Protocol Title:

Effects of Exercise on Glymphatic Functioning and Neurobehavioral Correlates in Parkinson's Disease

Protocol Date: June 4, 2024

NCT04140708

Effects of Exercise on Glymphatic Functioning and Neurobehavioral Correlates in Parkinson's Disease

Statistical Plan

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Sponsor: Vanderbilt University Medical Center

Funded by: Department of Defense & National Institute of Health

Version Number: v.5.0

4-JUNE-2024

1 STATISTICAL PLAN

1.1 AIM 1

Aim (1). To quantify the relationship between CNS lymphatic measures and beta-amyloid burden.

- Hypothesis (1A). Glymphatic flow and lymphatic collector velocity inversely correlate with beta amyloid measured from 18F-florbetapir PET in older adults with Parkinson's disease.
- Exploratory Hypothesis (1B). In Parkinson's subjects matched for age and sex, glymphatic flow and beta-amyloid burden will be worse in patients with versus without mild cognitive impairment (MCI).

A.1.1 Experiment. We will enroll participants with idiopathic Parkinson's disease (n=32; age range=55- 80 years), equally distributed and matched for age and sex between groups with (n=16) and without (n=16) mild cognitive impairment. These populations provide a cohort to evaluate the potential relationship between glymphatic flow and beta-amyloid burden, given the elevated risk of beta-amyloid accumulation associated with both conditions. These patients will form a cohort to be assessed before and after a community-based exercise intervention. Participants will be recruited from Vanderbilt University Medical Center Neurology's Behavioral and Cognitive and Movement Disorder Clinics by Drs. Claassen and Considine. Potential participants will undergo a neurological and neuropsychological examination to characterize their medical and neurobehavioral status. Patients will have medical and motor presentation indicated for the community-based exercise program. Those patients with clinically-defined MCI, will be without evidence of vascular cognitive impairment, primary mood disorder, Lewy body disease, or comorbid medical condition that could contribute to cognitive symptoms (e.g. pulmonary, hematologic, metabolic, or vitamin deficiency).

A.1.1.1. Clinical and neuropsychological examination. All potential participants will undergo baseline clinical and neuropsychological examination to determine eligibility, categorize MCI status, and provide quantification of neurocognitive functioning and neuropsychiatric symptomatology. The neuropsychological examination protocol will consist of memory measure (i.e., the Hopkins Verbal Learning Test – Revised [HVLT-R], consisting of Learning, Recall, and Recognition subcomponents). We will also administer three executive functioning measures (i.e., Trail Making Test – A & B57 , Verbal Fluency [FAS] , Stroop]), to facilitate exploratory analyses. To evaluate sleep disturbance symptomatology, PROMIS – Sleep Disturbance (SD) and Sleep-related Impairment (SRI) short forms will be administered.
A.1.1.2. PET. A single administration of approximately 370 MBq (10 mCi) Florbetapir 18F, then a 10- minute PET acquisition (2 × 5 minute frames) approximately 50 minutes later will be performed. Images will be reconstructed using an ordered subset estimation algorithm (4 iterations, 16 subsets, with a post-reconstruction Gaussian filter of 5 mm), and corrected for scatter and attenuation. Images will be co-registered to the high spatial resolution T1-weighted image as we have performed previously and a mean cortical:cerebellar SUVr will be calculated as the primary parameter of interest. For exploratory evaluation, we will also record SUVr in bi-temporal, bi-occipital, bi-frontal, and biparietal lobes.
A.1.1.3. MRI. The following imaging and angiography sequences will be completed. We will apply our previously reported 3T methods (16-channel neurovascular coil) with the below 50 min protocol:

Anatomical imaging. T1-weighted (MPRAGE; spatial resolution=1.0x1.0x1.0mm3 ; 3D turbo-gradient-echo; TR/TE=8.2/3.7ms), and T2-weighted (FLAIR; spatial resolution=0.9x1.1x3.0mm3 ; turbo-inversion-recovery; TR/TI/TE=11000/2800/120ms) imaging, and intracranial (spatial resolution = 0.5x0.8x1.4mm3 ; 3D gradient

echo; TR/TE=23/3.5ms) and extracranial (spatial resolution=0.9x0.9x3.0mm3 ; 2D gradient echo; TR/TE=18.6/3.2ms) time-of-flight (TOF) MR angiography (MRA).

Microvascular hemodynamic compliance. A single-shot gradient echo echo-planar-imaging (EPI) scan (TE=30 ms), multiband-factor=3, isotropic spatial resolution=2 mm, SENSE-factor=2.5, and TR=1s will be acquired with whole-brain coverage. The scan will utilize a hypercapnic normoxic (5% CO2 / 21% O2 / 74% N2) stimulus. The stimulus will be administered through a tight-fitting non-rebreathing oxygen facemask from compressed gas cylinders (flow rate=12 L/min) with the following paradigm: 180s normocapnic normoxia / 90s hypercapnia repeated once, followed by 180s normocapnic normoxia. Heart and respiration rate, EtCO2, and FiO2 will be recorded.

CSF flow. 2D-Qflow measurements will be performed in the cerebral aqueduct. Venc=12 cm/s, spatial resolution = 0.5x0.5x4 mm3 , field-of-view = 200x200x4 mm3 , and 30 frames per heart cycle. Scan durations vary with heart rate, but are generally 2-4 min. Reproducibility has been determined by CSF flow velocity. The CSF flow velocity over the cardiac cycle measured using the proposed sequence. Error bars extend to all data from six health subjects. The insert shows the location of the cerebral aqueduct (blue) and slice placement (orthogonal to flow). Velocity sign indicates direction. ICC=0.81. repeating the flow measurements in six subjects. Background correction will be performed with a method previously developed for quantification of blood flow velocity in small perforating arteries.

Lymphangiography for lymph vessel visualization. 3D turbospin-echo with variable refocusing angle sweep=40-110°, driven equilibrium module, dynamic DANTE flip angle sweep=0–8°, TE=600/TR=2500ms, slices=80, spatial resolution=1x1x1mm3. We have shown that this method provides sensitivity to long T2 slow flow species, such as lymph, and we have evaluated this method for sensitivity to lateralizing lymphedema and MLD therapy.

Susceptibility weighted imaging (SWI) for identification of medullary veins. Whole-brain 3D turbo-gradient-echo (TR/TE1/ΔTE=31/7.2/6.2 ms; flip angle=17°) with spatial resolution=0.6x0.6x2 mm3 (slices=130) will be acquired with the same slice angulation as DTI.

Diffusion tensor imaging (DTI) for perivascular flow determination. Whole-brain 2D spin echo EPI (TR/TE=10000/60 ms) in 32 directions (2 mm isotropic spatial resolution) using sequential acquisitions with b=0 s/mm2 , b=1000 s/mm2 , and b=2000 s/mm2 .

Spin labeling for cervical LN perfusion. Spin labeling will be performed with an 11-ms hypersecant inversion (labeling) prepulse, followed by an inversion time range of 300 to 2400 ms in 300 ms increments, which will enable blood transit time and perfusion quantification. Other parameters: labeling=STAR; TE=11 ms; spatial resolution=2x2x2 mm3 ; averages=14.

A.1.2. Analysis and statistical plan.

A.1.2.1 Clinical and neuropsychological examination. MCI will be defined based on established clinical criterion of cognitive performance below 1.5 standard deviations compared to age-based normative expectations in the memory domain and/or the executive functioning domain, with a Clinical Dementia Rating (CDR) of 0 or 0.5, indicating cognitive dysfunction has not contributed to functional dependence for activities of daily living61, 62. The memory domain by determining the mean memory score across performance on the HVLT-R (i.e., Learning Trials 1- 3, Delayed Recall, Delayed Recognition). Similarly, an executive functioning domain will be determined by the mean score across the TMT (part B), Stroop, and Letter Fluency. Clinical analyses will be overseen by Drs. Claassen and Considine.

A.1.2.2. Image analysis. Analysis will be overseen by the PI and radiology Co-Is Drs. McKnight and Donahue, who together have experience with all image types. Lymphatic spin labeling. Sequential images are acquired (A) without and (B) with magnetic labeling (adiabatic inversion of arterial water spins). Following labeling, blood water flows into LNs (C,D) and attenuates signal. The difference image is proportional to perfusion

(ml/100g/min), which can be quantified upon application of the flow modified Bloch equation (E: T2-weighted image of LNs; F: corresponding perfusion weighted signal). (G) The blood arrival (red) and lymph arrival (black) following labeling over six subjects and LNs (dimensions shown above). The blood signal peaks and decays first, with the magnitude indicating perfusion. The lymph signal peaks later, due to slower lymphatic flow; note that the lymphatic signal is detectable at such long delays due to the longer T1 of lymph (T1=3100 ms) relative to blood (T1=1650 ms).

Anatomical imaging. Gray and white matter volume will be recorded using FSL-FAST50. MRIs will be reviewed by radiology Co-I Dr. McKnight. Infarct presence will be recorded if there is an ischemic lesion≥3 mm in diameter visible on FLAIR using established criteria of hyperintense on FLAIR and hypointense on T1 approaching CSF signal. Dr. McKnight will also grade intracranial MRA for vasculopathy extent and abnormalities will be noted; the purpose is to ensure that all subjects have no radiological indicators of disease and no-to-mild (0-49%) stenosis.

Microvascular hemodynamic compliance. Data will be corrected for motion, baseline drift, and slice timing. Multiple regressors will be calculated for the data corresponding to the block hypercapnia paradigm but with different onset timing. This will allow for cross-correlation statistics, which describe the strength of the correlation between the regressor and the data, to be calculated on a voxel-wise basis for a series of regressors that are offset in time. The time shift for the regressor that provides the maximum correlation statistic will be chosen as the CVRDELAY as we have outlined previously, and the signal change normalized by the EtCO2 change at this point will be recorded as CVRMAX. Maps will be registered to a 2 mm atlas. The primary observables preserved for hypothesis testing will be the CVR metrics (CVRMAX and CVRDELAY) separately in gray matter parenchyma and CP.

CSF flow. Based on the standard deviation over time, voxels within the cerebral aqueduct showing significant flow above the noise floor will be recorded. Phase unwrapping will be performed. Net CSF flow will be determined by integrating the mean velocity over the heart cycle over all aqueduct voxels. The observable preserved for hypothesis testing will be the CSF flow velocity through the aqueduct.

Lymphangiography analysis. Lymph vessels will be visualized using a maximum intensity projection (MIP) of the 3D TSE data. The theory underlying flow-suppression in DANTE modules is that flowing spin magnetization is attenuated owing to a spoiling effect caused by flow along the applied gradient65. For slowly flowing spins, the spin magnetization is attenuated in a manner that depends on flow velocity. We will fit a model of signal decay as a function of flip angle, and we will record the mean lymphatic flow velocity in draining cervical vessels at the most distal level of the deep cervical LNs.

SWI analysis. Phase and magnitude data will be combined and a minimum intensity projection calculated. Images will be registered to DTI scans and used for region-of-interest selection.

DTI analysis. Data will be processed using FSL66, including motion- and eddy current-correction, and will be brain-extracted. Tensor eigenvalues and eigenvectors, as well as diffusivity (D) values will be calculated from the diffusion images, b-values, and b-vectors using dtifit. Radiology Co-I Dr. McKnight will draw regions-of-interest where the lateral projections of the medullary veins (from SWI) are orthogonal to the projection and association fibers, which occurs at approximately the location of the lateral ventricles. Within these regions the diffusivity in x, y, and z (Dxx, Dyy, Dzz) and DTIALPS score=mean(Dxx,proj,Dxx,assoc)/mean(Dyy,proj,Dzz,assoc) will be quantified67; the primary variable of interest will be bilateral DTI-ALPS score.

Spin labeling analysis. We will follow our previously reported procedures68 and utilize recentlyquantified 3T T1 and T2 LN values. Images will be corrected for motion, pair-wise subtracted, and the flow-modified Bloch equation will be applied to quantify perfusion in absolute units (ml/100g/min). Spin labeling images will be registered to the anatomical image, which will allow us to localize LNs. The primary outcome variable will be mean LN perfusion in cervical LNs.

A.1.3. Statistical concerns. Statistical analyses will be overseen by statistician Co-I Dr. Kang. Descriptive statistics, including means, standard deviations, and ranges for continuous parameters, as well as percent and frequency for categorical parameters, will be presented. Tests for normality and homoscedasticity will be made. To test Hypothesis (1A), Pearson or Spearman (depending on normality findings) will be applied to evaluate the relationship between glymphatic flow, measured as the DTIALPS score, and the cortical:cerebellar SUVr, measured from PET. As an exploratory analysis to understand whether the relationship is spatially dependent, correlation testing will be repeated using the regional SUVr measures and corrected p-values reported. Power calculations are based on preliminary data in six MCI subjects in which an inverse correlation (Spearman's alpha =-0.49) was found between SUVr and DTI-ALPS score. With a sample size of 32, we should have 82% power to detect a correlation of -0.49 with two-sided $p<0.05$. To test Exploratory Hypothesis (1B), we will apply a Student's t-test or Wilcoxon rank-sum test (depending on normality findings), to evaluate whether mean glymphatic flow, measured as the DTI-ALPS score, is different between age- and sex-matched participants with vs. without MCI. Motivation is based on our preliminary data, where we have observed a large effect size (Cohen's d=2.3) in DTI-ALPS score between age- and sex-matched subjects with vs. without MCI.

A.1.4. Impact. Results will for the first time evaluate how the proposed human CNS lymphatic system and levels of beta-amyloid burden are altered in Parkinson's disease. If confirmed, results will motivate the use of evaluating human CNS lymphatic dysfunction with proposed methods for understanding betaamyloid clearance disorders.

A.2. Aims (2). Quantify the glymphatic functioning and beta-amyloid burden response to 12 weeks of a community-based exercise course developed specifically for PD patients.

Exploratory Hypothesis (2A). Exercise treatment will result in improved glymphatic functioning.
Exploratory Hypothesis (2B). Exercise treatment will result in a reduction of cortical beta-amyloid uptake.
Exploratory Hypothesis (2C). The degree of improvements in glymphatic functioning and cortical beta-amyloid uptake in response to exercise therapy will be more significant in MCI versus non-MCI participants. D.2.1 Experiment. We will enroll the patients from Aim (1) (A.1.) all of whom are undergoing community-based exercise intervention to treat symptoms of Parkinson's.

A.2.1.1. Exercise intervention procedure. Following baseline assessment, all patients will participate in a community-based kick boxing group exercise class designed for those living with Parkinson's called "Rock Steady Boxing." Patients will complete 12 consecutive, 90-minute, once-weekly classes, then undergo follow-up assessment. Treatment compliance will be monitored by the collaborating exercise trainer. All participants, regardless of degree of treatment compliance will receive follow-up assessment.
A.2.1.2. Imaging procedure. PET and MRI methods as outlined in A.1.1.2. and A.1.1.3. will be performed.

A.2.2. Analysis and statistical plan. Our main hypotheses are that after 12 weeks of once-weekly exercise treatment for PD, glymphatic functioning (per DTI-ALPS) will improve and beta-amyloid burden (per PET) will decline. We expect that degree of treatment compliance standards will moderate these changes. We will also explore whether scalar degree of treatment compliance relates to degree of change in our assessment modalities. Statistical analysis will be overseen by statistics Co-I Dr. Kang and the same considerations outlined in Aims (1) will be applied. To test Exploratory Hypothesis (2A), a paired Student's t-test or Wilcoxon signed-rank test, depending on normality findings, will be applied to determine if glymphatic flow (DTI-ALPS score) is increased after, relative to before, exercise intervention. To test Exploratory Hypothesis (2B), an identical

procedure will be applied to evaluate differences in SUVr pre vs. post exercise intervention and corrected p-values will be reported. To gain additional information, a multiple linear regression will be performed using the change in glymphatic flow (DTI-ALPS score) as the dependent variable and degree of compliance and change in beta-amyloid burden as the independent variables. To test Exploratory Hypothesis (2C), we will perform these same comparisons separately for MCI and non-MCI subgroups. Aim 2 hypotheses are exploratory, given limited extant literature defining reliable effect sizes; however, one randomized study (n = 33) demonstrated a 30% divergence in mean plasma levels of a CSF beta-amyloid marker between exercise (-6%) and non-exercise participants (+24%) with MCI. Thus, with the proposed sample size of 32 (each with a measurement before and after intervention), we should have at least 80% power to detect a significant difference in mean DTI-ALPS score pre- vs. post-exercise with at least 80% power and two-sided Type I error=5%. We should also have sufficient power to include two independent variables in the regression model.
A.2.3. Impact. Results will provide evidence of how CNS lymphatic and beta-amyloid properties adjust in response to regular vigorous exercise in a PD sample.

A.3. Aim (3). Evaluate the degree to which neuroimaging-based response to exercise mediates clinical improvement in neurocognitive functioning and neuropsychiatric symptomatology.

Hypothesis 3.A. Neurocognitive functioning will improve following completion of the exercise intervention.
Exploratory Hypothesis (3.B.). Sleep disturbance symptoms will diminish following completion of the exercise intervention.
Exploratory Hypothesis (3.C.). The degree of change glymphatic function and cortical beta-amyloid uptake in response to exercise therapy will be correlated with the degree of improvements in neurocognitive functioning.
Exploratory Hypothesis (3.D.). The degree of change glymphatic function and cortical beta-amyloid uptake in response to exercise therapy will be correlated with the degree of improvements in sleep disturbance symptomatology.

A.3.1. Experiment. We will enroll the patients from Aim 2 all of whom completed the community-based exercise intervention and underwent pre/post neuroimaging protocols. ▪ Neuropsychological examination. Subjects will undergo follow-up neuropsychological examination to quantify neurocognitive functioning and neuropsychiatric symptomatology. The neuropsychological examination protocol will be the same outlined in A.1.1.1.
A.3.2. Analysis and statistical plan. Our main exploratory hypothesis is that the degree of improvement in glymphatic functioning (per DTI-ALPS) and decreased beta-amyloid burden (per PET) will significantly associate with neurobehavioral dysfunction improvements. We will also explore whether scalar degree of treatment compliance relates to degree of change in our assessment modalities. Statistical analysis will be overseen by statistics Co-I Dr. Kang and the same considerations outlined in Aims (1) will be applied. To test Hypothesis (3A) a paired Student's t-test or Wilcoxon signed-rank test, depending on normality findings, will be applied to determine if memory and executive functioning domain averages are improved after, relative to before, exercise intervention. We have powered this aim based on recent literature that has shown a medium improvement in executive functioning in between subject design (spatial working memory task, $\eta p2 = 0.17$; MoCA global cognitive screen, $\eta p2 = 0.133$; Treatment n = 24, Control n = 9) and large effect size within-subject design (TMT-B-A time, $\eta p2$'s = 0.34; n = 19)70 . To test Exploratory Hypothesis (3B), a paired Student's t-test or Wilcoxon signed-rank test, depending on normality findings, will be applied to determine if PROMIS – SRI and SD symptom scores are reduced is after, relative to before, exercise intervention. To test Exploratory Hypothesis (3C), two multivariate linear regressions will be employed, both will enter mean memory and

mean executive functioning domain scores as independent variables, with one regression using DTI-ALPS score and the other using cortical:cerebellar SUVr ratio as sole dependent variables. Exploratory Hypothesis (3D) analysis will be the same, with PROMIS – SRI and SD symptom scores substituted as independent variables. *A.3.3. Impact.* Results will provide evidence of how CNS lymphatic and beta-amyloid burden response to regular vigorous exercise in a PD sample relates to neurobehavioral functioning, as a clinical outcome metric.

B. Limitations and alternative strategies. BOLD CVR-weighted maps are derived from susceptibility induced changes around capillaries and veins, but periarterial spaces are also relevant. While the BOLD contrast directly originates around capillary and post-capillary vasculature, venules and veins are central to perivascular fluid clearance. Furthermore, the changes are dependent on compliance of the arteriolar microvasculature, as dHb in veins will not occur without arteriolar vasodilation. Thus, dysregulation of both vessel types will manifest in the observable, and we provide preliminary data on the relevance of these measures and their relationship to choroid plexus activity. Methods are too complex and will not be generalizable. A major focus of our work is to provide tools that are generalizable and can be implemented on different scanner platforms by non-specialists, and contrast evaluated using graphical software. We have also recently implemented our lymphangiography procedure on the clinical scanners at Vanderbilt Children's Hospital for evaluation of peri-operative cardiac surgeries (e.g., chylothorax). The diffusivity measure could be due to blood flow. At the chosen b=1000 s/mm2 , spins within fast flowing venous blood should be completely suppressed. Aim 1 focuses primarily on intracranial glymphatic flow; how will the other data be included? The imaging protocol includes multiple measurements of the CNS lymphatic system. To reduce concerns with multiple comparisons, Aim 1 has been powered based on our most promising preliminary data (DTI-ALPS score, obtained in 61 older subjects with and without aMCI). If Aim (1) findings suggest that other CNS lymphatic system measures are more promising, we will use these measures in Aims (2-3) as well to investigate their dependency on beta-amyloid burden.
Patient heterogeneity is a concern. Since other clinical factors can affect brain and cognitive functioning, we plan to control for age and screen for the most common medical conditions that might disproportionally affect outcome assessments. Furthermore, we have selected an age-cutoff that reduces clinical heterogeneity and has extant support for the presence of our conditions-of-interest. Patient recruitment. Vanderbilt University Medical Center's Neurology division has a large clinical volume of adult patients with Parkinson's. Thus, our recruitment target is felt to be reasonable. Patient attrition. We have accounted for a possible 10% attrition/exclusion in our protocol. We will use compliance as a scalar variable, allowing us to retain all patients at follow up, regardless of degree of compliance.

C. Sex as a biological variable. Given the relative infancy of the field of human CNS lymphatic imaging, to our knowledge there is no literature reporting sex differences for all proposed biomarkers of the lymphatic drainage system. Therefore, we have powered our study to specifically address this concern, and subjects will be recruited equally for both sexes and statistical measures are in place to evaluate how each observable varies with biological sex. This should be the first study to report how these properties of the lymphatic system differ with biological sex in humans.

D. Scientific rigor and reproducibility. The need to understand CNS small blood and lymphatic vessels is becoming increasingly recognized, with more than 150 manuscripts published in the last five years on this topic. However, nearly all studies utilize animal models only, and there is much less information available on how this system operates in humans in health or in the presence of clearance disorders such as Alzheimer's disease. One reason for this is that reliable methods for evaluating lymphatics in vivo are underdeveloped. Methods proposed here (e.g., CVR compliance, lymphangiography, glymphatic flow, and CSF flow velocity)

have already been evaluated by us for reproducibility, and lymphatic methods have been optimized in prior work in patients with lymphatic insufficiency of known etiology (e.g., A.1.3.). Finally, each aim includes a statistical analysis section in which power calculations are provided based on our preliminary data and available extant literature. The statistical analysis of all aims will be overseen by biostatistician collaborator Dr. Kang to ensure responsible analysis and interpretation of the results.