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Neurovascular Dysregulation and Acute Exercise Intolerance in ME/CFS: A Randomized, Placebo-Controlled Trial of Pyridostigmine

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Abbreviations List

Ca-vO2: arterial-venous oxygen content difference

iCPET: Invasive cardiopulmonary exercise test

MAP: Mean arterial pressure

mPAP: Mean pulmonary artery pressure

ME/CFS: Myalgic encephalomyelitis/chronic fatigue syndrome

PASC: Post-acute sequelae of SARS-CoV-2 infection

PAWP: Pulmonary arterial wedge pressure

POTS: Postural orthostatic tachycardia syndrome

Qc: Cardiac output

RAP: Right atrial pressure

SE: Standard error

SFN: Small fiber neuropathy

V_E/VCO2: Ventilatory efficiency

VO2: Oxygen uptake

ABSTRACT

Background

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is characterized by intractable fatigue, post-exertional malaise, and orthostatic intolerance, but its pathophysiology is poorly understood. Pharmacologic cholinergic stimulation was used to test the hypothesis that neurovascular dysregulation underlies exercise intolerance in ME/CFS.

Research Question

Does neurovascular dysregulation contribute to exercise intolerance in ME/CFS and can its treatment improve exercise capacity?

Methods

Forty-five subjects with ME/CFS were enrolled in a single-center, randomized, double-blind, placebo-controlled trial. Subjects were assigned in a 1:1 ratio to receive a 60 mg dose of oral pyridostigmine or placebo after an invasive cardiopulmonary exercise test (iCPET). A second iCPET was performed 50 minutes later. The primary end point was the difference in peak exercise oxygen uptake (VO2). Secondary end points included exercise pulmonary and systemic hemodynamics and gas exchange.

Results

Twenty-three subjects were assigned to pyridostigmine and 22 to placebo. The peak VO2 increased after pyridostigmine but decreased after placebo (13.3 ± 13.4 mL/min vs. -40.2 ± 21.3 mL/min, P<0.05). The treatment effect of pyridostigmine was 53.6 mL/min (95% CI, -105.2 to -2.0). Peak versus rest VO2 (25.9 ± 15.3 mL/min vs. -60.8 ± 25.6 mL/min, P<0.01), cardiac output

(-0.2 ± 0.6 L/min vs. -1.9 ± 0.6 L/min, P<0.05), and RAP (1.0 ± 0.5 mm Hg vs. -0.6 ± 0.5 mm Hg, P<0.05) were greater in the pyridostigmine group compared to placebo.

Interpretation

Pyridostigmine improves peak VO2 in ME/CFS by increasing cardiac output and right ventricular filling pressures. Worsening peak exercise VO2, Qc, and RAP after placebo may signal the onset of post-exertional malaise. We suggest treatable neurovascular dysregulation underlies acute exercise intolerance in ME/CFS.

Trial Registration number: NCT03674541

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a common, debilitating disorder that has a global impact of millions. Approximately 2.5 million Americans are diagnosed with ME/CFS, with a resulting loss in productivity amounting to $20,000 per patient, or $9.1 billion overall.[1] The National Academy of Medicine (formerly the Institute of Medicine) requires three major criteria for diagnosis: substantial impairment from fatigue for >6 months, post-exertional malaise, and unrefreshing sleep, plus either cognitive impairment or orthostatic intolerance.[2]

The pathophysiology underlying ME/CFS remains poorly understood. Proposed mechanisms include infectious,[3] inflammatory,[4] autoimmune,[5] neuroendocrine,[6] and genetic and environmental causes.[7] Due to considerable overlap among ME/CFS, postural orthostatic tachycardia syndrome (POTS) and fibromyalgia, small fiber neuropathy (SFN) has been implicated as a cause of these syndromes.[8-10] We recently showed that SFN was present in 31% of ME/CFS patients undergoing invasive cardiopulmonary exercise tests (iCPET),[11] similar to the reported 38% in POTS[12] and 50% observed in fibromyalgia.[13]

Immunohistochemical studies show small fibers regulate microvascular tone through sympathetic and parasympathetic cholinergic synapses on perivascular myocytes.[14] It was therefore hypothesized that SFN contributed to hemodynamic phenotypes of low cardiac preload[15] from impaired venous return and impaired peripheral oxygen extraction, analogous to neurovascular dysregulation observed in POTS[16] and fibromyalgia.[17] We were unable to link neurite density to vascular dysregulation during incremental exercise.[11] The skin biopsies were

epidermal and not designed to detect small fibers innervating sweat glands, and therefore, may not have captured the full spectrum of dysautonomia due to SFN.

An alternative interpretation is the presence of co-existing ganglionopathy, sometimes associated with anti-neuronal acetylcholine receptor antibodies and identical vascular dysregulation.[18] Pyridostigmine, an acetylcholinesterase inhibitor, is thought to enhance cholinergic stimulation of norepinephrine release at the post-ganglionic synapse and has shown significant improvement in both symptom burden and heart rate response in POTS.[19] The objective of this trial was to use pyridostigmine to determine if neurovascular dysregulation underlies exertional intolerance in ME/CFS.

METHODS

Trial Design and Oversight

This was a single-center, randomized, double-blind, placebo-controlled trial. Subjects were assigned in a 1:1 ratio to receive a 60 mg dose of oral pyridostigmine or placebo after an iCPET. A second iCPET was performed 50 minutes later. Hemodynamics were recorded throughout the epoch of exercise during both tests.

The trial was supported by the Open Medicine Foundation Eliassen Fund. The authors were responsible for the trial design, data collection, analysis, and writing of the manuscript. A full list of trial personnel is provided in Section S1 in the Supplementary Appendix. The study was approved by the Partners Human Research Committee (IRB 2018P001871). The study was registered at clinicaltrials.gov (NCT03674541).

Trial Population

The trial population consisted of patients 18 years of age or older with ME/CFS. All fulfilled the National Academy of Medicine requirement of three major criteria (i.e. chronic fatigue for > 6 months, post-exertional malaise, unrefreshing sleep) plus one minor criteria (i.e. either cognitive impairment or orthostatic intolerance).[2] They were required to have a peak right atrial pressure (RAP) ≤ 6.5 mmHg during their first, maximal iCPET,[15] along with the exclusion of resting or exercise pulmonary arterial or venous hypertension during their resting right heart catheterization and first iCPET.[20] Patients with conditions predictive of exercise limitation, such as anemia, BMI > 30 kg/m^2, and active cardiopulmonary disease, were excluded. A full list of inclusion and exclusion criteria are listed in Section S2.

Trial Procedures

Protocols for iCPET, hemodynamic measurements, and gas exchange measurements were described previously.[21,22] Briefly, the pulmonary and radial arteries were catheterized with ultrasound and fluoroscopic guidance, then a standard right heart catheterization was performed with oxygen saturation measurements to assess for intracardiac shunting.[23] Patients were transported to the cardiopulmonary exercise laboratory for maximum, incremental, upright exercise on a cycle ergometer as ventilation and pulmonary gas exchange were continuously measured (MGC Diagnostics, St. Paul, Minnesota). Hemodynamics, including RAP, mean pulmonary artery pressure (mPAP), and mean arterial pressure (MAP) were continuously recorded (Koninklijke Philips N.V., Amsterdam, Netherlands) and averaged throughout the respiratory cycle.[24] Pulmonary arterial wedge pressure (PAWP), and arterial and mixed-venous blood gases and pH were measured every minute and Qc calculated using the direct Fick principle. RAP and PAWP were measured as the mean of the "a" wave.

After confirmation of RAP ≤ 6.5 mmHg, a maximal exercise effort (respiratory exchange ratio > 1.05 and/or heart rate > 85% predicted), and exclusion of exercise pulmonary or venous hypertension,[20] subjects were administered 60 mg of oral pyridostigmine or placebo in a 1:1 ratio. A second iCPET was performed after a combined dosing and rest period of 50 minutes. The full two iCPET protocol is described in Section S3.

Modified Borg dyspnea and fatigue scales were administered immediately following both iCPETs. Subjects were asked to rate their dyspnea and fatigue from 0 ("nothing at all") to 10 ("maximal") during peak exercise.

Outcome Measures

The primary end point of the trial was the between group difference in peak exercise oxygen uptake (VO2) after pyridostigmine or placebo administration. Secondary end points included between group differences in peak versus rest for VO2, Qc, RAP, PAWP, ventilatory efficiency (V_E/VCO2), peak arterial-venous oxygen content difference (Ca-vO2), and modified Borg dyspnea and fatigue scales.

Statistical Analysis

After demonstrating a normal distribution of the data using the Kolmogorov-Smirnov test, a Welch's T test was used to compare exercise physiologic variables between the groups. Two-sided P values, standard errors (SE), and 95% confidence intervals were reported. For secondary end points, the P values and confidence intervals were not adjusted for multiplicity and cannot be used to infer definitive treatment effects for these secondary endpoints. Fisher's exact test was used to compare the baseline characteristics between the two groups. With 80% power to detect a 10% difference in oxygen uptake at peak exercise, we estimated the need to enroll 50 patients.[25] This number also accounted for screening failures. The analysis was performed using R Statistical Software (v4.1.0).

RESULTS

Patients

Of 362 subjects pre-screened for eligibility, 50 were enrolled to undergo iCPET. Five subjects were excluded after the initial iCPET, with the rest randomly assigned pyridostigmine (23 patients) or placebo (22 patients). Male subjects were removed from analysis due to a randomization error, yielding a 39-subject study sample (Figure 1). Baseline characteristics were similar in the two groups (Table 1). The mean age was 40 years. Few used diuretics or vasoactive drugs. Significant associated conditions included POTS, fibromyalgia, mast cell activation syndrome, and preceding infection. 38% of the study population had objective evidence of SFN. Thirty patients had a paraneoplastic antibody evaluation performed at the Mayo Clinic Clinical Laboratory. Striational antibody was detected in one placebo patient's panel and neuronal voltage-gated potassium channel antibody was detected in one treatment patient's panel. Acetylcholine receptor ganglionic neuronal antibodies were not detected in any patient panel.

Primary End Point

Peak VO2 increased after pyridostigmine but decreased after placebo (13.3 ± 13.4 vs. -40.3 ± 21.3, $P<0.05$). The treatment effect of pyridostigmine was 53.6 mL/min (95% CI, -105.2 to -2.0) (Table 2 and Figure 2).

Secondary End Points

Peak versus rest VO2 (25.9 ± 15.3 mL/min vs. -60.8 ± 25.6 mL/min, $P<0.01$), Qc (-0.2 ± 0.6 L/min vs. -1.9 ± 0.6 L/min, $P<0.05$), and RAP (1.0 ± 0.5 mm Hg vs. -0.6 ± 0.5 mm Hg, $P<0.05$) were

greater in the pyridostigmine group compared to placebo. There were no significant changes in PAWP, V_E/VCO2, and Ca-vO2 (Table 2 and 3).

DISCUSSION

ME/CFS is a common and often disabling disorder of unknown pathogenesis reported to affect 10-25% of patients in primary care practices,[26] 75-267/100,000 persons,[27] or 836,000-2.5 million people in the United States.[2] Nonspecific symptoms spanning multiple organ systems cause frequent evaluations by varied medical specialties, leading to combined direct and indirect US costs approaching $23 billion per year.[28] Emerging data suggest similar exercise pathophysiology[29] and an increased prevalence of ME/CFS[30] in patients with post-acute sequelae of SARS-CoV-2 infection (PASC). Thus, insights into pathogenesis and treatment of ME/CFS are needed.

There are no approved treatments for ME/CFS,[31] with prior studies having looked at cognitive behavioral therapy, graded exercise,[32] intravenous immunoglobulin,[33] and B-cell depletion.[34] While pyridostigmine has shown improvement in symptom burden and heart rate response in POTS,[19] its effect on ME/CFS patients are limited to case reports.[35] This is the first blinded, randomized, placebo-controlled trial to evaluate pyridostigmine's effects on acute exercise hemodynamics in ME/CFS.

Pyridostigmine Improves Exercise Hemodynamics

There was an increase in peak VO2 after pyridostigmine due to an increase in Qc and in turn, was related to improved RAP. Hence, our results suggest acute treatment with pyridostigmine improves aerobic capacity by an increase in cardiac output from augmented preload. This is consistent with studies showing deficient preload as a cause of exertional intolerance in ME/CFS.[11,15]

These data suggest neurovascular dysregulation underlies preload failure in ME/CFS. Decreased sympathetic outflow has been demonstrated during orthostatic challenge and isometric exercise in ME/CFS.[36-38] In the similar syndrome of POTS, abnormal lower extremity venous pooling occurs upon standing.[39] Infused norepinephrine and phenylephrine resulting in excess peripheral venoconstriction is consistent with adrenergic receptor upregulation from denervation,[40,41] and is further supported by low norepinephrine release after sympathetic nervous system stimulation in POTS patients.[42]

Pyridostigmine is a reversible acetylcholinesterase inhibitor which acts by increasing levels of acetylcholine at the pre-ganglionic sympathetic synapse, resulting in a downstream increase in norepinephrine at post-ganglionic receptors. Norepinephrine release leads to venoconstriction and improved vascular tone, with subsequent augmentation of cardiac preload, Qc, and aerobic capacity (Figure 3). In the related syndrome of POTS, a single 30 mg dose of pyridostigmine was previously shown to mitigate the heart rate increase during upright tilt.[19] Priming exercise has been shown to reduce intracardiac filling pressures in patients with heart failure with preserved ejection fraction, potentially explained by the shift of venous blood volume from stressed to unstressed compartments.[43] This may contribute to the decrease in peak VO2, Qc, and preload in the placebo group, reinforcing the steep portion of the Starling curve that these patients lie on.

Prior work suggests impaired systemic oxygen extraction from microcirculatory or mitochondrial dysfunction may contribute to decreased aerobic capacity in ME/CFS.[11,44] While there was a borderline reduction in systemic oxygen extraction in both treatment and placebo groups, this did not improve with acute pyridostigmine administration.

New Insights into Post-exertional Malaise

Post-exertional malaise, a hallmark symptom of ME/CFS, is described as "flu-like" debilitating fatigue that typically involves loss of physical stamina, cognitive impairment, impaired sleep, myalgias, arthralgias, and headaches.[45] Keller's et. al., noninvasive, two-day CPET protocol documented a significant decrease of peak VO_2 on day 2, hypothesized to be related to post-exertional malaise.[46] This study adds to the Keller et. al. data with invasive hemodynamics demonstrating the subsequent decrease in peak VO2 is driven by a decrease in Qc and cardiac preload. We hypothesize post-exertional malaise is related, in part, to neurovascular dysregulation precipitated by prior exercise.

In the placebo group, resting VO2 and Qc were increased prior to the second iCPET. Prior research suggests that immune-inflammatory mechanisms may play a role in the pathogenesis of ME/CFS by activating immune-inflammatory oxidative and nitrosative stress pathways.[47] Proinflammatory cytokines may be correlated with fatigue duration and disease severity, and cytokine profiling following submaximal exercise may help differentiate patients with ME/CFS from sedentary controls.[47,48] Additionally, increased resting metabolism from increased sympathetic outflow in ME/CFS is supported by elevated plasma catecholamine levels and

increased sympathetic nerve activity to the heart, skeletal muscle arterioles, and adrenals during rest. Moreover, adrenergic receptor upregulation may be contributing to sustained, resting sympathetic activity.[40,41] We speculate that an exaggerated immune-inflammatory and sympathetic response followed the first exercise test, resulting in elevated rest VO2 and Qc. Further studies are required to investigate the metabolomic, proteomic, and inflammatory cytokine signatures for ME/CFS and provide insight into post-exertional malaise.

Ventilatory Response

Dysfunctional breathing patterns may contribute to exercise limitations and impaired systemic oxygen extraction. Both groups demonstrated ventilatory inefficiency by elevated VE/VCO2 values. Hyperventilation or increased physiologic dead space ventilation cause increases in VE/VCO2, the latter not observed in this study. Thus, hyperventilation is the likely cause of dysfunctional breathing in this population, similarly observed in patients with PASC, some of whom have clinical overlap with ME/CFS.[29,30] Resulting alkalemia contributes to impaired systemic oxygen extraction by limiting the Bohr effect and causing a leftward shift of the oxygen-hemoglobin dissociation curve.[49] Ventilatory inefficiency may partly explain the lack of improvement in Borg fatigue and dyspnea scores, though underlying mechanisms remain unknown. Pyridostigmine did not influence ventilatory inefficiency. This may be due to a type II error. Another possibility is that increased cholinergic synaptic transmission in the sympathetic ganglion plays no role in the ventilatory response to acute exercise in ME/CFS.

Limitations

The physiologic changes we describe are small and are not clinically relevant, but within groups are concordant and between groups are statistically significant. The small changes in VO2, approximately 4% of peak values, may be a result of the use of a single dose of pyridostigmine studied acutely. It is possible that if we used higher doses of pyridostigmine for a longer period, there may have been greater physiologic changes. We justified this experimental protocol based on a prior tilt table study of POTS using a very similar dosing regimen and a desire to use existing pulmonary and radial artery catheters.[19] Future studies should assess the chronic effects of pyridostigmine in subsets of ME/CFS patients, varying in age, BMI, and pyridostigmine dose and duration needed to achieve clinically significant results.

Our prior work suggests two phenotypes of neurovascular dysregulation in ME/CFS– depressed Qc from impaired venous return and impaired peripheral oxygen extraction.[11] While the latter was observed in this study, acute administration of pyridostigmine did not influence peak (Ca-vO2) values. We have found improved indices of aerobic capacity using long-term, increased dose of pyridostigmine in two published abstracts.[50,51] Future studies should investigate long-term use and higher doses of pyridostigmine and its effect on peripheral oxygen extraction through arteriolar regulation in the muscle bed.

The study population had a 38% prevalence of SFN, similar to the prevalence reported in POTS and fibromyalgia.[12,13] There was asymmetry in the distribution in length-dependent SFN between groups, but this did not reach significance. Lower leg biopsies designed to detect distal length-dependent SFN are less sensitive for patchy, proximal, or non-length-dependent SFN and

is not expected to detect ganglionopathy,[52] the target of pyridostigmine.[19] The current study suggests that ganglionopathy and sympathetic outflow to systemic blood vessels is relevant to exertional intolerance in ME/CFS. It is also possible that enhancing sympathetic outflow from the ganglion with pyridostigmine overcomes the vasodilatory effects of SFN. Both pathways suggest that neurovascular dysregulation undermines exercise tolerance in ME/CFS.

Despite an improvement in peak VO2 and exercise hemodynamics, Borg fatigue scale worsened slightly after pyridostigmine. We speculate that this may be due to more work achieved during the second iCPET.

This study consisted of 50 participants, 5 of whom were excluded (Figure 1). Of the 45 remaining subjects, 39 were female and 6 were male. Although this gender distribution is reflective of the gender differences seen in ME/CFS,[53] our small sample size produced a randomization error where all 6 male participants were placed in the placebo group. We elected not to include males in the primary analysis as their response to exercise was discordant compared to females who received placebo. Peak VO2, Qc, and RAP increased with serial iCPET. Based on Qc/VO2 slopes and preserved systemic oxygen extraction, these subjects were characterized as the “low-flow” phenotype (e-Table 1). In addition, reducing the sample size increases the likelihood of type II errors. Studies with larger population sizes are required to ensure appropriate randomization and to assess the efficacy of pyridostigmine and differential exercise responses to both medication and placebo in males.

INTERPRETATION

Neurovascular dysregulation underlies acute exertional intolerance in ME/CFS. Pyridostigmine improves aerobic capacity in ME/CFS by increasing cardiac output through augmented right ventricular preload. A decrease in peak exercise VO2, Qc, and RAP after placebo suggests a physiologic mechanism underlying post-exertional malaise. A similar approach utilizing iCPET and pharmacologic intervention may prove useful in the study and treatment of PASC.[29,30]

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Author contributions: PJ and DMS take responsibility for the concept and design of this study. RP, SM, AW, MCS, JS, ABW, and DMS take responsibility for the implementation of this study. CJC and WX contributed to statistical analysis of the data. PJ, AW, MCS, JS, WX, ABW, and DMS contributed to the writing and revision of the manuscript.

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Take Home Points

Study Question: Does neurovascular dysregulation contribute to exercise intolerance in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) and can cholinergic stimulation with pyridostigmine improve exercise capacity?

Results: Comparing serial invasive cardiopulmonary exercise tests (iCPET), peak oxygen uptake (VO2) and associated changes in cardiac output (Qc) and right atrial pressure (RAP) were greater in the pyridostigmine group compared to placebo, driven by both improvement in the pyridostigmine group and worsening in the placebo group.

Interpretation: Pyridostigmine improves aerobic capacity by increasing Qc and right ventricular filling pressures, while worsening VO2 and hemodynamics after placebo may signal the onset of post-exertional malaise.

Table 1. Baseline Characteristics

Characteristic	All (N=39)	Pyridostigmine (N=23)	Placebo (N=16)
Age (year)	40 ± 14	40 ± 16	40 ± 11
Female (%)	39 (100%)	23 (100%)	16 (100%)
White Race (%)	33 (85%)	21 (91%)	12 (75%)
BMI ($kg*m^{-2}$)	23.5 ± 3.4	23.8 ± 2.7	23.0 ± 4.1
Hb (g/dL)	14.0 ± 1.2	13.9 ± 1.3	14.0 ± 1.1
Comorbidities (%)			
Hypertension	4 (10%)	3 (13%)	1 (6%)
Dyslipidemia	2 (5%)	1 (4%)	1 (6%)
Obesity	0	0	0
CV Family History	24 (62%)	13 (57%)	11 (69%)
Diabetes Mellitus	0	0	0
Previous Myocardial Infarction	0	0	0
Coronary Artery Disease	0	0	0
Medications (%)			
Characteristic	**All (N=39)**	**Pyridostigmine (N=23)**	**Placebo (N=16)**
Statins	2 (5%)	2 (9%)	0
Beta Blockers	3 (8%)	2 (9%)	1 (6%)
ASA	2 (5%)	2 (9%)	0
Calcium Channel Blockers	1 (2%)	1 (4%)	0
Diuretics	1 (2%)	1 (4%)	0
ACE Inhibitors	0	0	0

Associated Conditions (%)			
Characteristic	**All (N=39)**	**Pyridostigmine (N=23)**	**Placebo (N=16)**
Objective Evidence of SFN by Morphological and/or Functional Testing	14/37 (38%)	11/22 (50%)	3/15 (20%)
Epidermal Skin Biopsy Evidence of SFN (Neurite Density ≤ 5th Percentile)	7/36 (19%)	5/21 (24%)	2/15 (13%)
Sweat Gland Skin Biopsy Evidence of SFN	5/12 (41%)	5/8 (63%)	0/4 (0%)
Functional Testing (QSART and/or ESC) Evidence of SFN	2/16 (13%)	1/10 (10%)	1/6 (17%)
POTS	18 (46%)	13 (57%)	5 (31%)
Fibromyalgia	11 (28%)	6 (26%)	5 (33%)
MCAS	7 (18%)	4 (17%)	3 (19%)
Preceding Infection	20 (51%)	12 (52%)	8 (50%)
Positive ANA	10 (26%)	7 (30%)	3 (19%)

BMI: body mass index; Hb: hemoglobin; CV: cardiovascular; ASA: acetylsalicylic acid, ACE: angiotensin converting enzyme; SFN: small fiber neuropathy; QSART: quantitative sudomotor axon reflex test; ESC: electrochemical skin conductance; POTS: postural orthostatic tachycardia syndrome; MCAS: mast cell activation syndrome; ANA: Antinuclear Antibody

Table 2: Primary and Secondary Outcomes, changes between first and second iCPET

End Point	Pyridostigmine (N = 23)	Placebo (N = 16)	Treatment Effect (95% CI)	P Value
Primary End Point				
Peak VO_2 (mL/min)	13.3 ± 13.4	-40.3 ± 21.3	53.6 (-105.2 to -2.0)	**0.043**
Peak VO2 (mL/kg/min)	0.2±0.2	-0.8±0.4	1.0 (-1.9 to -0.7)	**0.035**
Secondary End Points				
Peak - Rest VO_2 (mL/min)	25.9 ± 15.3	-60.8 ± 25.6	86.7 (-148.1 to -25.2)	**0.008**
Peak Qc (L/min)	0.2 ± 0.2	-0.2 ± 0.3		0.263
Peak - rest Qc (L/min)	-0.2 ± 0.6	-1.9 ± 0.6	1.7 (-3.4 to -0.1)	**0.039**
Peak RAP (mm Hg)	1.2 ± 0.3	0.1 ± 0.5		0.068
Peak - rest RAP (mm Hg)	1.0 ± 0.5	-0.6 ± 0.5	1.5 (-3.0 to -0.04)	**0.045**

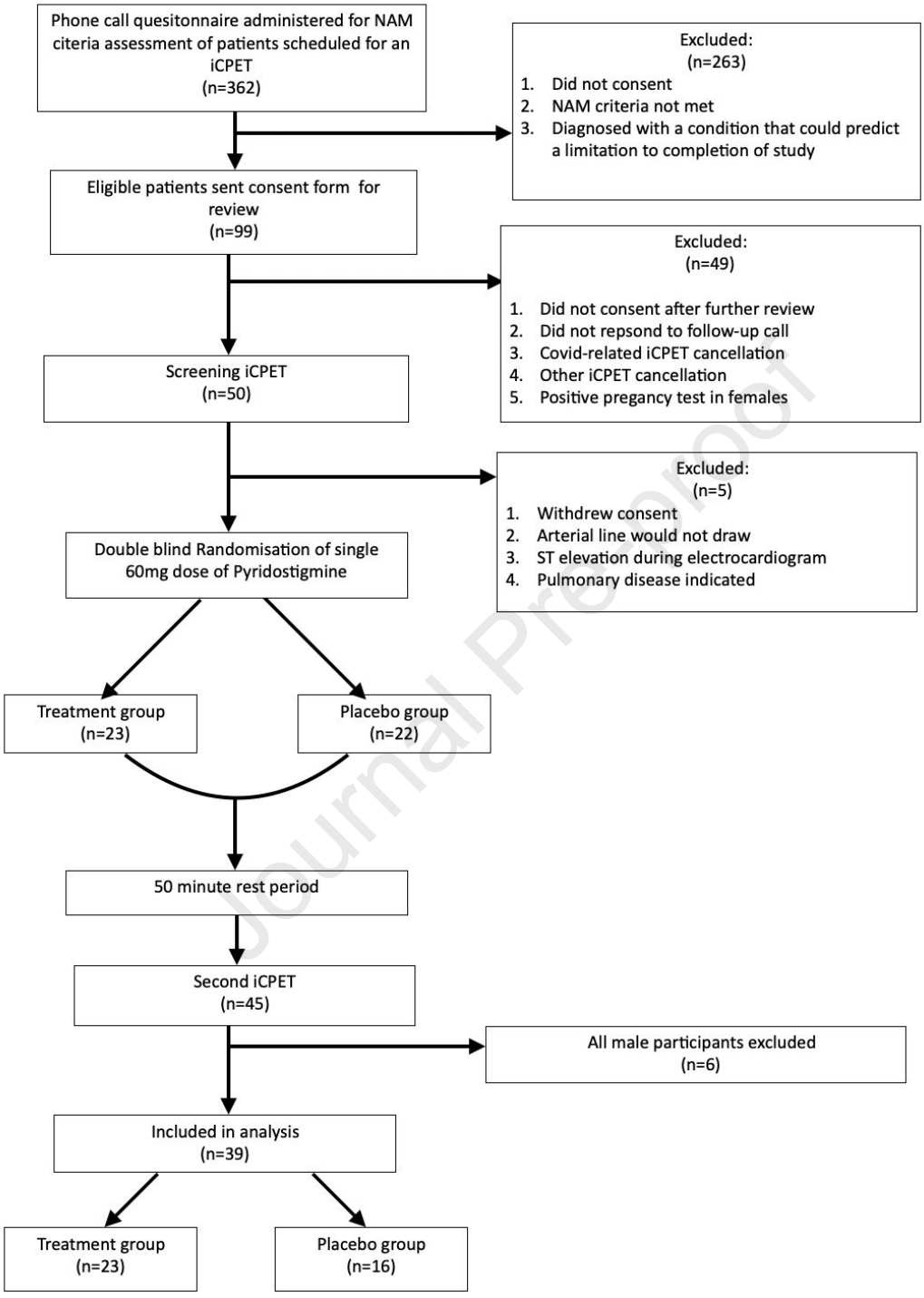
Peak PAWP (mm Hg)	1.0 ± 0.8	1.0 ± 0.5		1.000
Peak Stroke Volume (mL)	3.0 ± 1.4	-1.1 ± 1.9		0.093
Peak (Ca-vO2)/[Hb]	0.0 ± 0.0	0.0 ± 0.0		0.427
VE/VCO_2	-0.2 ± 0.8	1.0 ± 0.6		0.262
Borg fatigue scale	0.1 ± 0.2	-0.6 ± 0.3	0.8 (-1.5 to -0.1)	**0.038**
Borg dyspnea scale	-0.1 ± 0.2	-1.0 ± 0.5		0.147

Data are represented as mean ± SD. VO_2 = oxygen consumption; Qc= cardiac output; RAP= right atrial pressure; PAWP = pulmonary artery wedge pressure; Ca-vO_2 = arterial venous oxygen content difference; Hb = hemoglobin; VE/VCO2 = ventilatory efficiency.

		Pyridostigmine (N=23)		Placebo (N=16)	
	Test	Rest	Peak	Rest	Peak
Primary End Point					
VO_2 (mL/min)	1	290.2(62.8)	1221.5(396.3)	269.7(54.0)	1304.3(301.2)
	2	277.6(43.7)	1234.8(404.1)	290.2(68.6)	1264.1(309.4)
VO_2 (mL/kg/min)	1	4.7(1.1)	19.7(6.7)	4.3(0.6)	21.2(5.7)
	2	4.5(0.9)	19.9(6.8)	4.6(0.8)	20.5(5.4)
Secondary End Points					
Qc (L/min)	1	6.1(1.6)	11.0(2.4)	5.0(1.1)	11.5(2.1)
	2	6.4(2.2)	11.1(2.5)	6.6(2.0)	11.4(1.8)
RAP (mmHg)	1	-0.4(1.6)	-0.1(2.1)	-1.6(2.3)	0.4(2.1)
	2	-0.2(1.4)	1.1(2.4)	-0.9(1.8)	0.5(2.9)
PAWP (mmHg)	1	1.3(2.0)	5.1(3.7)	1.1(1.3)	4.4(2.9)
	2	2.0(1.8)	6.1(4.5)	1.6(1.8)	5.4(2.5)
SV (mL)	1	73.2(14.6)	68.8(10.5)	63.1(15.8)	72.7(11.4)
	2	77.2(27.7)	72.0(11.1)	77.5(26.4)	73.8(12.5)
$(Ca\text{-}vO_2)/[Hb]$	1		0.8(0.1)		0.8(0.1)
	2		0.8(0.1)		0.8(0.1)
HR (bpm)	1	84.0(15.0)	159.2(24.9)	80.7(9.9)	156.9(14.5)
	2	85.3(14.4)	154.1(25.6)	86.6(11.1)	155.7(14.1)
VE/VCO_2	1	34.1(7.7)		28.3(3.6)	
	2	33.9(8.2)		29.3(4.8)	
Additional Measures					
VO_2 at AT (mL/min)	1	647.6(180.0)		737.2(184.3)	
	2	699.0(202.3)		776.1(165.4)	
O_2 Pulse % Predicted	1	82.6(20.1)		91.7(22.6)	
	2	85.9(20.0)		88.2(21.9)	
$\Delta Qc/\Delta VO_2$	1	5.3(2.0)		6.0(2.2)	
	2	4.2(2.8)		5.2(2.5)	
Peak VO_2 % Predicted	1	74.1(24.9)		79.8(22.2)	
	2	74.7(25.2)		77.2(22.4)	
VO_2 at AT % Predicted	1	39.5(13.1)		45.3(14.5)	
	2	42.8(15.5)		47.6(13.5)	
VD/VT	1	0.3(0.1)	0.2(0.1)	0.3(0.0)	0.2(0.1)
	2	0.3(0.1)	0.2(0.1)	0.3(0.1)	0.2(0.1)

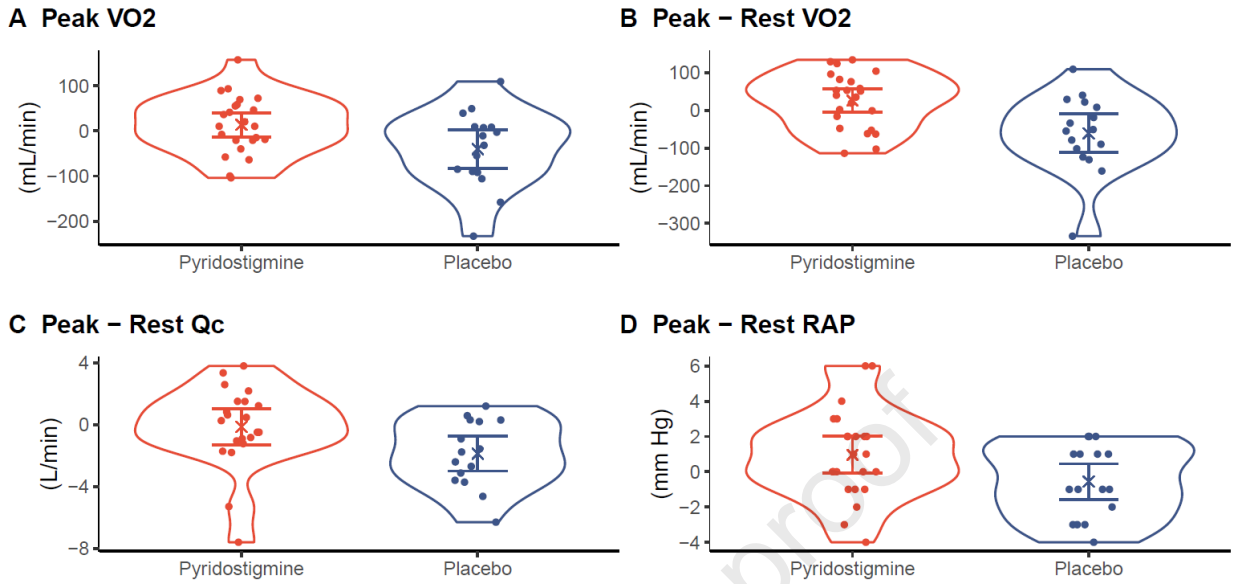
Data are represented as mean ± SD. VO_2 = oxygen consumption; Qc= cardiac output; RAP= right atrial pressure; PAWP = pulmonary artery wedge pressure; SV = stroke volume; $Ca\text{-}vO_2$ = arterial venous oxygen content difference; Hb = hemoglobin; HR = heart rate; VE/VCO2 = ventilatory efficiency; AT = anaerobic threshold; VD/VT = ratio of dead space to tidal volume.

Figure 1: Screening, Randomization, and Assessment



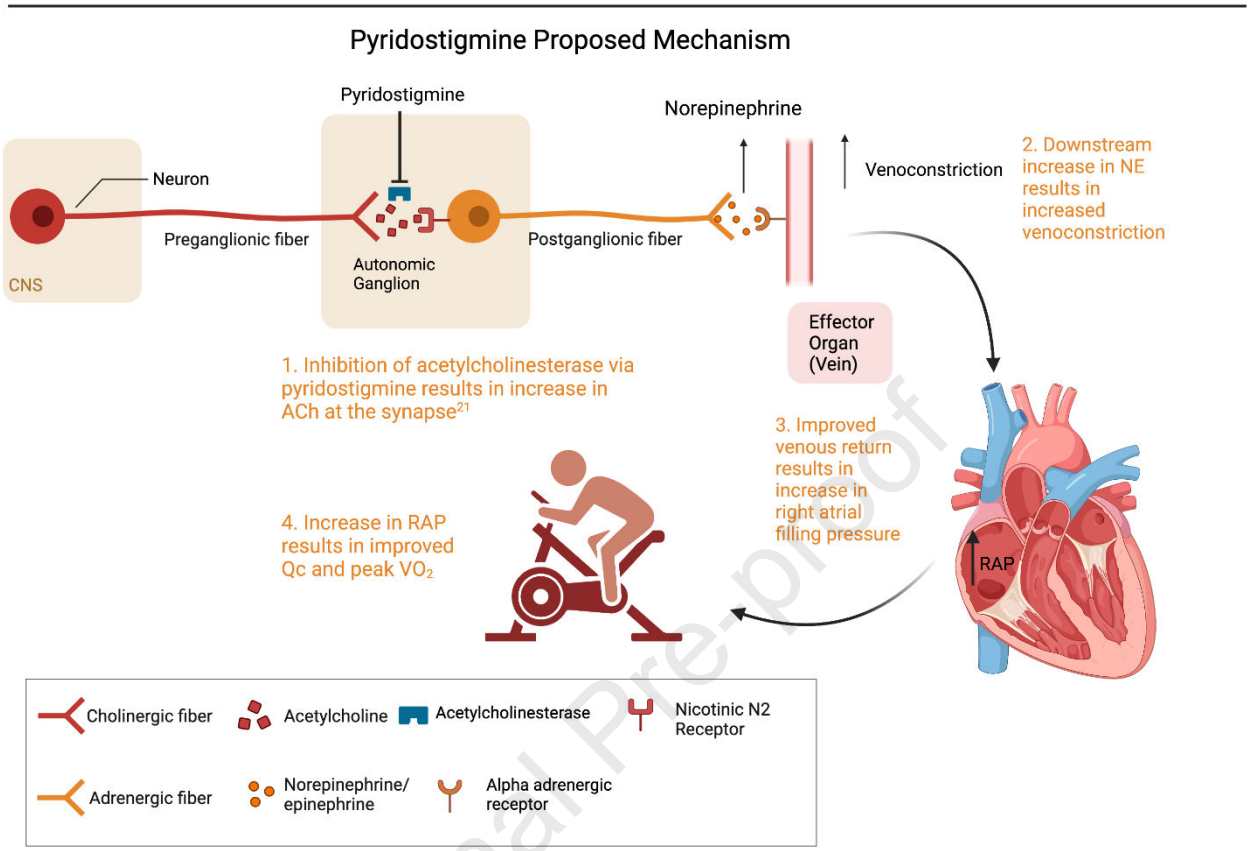
ME/CFS: Myalgic encephalomyelitis/chronic fatigue syndrome. iCPET: invasive cardiopulmonary exercise test. NAM: National Academy of Medicine. ST: segment of electrocardiogram wave that represents the end of ventricular depolarization and the beginning of ventricular repolarization during the cardiac cycle. Elevation indicates potential myocardial ischemia or infarction.

Figure 2: Primary and Secondary Outcomes, changes between iCPETs



Shown are mean ± 2xSEM and the violin plot distributions of the changes between the two consecutive iCPETS. iCPET: invasive cardiopulmonary exercise test; VO2: oxygen uptake; Qc: cardiac output; RAP: right atrial pressure

Figure 3



Adapted from "Organization of the Sympathetic and Parasympathetic Nervous System", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates

Section S1: Trial Personnel

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Section S2: Inclusion and Exclusion Criteria

Pre-Screening Eligibility Criteria
Inclusion Criteria
1. Meets the National Academy of Medicine criteria for myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS), or 2. Has medical comorbidities associated with ME/CFS such as dysautonomia, low ventricular filling pressures, postural orthostatic tachycardia syndrome, orthostatic hypotension, or fibromyalgia. Further confirmation that a subject meets the National Academy of Medicine criteria for ME/CFS by the telephone pre-screening questionnaire will be needed for subjects that meet this initial pre-screening inclusion criteria.
Exclusion Criteria
1. Obesity (body-mass index > 30 kg/m^2) 2. Non-controlled asthma 3. Anemia (hemoglobin < 10 g/dL) 4. Active or treated cancer 5. History of interstitial lung disease 6. Chronic obstructive pulmonary disease 7. Pulmonary hypertension 8. Congestive heart failure 9. Active arrhythmias 10. Valvular heart disease 11. Coronary artery disease 12. Other conditions that could predict a limitation or not completion of the study (as determined by the PI).

Screening Eligibility Criteria
Inclusion Criteria
1. Completing the clinically indicated iCPET
Exclusion Criteria
1. Pregnancy test positive in female subjects. 2. Submaximal testing in clinically iCPET: peak heart rate ≤ 85 percent predicted OR peak RER ≤ 1.05. 3. Pulmonary mechanical limitation to exercise in clinically indicated iCPET: VE /MVV > 0.7 at AT. 4. Pulmonary arterial hypertension in clinically indicated RHC rest mPAP > 20 mmHg, rest PAWP ≤ 15 mmHg, and PVR ≥ 3 Wood Units (WU). 5. Pulmonary venous hypertension in clinically indicated RHC: rest mPAP >20 mmHg and rest PAWP >15 mmHg.

6. Exercise pulmonary arterial hypertension in clinically indicated iCPET: In subjects ≤ 50 years of age: peak mPAP > 30 mmHg and PVR > 1.34 WU; and in subjects > 50 years of age: peak mPAP > 33 mmHg and PVR > 2.10 WU
7. Exercise pulmonary venous hypertension in clinically indicated iCPET: In subjects ≤ 50 years of age: PAWP > 19 mmHg; in subjects > 50 years of age peak PAWP > 17 mmHg.
8. Persistent hypotension during or after the clinically indicated iCPET: SBP < 90 mmHg for more than 5 minutes.
9. Refractory arrhythmia during or after the clinically indicated iCPET.

Section S3: Full Two iCPET Protocol

Two maximum symptom-limited incremental iCPETs were performed using an upright cycle ergometer and a breath-by-breath metabolic cart (ULTIMA CPX; Medical Graphics, St Paul, MN, USA) with subjects breathing room air. Prior to the first iCPET, 2 catheters were placed in the catheterization suite. A flow-directed, 4-port pacing pulmonary arterial catheter was placed via the internal jugular vein using ultrasound and fluoroscopic guidance. An arterial line was inserted into the radial artery in the wrist using a 5 French micropuncture catheter. After the catheters were placed, patients were transported via wheelchair to the exercise lab where they were assisted onto the upright cycle ergometer. Patients began with 3 minutes of unloaded cycling at 55-65 rpm, during which EKG was monitored and BP, intracardiac pressures, and pulmonary gas exchange were recorded. The work ramp was individually selected based on the history of exercise tolerance in the field, ranging from 10 to 25 Watts/min. During the last 15 s of each minute of exercise, systemic arterial and mixed venous blood samples were simultaneously collected from the radial artery and distal pulmonary artery, respectively. By co-oximetry, oxygen saturation, hemoglobin concentration, and arterial and mixed venous oxygen content were measured for each blood sample. Qc was then calculated by the direct Fick principle using a simultaneously measured VO_2. Test termination was determined by patient safety and indication of maximum effort, which is defined by peak respiratory exchange ratio (RER) ≥1.05 and/or peak heart rate (HR) ≥85% predicted. After the first iCPET, patients who met the screening criteria were administered 60 mg pyridostigmine or placebo. The pulmonary and radial artery catheters remained in place during the combined dosing and rest period. After 50 minutes, patients performed a second iCPET. The second iCPET was identical to the protocol outlined above, with one key difference. iCPET practice dictates that one-milliliter blood samples are collected from the radial arterial catheter and distal port of the non-wedged pulmonary arterial catheter every minute during exercise. However, during iCPET 2, mixed venous and arterial samples were only obtained at rest and peak exercise. For patient safety purposes,

we also minimized PAWP measurements. PAWP and other hemodynamic parameter measurements were only taken at two time points: resting baseline and peak exercise. After completion of the second iCPET, patients were transported to the recovery room via wheelchair. Patients spent one hour under the supervision of recovery room staff prior to discharge and the radial and pulmonary artery catheters were removed during this time.

e-Table 1: Exercise Variables for Males Receiving Placebo

	Test	Placebo (N=6)	
Peak VO2 (mL/min)	1	2100.3(530.1)	
	2	2212.2(545.9)	
Peak VO2 % Predicted	1	84.0(16.3)	
	2	88.4(15.6)	
(Ca-vO2)/[Hb]	1	0.9(0.1)	
	2	0.9(0.1)	
ΔCO/ΔVO2	1	4.4(1.2)	
	2	4.4(1.1)	
		Rest	**Peak**
Qc (L/min)	1	8.0(2.1)	14.5(3.1)
	2	8.0(1.1)	16.0(3.2)
RAP (mmHg)	1	-0.5(2.6)	2.7(2.2)
	2	1.2(1.1)	4.3(1.9)