The test requires a wooden board with nine holes of standardized diameter (0.71 cm), depth (1.3 cm), and spacing (3.2 cm), along with nine cylindrical pegs of standardized diameter (0.64 cm) and length (3.2 cm). During the test, the participant is timed while placing the pegs into the holes and subsequently removing them. Timing begins when the participant first touches a peg and ends when the last peg is released. The 9HPT is a valid and reliable test sensitive to clinical changes, and its validity and reliability have been established in individuals with MS (Feys et al., 2017).

Timed Up and Go Test (TUG):

TUG is widely used in clinical practice and has been shown to be a valid and reliable measure for assessing functional mobility and fall risk in individuals with MS. In a study conducted by Kalron et al., individuals with MS who experienced recurrent falls (at least one fall) had significantly longer TUG times compared with those without falls. Moreover, prolonged TUG time in recurrent fallers was identified as an excellent predictor of fall risk, supporting its use in fall risk assessment in this population (Kalron et al.,2017).

Sample Size and Power

The sample size calculation and power analysis of the study were conducted using the G*Power software (version 3.0.10; Universität Düsseldorf, Düsseldorf, Germany). For

the comparison of changes in the 6MWT, which was defined as the primary outcome measure of the study, an analysis of covariance (ANCOVA) was planned in order to control for the effect of baseline measurements. For a study design consisting of two groups and two measurement time points (pre-test and post-test), assuming an expected effect size of f = 0.70, the required sample size was calculated as at least 15 participants per group and 30 participants in total, with a Type I error rate (alpha) of 5% and a statistical power (1–beta) of 95%. Considering potential drop-outs that might occur during the study period, the sample size was increased by approximately 25%, and the study was initiated with a total of 38 participants, including 19 participants in each group. After completion of the study, a post-hoc power analysis was performed based on the primary outcome measure (6MWT values). For an analysis with a Type I error rate of 5%, a sample size of 30, and an effect size (f) of 2.16, the post-hoc statistical power (1–β) was found to exceed 99.99%.

Intervention

The aerobic exercise program was developed in accordance with the existing literature with the aim of improving functional capacity, reducing disease progression, and promoting favorable changes in NVU-related biomarkers in individuals with MS (Learmonth and Givon, 2017). The program designed for individuals with MS was scheduled for eight weeks, with two sessions per week. Participants who met the inclusion criteria, had SMMT scores ≥24, EDSS scores between 0 and 4.0, and provided written informed consent were randomly assigned to one of two groups: the control group (C) or the exercise group (E).

Following group allocation, baseline assessments—including the 6MWT, TUG, and 9HPT were conducted. After completion of baseline assessments, participants in the exercise group received an individualized aerobic exercise program tailored to their functional capacities and were supervised for eight weeks, with two sessions per week lasting 30–40 minutes each. In addition, a conventional exercise program tailored to individual needs was prescribed as a home exercise program. Participants in the control group were advised to perform only the conventional exercise program at home, according to their individual needs. At the end of the eighth week, both groups underwent

post-intervention assessments, including the 6MWT, TUG, and 9HPT, and blood samples were collected to complete the evaluations.

Aerobic Exercise Program

The aerobic exercise program was implemented under the supervision of an expert physiotherapist using a treadmill, with two sessions per week over a total period of eight weeks. Participants' functional capacities were determined by estimating peak oxygen consumption (VO_2peak) based on walking distance obtained from the 6MWT, heart rate, and blood pressure measurements (Ross et al., 2010). During the aerobic exercise sessions, heart rate and oxygen saturation were continuously monitored using a pulse oximeter, and exercise intensity was adjusted to maintain a perceived exertion level between 11 and 14 on the Borg Rating of Perceived Exertion Scale.

Following completion of each aerobic exercise session, participants were monitored for at least five minutes until heart rate, blood pressure, and Borg scale ratings returned to baseline values. Session duration was planned in accordance with the literature and consisted of three phases: warm-up, training, and cool-down, with a total duration ranging from 30 to 40 minutes. The warm-up phase lasted 5 minutes, followed by a moderate-intensity training phase lasting 20–30 minutes at 50–75% of VO_2peak, and a 5-minute cool-down phase. When necessary, the exercise duration was divided into two bouts based on participants' fatigue levels. Progression of exercise intensity (i.e., walking speed) was increased gradually by 0–5% per week, based on heart rate responses and perceived exertion (Halabchi et al., 2017).

Conventional Exercise Program

The conventional exercise program prescribed according to individual needs included exercises aimed at strengthening the lower extremities and trunk, balance and gait training exercises (progressing from firm to soft surfaces, from eyes open to eyes closed, and from wide to narrow base of support), gait training exercises (marching gait, walking with reduced base of support, and lateral walking), and coordination exercises (Frenkel coordination exercises performed in sitting or supine positions, depending on the participant's condition). Each individualized program consisted of approximately 8–12

exercises and was recommended to be performed at least two days per week, with each session not exceeding one hour in duration (Moeinzadeh et al., 2023). Participants were informed about the importance of adhering to the exercise program regularly.

NfL, GFAP, and VEGF-a Analyses

Blood samples required for biomarker analyses were collected from all participants after a minimum fasting period of 12 hours, between 09:00 and 11:00 a.m. The samples were transferred into gel-containing biochemical tubes and centrifuged at 4000 rpm for 5 minutes. After centrifugation, serum samples were aliquoted into 1.5 mL microtubes and stored at −80°C in the Department of Medical Biochemistry, Ondokuz Mayıs University Hospital, until the day of analysis.

On the day of analysis, serum samples were thawed under appropriate conditions and diluted at a ratio of 1:2 using the sample dilution buffer provided in the assay kits. Analyses were performed using commercially available enzyme-linked immunosorbent assay (ELISA) kits based on the sandwich method. Each sample was analyzed in duplicate, and the coefficient of variation (CV) between duplicates was calculated. Samples with a CV greater than 10% were reanalyzed. As all analyses were conducted in a single run, inter-assay variability could not be directly assessed; instead, intra-assay repeatability was evaluated. The following ELISA kits were used to measure protein levels of the study parameters: Human GFAP (FineTest, Cat. No. EH0410, China), Human VEGF-a (FineTest, Cat. No. EH0327, China), and Human NfL (FineTest, Cat. No. EH1205, China).

Ethical Considerations

Ethical approval for the implementation of this study was obtained from the Scientific Research Ethics Committee of Lokman Hekim University (Approval No: 2024313; dated 31 January 2025), and written permission was granted by Ondokuz Mayıs University Faculty of Medicine, where the study was conducted. Prior to participation, all individuals were verbally informed about the study procedures, and written informed consent was obtained from each participant.

Outcome Measurements

Outcome measures of the study were structured into two main categories primary and secondary outcomes in order to evaluate the effects of the intervention on clinical functional outcomes and NVU-related biomarkers. All measurements were performed before randomization (baseline) and at the end of the eight-week intervention period. Clinical assessments were conducted by physiotherapists experienced in neurological rehabilitation, and the assessors were blinded to the biochemical analysis results.

The primary outcome of the study was the assessment of clinical progression in individuals with MS. Clinical progression was evaluated by measuring functional mobility, walking capacity, and upper extremity function using the 6MWT, the 9HPT, and the TUG, respectively. In the exercise group, significant reductions were observed in dominant and non-dominant hand 9HPT times and TUG time, along with a significant increase in 6MWT distance ($p < 0.001$). No significant changes were observed in these parameters in the control group ($p > 0.05$). Between-group comparisons revealed that improvements in the exercise group were significantly greater than those observed in the control group ($p < 0.001$). These findings indicate that the eight-week aerobic exercise program had a positive effect on clinical progression markers in individuals with MS. Notably, the observed increase in 6MWT distance exceeded the previously reported minimal clinically important difference for individuals with multiple sclerosis.

The secondary outcomes consisted of biomarkers reflecting NVU function. Serum levels of NfL, VEGF-a, and GFAP were measured as NVU biomarkers. Measurements were performed using the ELISA method on fasting morning blood samples collected from all participants. Secondary outcome analyses demonstrated no significant within-group or between-group differences in serum NfL, VEGF-a, or GFAP levels ($p > 0.05$). These results suggest that the eight-week aerobic exercise program did not produce a significant effect on NVU-related biomarkers.

In addition, clinical and sociodemographic characteristics, including age, sex, MS type, disease duration, relapse history, and exercise habits were recorded using the demographic data form. These data confirmed the absence of significant baseline

differences between groups and allowed for control of potential confounding variables in the analyses.

Data Analyses

Statistical analyses were performed using IBM SPSS Statistics version 26.0 (SPSS Inc., Chicago, IL, USA). The normality of continuous variables was assessed using visual (histograms and probability plots) and analytical methods. Descriptive statistics were presented as frequencies and percentages for categorical variables, and as medians with percentiles or means with standard deviations for continuous variables, as appropriate.

The Chi-square test was used to compare independent categorical variables. For within-group (pre-treatment vs. post-treatment) comparisons of continuous variables in dependent samples, the Paired Samples t-test was applied for normally distributed data, while the Wilcoxon signed-rank test was used for non-normally distributed data. For between-group comparisons of continuous variables in independent samples, the Independent Samples t-test was used for normally distributed data, and the Mann–Whitney U test for non-normally distributed data (Barton and Peat, 2014; Field, 2024).

One-way analysis of covariance (ANCOVA) was used to compare the effectiveness of the intervention methods. The assumptions of ANCOVA—normal distribution of the dependent variable in both groups, homogeneity of variances between groups, normal distribution of residuals, a linear relationship between the covariate and the dependent variable, and homogeneity of regression slopes—were tested. When these assumptions were not met, Quade's ANCOVA was applied. For both analyses, post-treatment values were assigned as the dependent variable, the grouping variable as the independent variable, and pre-treatment values as the covariate.

Partial eta squared (η^2) values obtained from the analyses were used to determine effect sizes (Barton and Peat, 2014; Field, 2024). Effect sizes were classified as small ($0.01 < \eta^2 < 0.06$), medium ($0.06 < \eta^2 < 0.14$), and large ($\eta^2 > 0.14$) (Cohen, 2013). A p-value of less than 0.05 was considered statistically significant for all analyses.